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Altered Catalytic Activity of and DNA Cleavage by DNA Topoisomerase II from Human Leukemic Cells Selected for Resistance to VM-26†

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ABSTRACT: The simultaneous development of resistance to the cytotoxic effects of several classes of natural product anticancer drugs, after exposure to only one of these agents, is referred to as multiple drug resistance (MDR). At least two distinct mechanisms for MDR have been postulated: that associated with P-glycoprotein and that thought to be due to an alteration in DNA topoisomerase II activity (at-MDR). We describe studies with two sublines of human leukemic CCRF-CEM cells ≈50-fold resistant (CEM/VM-1) and ≈140-fold resistant (CEM/VM-1-5) to VM-26, a drug known to interfere with DNA topoisomerase II activity. Each of these lines is cross-resistant to other drugs known to affect topoisomerase II but not cross-resistant to vinblastine, an inhibitor of mitotic spindle formation. We found little difference in the amount of immunoreactive DNA topoisomerase II in 1.0 M NaCl nuclear extracts of the two resistant and parental cell lines. However, topoisomerase II in nuclear extracts of the resistant sublines is altered in both catalytic activity (unknotting) of and DNA cleavage by this enzyme. Also, the rate at which catenation occurs is 20–30-fold slower with the CEM/VM-1-5 preparatons. The effect of VM-26 on both strand passing and DNA cleavage is inversely related to the degree of primary resistance of each cell line. Our data support the hypothesis that at-MDR is due to an alteration in topoisomerase II or in a factor modulating its activity.

We have described a human leukemic cell line selected for resistance to VM-26 (CEM/VM-1)[1] that has an "atypical" multiple drug resistance (at-MDR) phenotype (Danks et al., 1987; Beck et al., 1987). Cells with this phenotype are cross-resistant to many of the same natural product drugs used to select P-glycoprotein-overexpressing MDR cells, but other characteristics of these cells differ markedly from those of the

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[1] Abbreviations: MDR, multiple drug resistance, multiple drug resistant; at-MDR, MDR associated with altered topoisomerase II activity; VM-26, teniposide [9-[4,6-*O*-(2-thenylidene)-β-D-glucopyranosyl]-4′-demethylepipodophyllotoxin]; VP-16, VP-16-213, etoposide [9-(4,6-*O*-ethylidene-β-D-glucopyranosyl)-4′-demethylepipodophyllotoxin]; DMSO, dimethyl sulfoxide; mAMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; CEM/VM-1, cloned CEM subline, ≈50-fold resistant to VM-26; CEM/VM-1-5, CEM/VM-1 subline, ≈140-fold resistant to VM-26; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BCIP, 5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt; BSA, bovine serum albumin; Pgp, P-glycoprotein; VCR, vincristine.

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well-characterized Pgp-MDR cells. At-MDR cells show little or no cross-resistance to the *Vinca* alkaloids, do not overexpress the *mdr*1 gene, and have about the same steady-state levels of drug compared to drug-sensitive parents (Danks et al., 1987; Beck et al., 1987). We suggested that the basis for at-MDR may be an alteration of DNA topoisomerase II activity in these cells.

While the precise enzymatic mechanism of DNA topoisomerase II is not fully defined, its overall function has been described [for reviews, see Vosberg (1985), Wang (1985), and Maxwell and Gellert (1986)]: The enzyme binds to double-stranded DNA, cleaves both strands, passes a second strand of DNA through the cleaved site, and rejoins the strands at the original site of cleavage. This process produces a species of DNA altered only in its topological configuration. Eukaryotic DNA topoisomerase II can unwind supercoiled DNA (Hsieh & Brutlag, 1980), catenate or decatenate circular DNA (Hsieh & Brutlag, 1980), and knot (Hirose et al., 1988; Hsieh, 1983a) or unknot DNA (Liu et al., 1981). Each of these topological alterations is accomplished by the DNA strand passing activity of the enzyme. This activity can be measured by separating different forms of DNA on agarose gels. Also, it is possible to quantitate the amount of DNA topoisomerase II covalently bound to DNA by measuring the number of stable DNA–protein complexes formed between the enzyme and radiolabeled DNA. These "cleavable complexes" can be selectively precipitated by treatment with detergent and high salt concentrations (Liu et al., 1983). This method does not measure strand passing activity but rather, by denaturing the enzyme and stabilizing the enzyme–DNA complex, reflects the equilibrium between topoisomerase II–DNA binding and DNA cleavage. Drugs such as VM-26 and m-AMSA stabilize this enzyme–DNA complex (Chen et al., 1984; Nelson et al., 1984) and inhibit DNA topoisomerase II activity (Chen et al., 1984; Minocha & Long, 1984).

We describe here the activities of and complex formation by topoisomerase II in nuclear extracts from two sublines of CCRF-CEM human leukemic cells selected for resistance to VM-26. To test the hypothesis that the resistance of these lines to VM-26 is due to an alteration in DNA topoisomerase II activity, we measured amounts of immunoreactive DNA topoisomerase II and strand passing activities of nuclear extracts from the two drug-resistant lines and compared them with the drug-sensitive parent CEM line. We also measured complex formation by the nuclear extracts from each of the cell lines.

Experimental Procedures

Chemicals and Supplies. Minimal essential medium (Earle's salts) and trypan blue were purchased from Gibco (Grand Island, NY), and fetal bovine serum was from Hyclone Laboratories (Logan, UT). VM-26 and VP-16 were provided by Bristol-Myers Laboratories (Wallingford, CT), and VCR and VLB were from Eli Lilly and Co. (Indianapolis, IN). Supercoiled (form I) pBR322 DNA dimer, Klenow fragment of DNA polymerase I, and *Hin*dIII restriction enzyme were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Propidium iodide was obtained from Calbiochem (San Diego, CA), and DMSO was from Fisher Scientific Co. (Fair Lawn, NJ). Nitrocellulose paper (0.45-μM pore size) was purchased from Schleicher and Schuell (Keene, NH). Agarose and Bio-Rad dye reagent were purchased from Bio-Rad Laboratories (Richmond, CA). [α-^{32}P]dATP was obtained from Du Pont/NEN (Boston, MA). Hoecsht dye 33258 was purchased from Polysciences, Inc. (Warlington, PA). Alkaline phosphatase linked rabbit anti-rat and goat anti-rabbit IgG + IgM + IgA was purchased from Zymed Laboratories (S. San Francisco, CA), and YOL 1/34 anti-tubulin was from Accurate Chemical Co. (Westbury, NY). Immunoglobulin-free BSA was obtained from Boehringer Mannheim (Indianapolis, IN). *Hin*dIII digests of λ phage DNA and all other chemicals were purchased from Sigma Chemicals (St. Louis, MO). Drug solutions were prepared as previously described (Conter & Beck, 1984). Antiserum IID3 against the 3′ end of recombinant mammalian DNA topoisomerase II was a generous gift from Dr. L. F. Liu, Johns Hopkins University, Baltimore, MD. We are grateful to Dr. F. H. Drake, SmithKline Beckman Laboratories, King of Prussia, PA, for providing a sample of purified DNA topoisomerase II.

Cells and Culture Conditions. CEM cells were cultured and CEM/VM-1 cells were selected, cloned, and cultured as described previously (Beck et al., 1979; Danks et al., 1987). The CEM/VM-1-5 cells were selected from the CEM/VM-1 line by intermittent exposure to increasing concentrations of VM-26. Resistance of the CEM/VM-1 cells to VM-26 is stable for at least 12 months in the absence of drug. The CEM/VM-1-5 line was developed recently, and the stability of its resistance has only been confirmed through 15 weeks.

Growth Inhibition Assay, Measurement of Doubling Time, and Cell Cycle Analysis. Drug cytotoxicity was assayed in a 48-h growth inhibition assay as described previously (Conter & Beck, 1984). DMSO controls in the absence of drug had no measurable cytotoxicity. Doubling time is defined as the number of hours required for the cell concentration to change from 4×10^5 to 8×10^5 cells/mL. These particular cell densities were chosen because all three cell lines are in log phase growth in this interval. Cell cycle analysis was done by flow cytometry using propidium iodide stained cells (Beck et al., 1986).

Preparation of Nuclear Extracts. Nuclear extracts were prepared according to the method of Sullivan et al. (1987) with the following modifications: 1×10^8 exponentially growing cells were permeabilized by incubation for 8–12 min in 1.75 mL of hypotonic buffer [buffer G of Sullivan et al. (1987), 5 mM KH_2PO_4, 2 mM $MgCl_2$, 4 mM DTT, 0.1 mM Na_2EDTA, and 1 mM PMSF, pH 7.0]. When ≈90% of the cells stained with trypan blue, they were centrifuged at 400*g* for 5 min. The cells were then suspended in buffer H (buffer G with 0.25 M sucrose) at a concentration of $\approx 5 \times 10^8$/8 mL. Each 8-mL aliquot was layered over 3 mL of buffer I (buffer G with 0.3 M sucrose) and centrifuged at 2000*g* for 20 min. All the supernatant was then removed with a Pasteur pipet and the pellet volume determined. The pellet was resuspended in a volume of buffer J (Sullivan et al., 1987; 5 mM KH_2PO_4, 4 mM DTT, 1 mM Na_2EDTA, and 1 mM PMSF, pH 7.0) equal to half the volume of the pellet and incubated on ice for 15 min. Nuclear topoisomerase II was then extracted by adding half the pellet volume of buffer L (40 mM Tris, 0.7 M NaCl, 1 mM PMSF, 4 mM DTT, and 20% glycerol, pH 7.5) for 0.35 M salt extracts or buffer M (40 mM Tris, 2.0 M NaCl, 1 mM PMSF, 4 mM DTT, and 20% glycerol, pH 7.5) for 1.0 M salt extracts. The solutions were vortexed and incubated on ice for 30 min. The solution with buffer M added is very viscous. These solutions were centrifuged at 100000*g* for 60 min, and the clear supernatant was removed. The remaining "pellet" was centrifuged again, and the second supernatant was pooled with the first one. CEM nuclei prepared in this way do not contribute any significant volume to the final volume of supernatant.

The first three steps are critical for the reproducibility of the nuclear extractions and may need to be modified for different cell lines. First, incubation in the hypotonic buffer (buffer G) must be long enough to permeabilize the cells but short enough that the nuclei remain intact. Second, the permeabilized cells must be centrifuged hard enough to give a packed pellet, but not so hard as to disrupt the nuclei. If the nuclei have been disrupted, resuspension of the 400*g* pellet in buffer H is very difficult because of DNA release resulting in a "stringy" consistency of the cell pellet. Third, when the resuspended cell solution is layered over buffer I, volumes and test tube sizes must be such that after the 2000*g* centrifugation the cell pellet does not obscure the interface of the 0.25 and 0.30 M sucrose buffers. (We used a 15-mL plastic Falcon tube with a conical bottom.) The 2000*g* centrifugation must result in a packed nuclear pellet so that an accurate pellet volume to within 0.1 mL can be determined. We have found that if the above procedures are followed exactly, preparations made with 1.0 M NaCl reproducibly yield ≈5–7 mg of nuclear protein/mL and those made with 0.35 M NaCl reproducibly yield ≈3–4 mg of nuclear protein/mL. Also, in order to minimize proteolysis, protease inhibitors (benzamidine, soybean trypsin inhibitor, and leupeptin) were included in buffers G–J, L, and M as outlined by Drake et al. (1987). All solutions of protease inhibitors were prepared immediately before use and kept at 4 °C. Protein concentrations of the nuclear extracts were determined by using Bio-Rad dye reagent according to the directions of the manufacturer. Extracts were stored in aliquots at −80 °C with no loss of topoisomerase II activity for at least 6 months. One experiment (Figure 5) including the effect of VM-26 on enzyme activity in both 0.35 and 1.0 M salt extracts is shown for comparison.

The salt concentration referred to in assay conditions or extractions indicates added salt (KCl and NaCl) and does not take into account any salts extracted simultaneously from the nuclei with the DNA topoisomerase II. Nuclear extracts obtained by the above method contain DNA topoisomerase I activity that relaxes supercoiled DNA dimers in the absence of ATP. However, since topoisomerase I cannot efficiently catenate pBR322 (Hsieh, 1983b) or unknot P4 DNA (Liu & Davis, 1981) and its effect on DNA is ATP independent, the activities of DNA topoisomerase I and DNA topoisomerase II are easily distinguished.

Western Blots. Protein (75 μg) was loaded in each lane of a 5% SDS–PAGE gel and electrophoresed by the method of Laemmli (1970). The proteins were transferred to nitrocellulose paper by using a PolyBlot apparatus (American Bionetics, Emeryville, CA) and the method suggested by the manufacturer. The antibody reaction was done essentially according to the method of Towbin et al. (1979), with specific reactions as follows: IID3 antiserum to DNA topoisomerase II was used as the primary antibody at a 1:1000 dilution with 3% immunoglobulin-free BSA. The incubation was done at 4 °C for ≈14 h. The blot was then washed, and a second incubation was done with an alkaline phosphatase linked goat anti-rabbit IgG + IgM + IgA with 3% BSA in Tris–NaCl wash solution. After this second incubation for 3 h at room temperature, the blot was washed again. Fifty milligrams of BCIP was dissolved in ≈300 μL of DMSO and diluted to 100 mL with 1 M Tris-HCl, pH 8.8. The nitrocellulose papers were then incubated in substrate solution until blue bands appeared on the paper. Control blots, to assure equal protein loading, were incubated with a 1:400 dilution of YOL1/34 antibody to tubulin followed by a 1:200 dilution of rabbit anti-rat IgG + IgM + IgA as the second antibody.

DNA Substrates. P4 DNA is a knotted phage DNA and was prepared according to the method of Liu and Davis (1981) with the following modifications. An overnight culture of C117 *Escherichia coli* was fed with an equal volume of media. One hour later 1.3×10^9 PFU of P4 phage was added per 100 mL of *E. coli* culture. The P4/*E. coli* suspension was incubated at 37 °C for 30 min without shaking, after which time 20 mL of suspension was added to 1 L of media. Cultures were then grown in a shaker apparatus until bacterial lysis occurred. The rest of the procedure was done as described by Liu and Davis (1981). After the phage were harvested, the tailless capsids were separated from the complete phage by adding 0.632 g of CsCl/mL of phage suspension and centrifuging for 4 days at 30000 rpm (≈80000*g*) in a Beckman Ti60 rotor. By use of this protocol, the two bands of DNA separated by several centimeters.

The dimeric form of pBR322 is an 8.6-kb double-stranded, negatively supercoiled DNA. When digested with a restriction enzyme that has one restriction site/pBR322 monomer, 4.3-kb linear pBR322 monomers are formed. When cleaved by topoisomerase II, the linear dimer migrates at 8.6 kb. Like the monomer, the rate of migration of each topological form of dimeric pBR322 is dependent on the percent agarose in the gel, the buffer, and the current or voltage used. The migration characteristics of pBR322 dimers have been well characterized (Sander & Hsieh, 1983; Hsieh, 1983a).

Strand Passing Assay—Unknotting Activity. Unknotting activity was measured by using P4 DNA as a substrate, nuclear preparations prepared as described above, and reaction conditions of Liu and Davis (1981). Reactions were terminated in one of two ways, with the amounts of reagents indicated in final concentrations. Experiments showing the protein concentration activity profile (Figure 2) and effect of VM-26 on strand passing activity (Figure 4) were terminated by a 2.5-h incubation in 1% SDS and 150 μg of proteinase K/mL at 37 °C. Reactions showing the effect of VM-26 on equal enzyme activities (Figure 5) were stopped by a 2.5-h incubation in 10 mM EDTA and 150 μg of proteinase K/mL at 37 °C. When an incubation with proteinase K was done, the samples were adjusted to 10% glycerol and 0.02% bromophenol blue immediately before putting them on agarose gels. With 1.0 M NaCl extracts, the final added salt concentration was ≈113 mM; with 0.35 M NaCl extracts, the final added salt concentration was ≈109 mM.

Strand Passing Assay—Catenating Activity. Catenating activity was measured by using the negatively supercoiled pBR322 8.6-kb dimer DNA as a substrate and nuclear preparations extracted with 1.0 M NaCl. Assays were run for the length of time indicated in Figure 3. Reactions were terminated by bringing the final concentrations to 1% SDS, 10% glycerol, and 0.02% bromophenol blue. The reactions shown in Figure 3 were not treated with proteinase K. A total of 0.3 μg of DNA was used for each assay. Total added salt was the same as for the experiments with P4 DNA. In the strand passing assays in which VM-26 was added to the reaction, the DMSO controls without drug showed no effect on strand passing activity or DNA cleavage.

Agarose Gel Electrophoresis. Reaction products in the unknotting experiments (P4 DNA) were electrophoresed in 0.7% agarose gels under the TBE buffer and electrophoresis conditions of Miller et al. (1981). The gels were stained with ethidium bromide and photographed under UV light.

Reaction products in the experiment measuring rate of catenation of pBR322 DNA were electrophoresed in 1% agarose gels, using a modified TBE buffer (pH ≈8.1) of the

Table I: Cross-Resistance of CCRF-CEM Sublines Selected for Resistance to VM-26

drug	CEM IC_{50}[a] (nM)	degree of resistance[b] CEM/VM-1	CEM/VM-1-5
VM-26	48.0 ± 39.0	47[c]	141[c]
VP-16	416 ± 200	41[c]	129[c]
vinblastine	2.8 ± 2.5	1.4	0.9
vincristine	2.4 ± 2.1	1.5	7.2[c]
doxorubicin	29.0 ± 24.0	16[c]	116[d]
mAMSA	12.1 ± 14.7	21[d]	87[d]
actinomycin D	5.9 ± 7.7	3.2[d]	11.4[d]

[a] 50% inhibitory concentration in a 48-h growth inhibition assay ± SD. Values shown for CEM cells are means of 14–18 separate experiments. For CEM/VM-1 and CEM/VM-1-5, values are means of 3–4 separate experiments. [b] IC_{50} of the resistant cell line divided by that of the parent CEM line. [c] Significantly different from CEM cells, $p < 0.001$. [d] Significantly different from CEM cells, $p < 0.01$.

following composition in grams/liter: Tris base, 10.9; boric acid, 5.6; and Na_2EDTA, 0.042. The gels were run at 100 mA until the tracking dye had moved 5.5 cm (about 40 min). The migration properties of the different forms of pBR322 dimers were confirmed by the method of Sander and Hsieh (1983). As supplied, the pBR322 is ≈90% supercoiled and ≈10% nicked circular DNA, with <1% linear DNA contaminant. In the above system, the nicked DNA separates well from supercoiled DNA only if the gels are run longer than 1 h. Since the gels, when run for 40 min, clearly separated catenated DNA from all noncatenated forms, this method was adequate for measuring formation of catenanes. The method was used because it is rapid and simple and the substrate is available commercially, but it is not adequate to separate supercoiled from various partially relaxed pBR322 topoisomers. Since the slight differences in migration of the supercoiled DNA (shown in Figure 3) could be due to a simultaneous relaxation of the supercoiled substrate by topoisomerase I or II, the TPE gel and buffer system of Sander and Hsieh (1983) was used to confirm that DNA topoisomerase I activity was present in the nuclear extracts. At the later time points in Figure 3 all the remaining substrate DNA is relaxed. After electrophoresis, pBR322 gels were stained with ethidium bromide and photographed under UV light.

^{32}P-Labeling of DNA. pBR322 dimer was digested with *Hin*dIII under reaction conditions recommended by the manufacturer. The linear 4.3-kb reaction product was then labeled at the 3′ ends with [^{32}P]dATP by the Klenow fragment of DNA polymerase I. Unincorporated nucleotides were removed by three sequential precipitations with ethanol–2 M ammonium acetate as described by Maniatis et al. (1982). Final DNA concentration was measured by using Hoechst dye 33258 with a fluorometer (Model TKO100, Hoefer Scientific Instruments, San Francisco, CA) according to the manufacturer's instructions. The specific activity of the DNA was approximately 1×10^6 cpm/μg of DNA.

Cleavable Complex Formation. These assays were done according to the method of Liu et al. (1983) for precipitation of single-stranded DNA–protein complexes but using the reaction mixture for the P4 unknotting reaction (Liu & Davis, 1981). Where indicated, the reaction mix contained 1 mM ATP. DMSO (1.5–3%) had no effect on cleavable complex formation.

Results

Resistance and Cross-Resistance Properties of at-MDR Cells. Table I shows the resistance and cross-resistance of the two sublines (CEM/VM-1 and CEM/VM-1-5) to VM-26 and several other natural product drugs. CEM/VM-1 cells are ≈50-fold resistant and CEM/VM-1-5 cells are ≈140-fold resistant to VM-26. The cross-resistance to VP-16 in both cell lines was expected because of the similarity of VM-26 to VP-16. Both lines are also cross-resistant to three other drugs known to interfere with DNA topoisomerase II activity, doxorubicin, mAMSA, and actinomycin D (Tewey et al., 1984). Neither cell line is cross-resistant to the *Vinca* alkaloid vinblastine, a property that distinguishes at-MDR from Pgp-MDR (Danks et al., 1987). As we reported earlier, the CEM/VM-1 cells are also as sensitive to VCR as the parent CEM line (Danks et al., 1987). However, the CEM/VM-1-5 line is ≈7-fold resistant to VCR. A lesion other than altered DNA topoisomerase II activity may account for this cross-resistance to VCR in the CEM/VM-1-5 cells, but it does not appear to be due to overexpression of the P-glycoprotein mRNA (J. S. Wolverton, unpublished observation).

Growth Characteristics of CEM, CEM/VM-1, and CEM/VM-1-5 Cells. While the cross-resistance data indicate a significant difference in degree of resistance to VM-26 between the two resistant cell lines, interpretation of these data should include cell growth characteristics in the absence of drug. All three cell lines are in exponential growth at densities of 4–8 × 10^5 cells/mL (data not shown); however, when seeded at 4.0 × 10^5 cells/mL, the growth rates are different. The doubling time of the CEM cells is 26 ± 6 h, that of the CEM/VM-1 cells is 33 ± 9 h, and that of the CEM/VM-1-5 cells is 37 ± 8 h. The cell density at which the growth of each line plateaus is also different: CEM cells are no longer in log growth at ≈1.2 × 10^6 cells/mL, while the CEM/VM-1 and CEM/VM-1-5 cells plateau at ≈1.0 × 10^6 and ≈9.0 × 10^5 cells/mL, respectively. The resistance and cross-resistance studies were all done with cells in exponential growth, but the slower growth rates of the resistant cell lines may modify slightly the apparent degree of resistance. Among all three cell lines, the percentage of cells in each phase of the cell cycle (G1, S, and G2 + M) as determined by cytofluorometric analysis of propidium iodide stained cells was the same: 49.0–53.3% in G1; 38.1–38.3% in S; and 8.4–12.9% in G2 + M.

Extraction of Immunoreactive Topoisomerase II. Since interaction with DNA topoisomerase II is a property common to most of the drugs in Table I to which the at-MDR cell lines are resistant, changes in either the amount or the activity of this enzyme are possible explanations for at-MDR. To test the first of these hypotheses, we measured the amount of immunoreactive topoisomerase II in nuclear preparations from each of the cell lines (Figure 1).

With anti-topoisomerase II antiserum (IID3) as the primary antibody, the reactive band from the 1.0 M salt nuclear extracts of each cell line and the purified DNA topoisomerase II standard migrated at 170 kDa. These data show that similar amounts of enzyme were extracted from each of the three cell lines. Less immunoreactive material and activity were extracted from nuclei with 0.35 M salt in each of the three cell lines (not shown). Several different methods for preparing nuclear extracts have been published. One of the major differences among them is the concentration of salt used for the final extraction of enzyme activity: 0.35 M (Filipski et al., 1983; Sullivan et al., 1986); or 1.0 M (Sullivan et al., 1987; Nelson et al., 1987). After several preliminary experiments, we chose to use 1.0 M NaCl for enzyme extraction because the lower concentration of NaCl yielded smaller amounts of DNA topoisomerase II activity as determined by catenation assay and smaller amounts of enzyme as determined by Western blots. Also, seven Western blots showed the amount of topoisomerase II extracted from three separate preparations

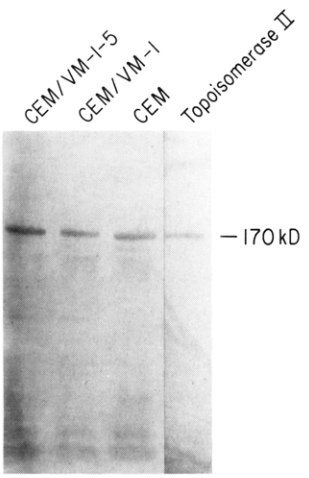

FIGURE 1: Western blots of DNA topoisomerase II in nuclear extracts of CEM, CEM/VM-1, and CEM/VM-1-5 cells. DNA topoisomerase II was extracted from nuclei with 1.0 M NaCl. Equal amounts of protein (75 μg) from each of the preparations were separated on a 5% SDS–PAGE gel, transferred to nitrocellulose paper, and reacted with IID3 DNA topoisomerase II antiserum. Purified topoisomerase II served as a control. A representative blot is shown. See Experimental Procedures for details of the method.

with 1.0 M salt was very reproducible. In two of the 0.35 M preparations a smaller proportion of the enzyme was extracted from the resistant cells, but the amount of enzyme extracted with 0.35 M salt from drug-sensitive CEM cells was consistent among the three preparations. Duplicate blots were probed with anti-tubulin antibody and confirmed that equal amounts of protein were present in each lane.

Unknotting Activities of Nuclear Extracts. We measured the strand passing activities of topoisomerase II in our nuclear preparations using P4 DNA as a substrate. Unknotting activity is specific for topoisomerase II even in crude cell preparations (Liu & Davis, 1981). Figure 2 shows the amount of unknotting (and catenating) activity of 0.025–3.5 μg of protein from 1.0 M NaCl extracts after a 30-min incubation followed by proteinase K treatment. At 0.05 and 0.1 μg, the CEM extract formed nicked DNA. Between 0.1 and 0.5 μg we only saw unknotting of DNA, and catenation was evident at protein concentrations above 0.5 μg. Strand passing was inhibited at protein concentrations greater than 2.0 μg; the mechanism of this inhibition is not known.

The nicked DNA migrated more slowly than unknotted DNA and was present in several lanes in which the reaction included ATP and relatively low protein concentrations. In a similar experiment (not shown) in which we did not treat extracts with proteinase K, these bands did not separate but appeared as a fluorescent "tail" above the band of unknotted DNA, indicating that there was protein associated with the bands. After treatment with T4 ligase, these bands migrated faster than knotted P4 DNA (data not shown) and therefore are interpreted to be nicked knotted P4 monomers.

With the CEM/VM-1 and CEM/VM-1-5 preparations, the two bands of nicked DNA were present over a greater concentration range of nuclear protein than in the CEM cell preparations (0.05–0.25 μg for CEM/VM-1 and 0.05–1.5 μg for CEM/VM-1-5). The CEM/VM-1-5 extract formed less intact unknotted monomer and less catenated DNA than the other two nuclear preparations. The lowest concentration of nuclear extract catalyzing complete unknotting of DNA was 0.25, 0.5, and 1.0 μg in the CEM, CEM/VM-1, and CEM/VM-1-5 nuclear preparations, respectively. Overall, the unknotting data show differences in activity among the preparations, the most apparent of which was the increase in

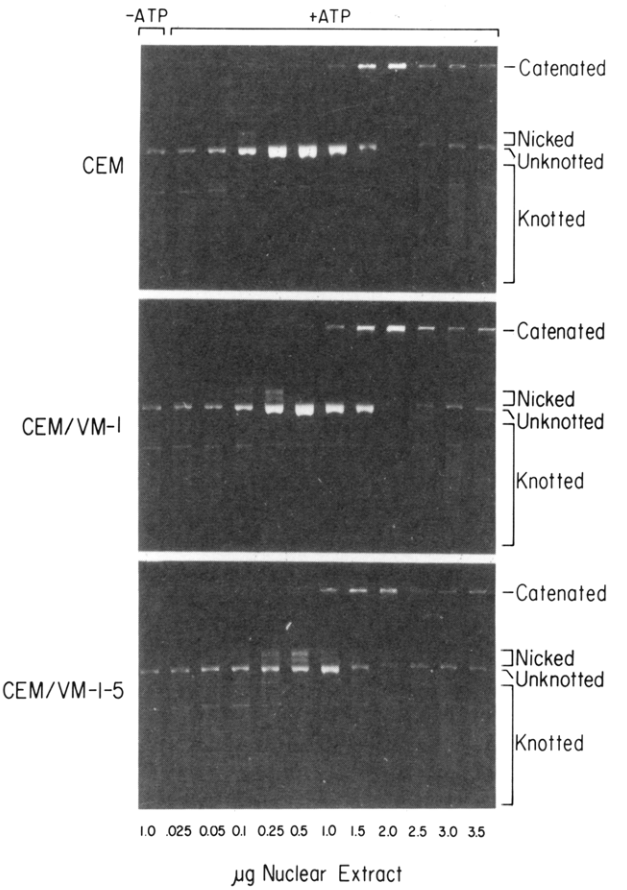

FIGURE 2: Unknotting (strand passing) activity of 1.0 M NaCl extracts of nuclei from CEM, CEM/VM-1, and CEM/VM-1-5 cells. The indicated amount of protein from each preparation was incubated with 0.75 μg of P4 knotted DNA for 30 min. The samples were treated with proteinase K and electrophoresed on a 0.7% agarose gel as detailed under Experimental Procedures.

nicked DNA with extracts from the resistant cells.

Catenating Activities of Nuclear Extracts. As seen in Figure 2, topoisomerase II can catenate as well as unknot DNA. The catenating reaction is less specific than the unknotting reaction in that factors other than topoisomerase II can be involved (Krasnow & Cozzarelli, 1982). Since the data in Figure 2 suggested that the enzyme from the CEM/VM-1-5 cells formed fewer catenanes than enzyme preparations from the other two cell lines, we further characterized the catenating activity in our nuclear preparations by determining the rate at which extracts from each of the three cell lines formed catenanes of pBR322 DNA (Figure 3). We measured catenating activity of 1.75 μg of protein at time intervals between 1 and 50 min. We found that the nuclear extract from CEM and CEM/VM-1 cells catenated 0.3 μg of DNA within 5 min (Figure 3). In contrast, the reaction catalyzed by the nuclear extract from CEM/VM-1-5 cells did not reach equilibrium until 20–30 min. Some catenated DNA product was evident as early as 1 min with the CEM/VM-1-5 preparation, but maximum conversion of supercoiled to catenated DNA was not seen until later. We conclude that rates of catenation differ among the cell lines and may be due either to alterations in catalytic activity of topoisomerase II or to differential extraction of other nuclear proteins that modulate this enzyme activity.

To verify that the DNA remaining in the wells was catenated, we treated the samples with proteinase K (150 μg/mL for 2.5 h). This treatment did not change the migration of any of the DNA (not shown). The slight change with time

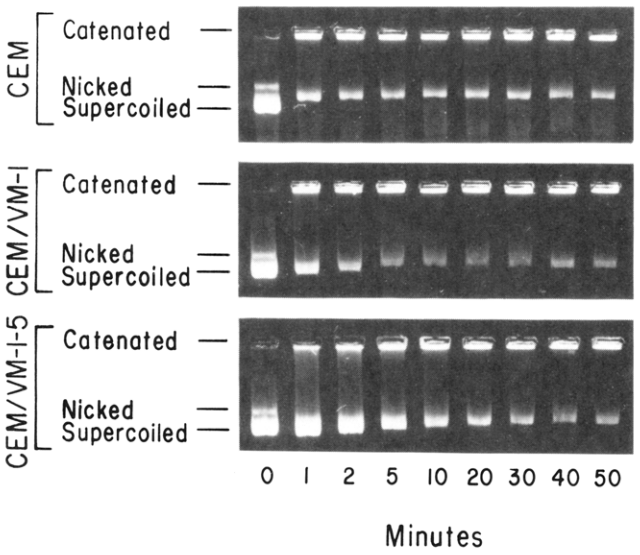

FIGURE 3: Time course of catenating activity. pBR322 DNA (0.3 μg) and 1.75 μg of nuclear extract from each of the cell lines were incubated for the indicated times. The reactions were stopped with SDS and run on a 1% agarose gel. ATP was included in all reactions shown. See Experimental Procedures for details.

in migration of noncatenated DNA can be accounted for by the conversion of supercoiled DNA to nicked or relaxed DNA by topoisomerase I or II activities.

Effect of VM-26 on Strand Passing and Cleavage by Topoisomerase II. Drugs that interact with DNA topoisomerase II have been shown to inhibit strand passing (catalytic) activity (Minocha & Long, 1984) and increase cleavable complex formation (Tewey et al., 1984). Strand passing is a functional assay of topoisomerase II, while "DNA cleavage" measures covalent binding of topoisomerase II to DNA in the presence of denaturing agents (Liu et al., 1983). Measurement of the effects of these drugs on strand passing and complex formation is critical to the hypothesis that at-MDR is due to an alteration of topoisomerase II activity. Therefore, both functional (strand passing) and binding (cleavage) assays were done in the presence of VM-26.

Inhibition of Nicking and Unknotting of P4 DNA by VM-26. Using equal amounts (0.5 μg) of protein from nuclear extracts of each cell line, we found that VM-26 influenced, in a dose-dependent manner, the amount of both nicked and knotted DNA in a strand passing assay (Figure 4). The first lane shows unreacted P4 DNA in the absence of ATP; without ATP, VM-26 from 0 to 160 μM (only 160 μM shown) had no effect on the P4 DNA. The second lane shows the uninhibited reaction for each cell line. VM-26 at 20 μM inhibited the enzyme activity from each of the three cell lines. With the CEM and CEM/VM-1 extracts, the first effect of VM-26 was to decrease unknotted and increase nicked DNA; further increases in VM-26 concentrations decreased nicked DNA. With the CEM/VM-1-5 extract, the effect on unknotting was difficult to assess since less unknotted monomer was formed by this extract, but the nicking activity was definitely affected by VM-26 in a dose-dependent manner. The reaction was stopped with SDS–proteinase K so that the individual bands of nicked DNA would be clear. We used a low concentration of nuclear extract so that there was no DNA cleavage to obscure the effect of VM-26 on the formation of nicked and unknotted DNA even though SDS was used to stop the reaction. The lowest concentration of drug that inhibited enzyme activity was the same (20 μM) with all three cell lines, but the amount of extracts used had not been equalized for enzyme activity.

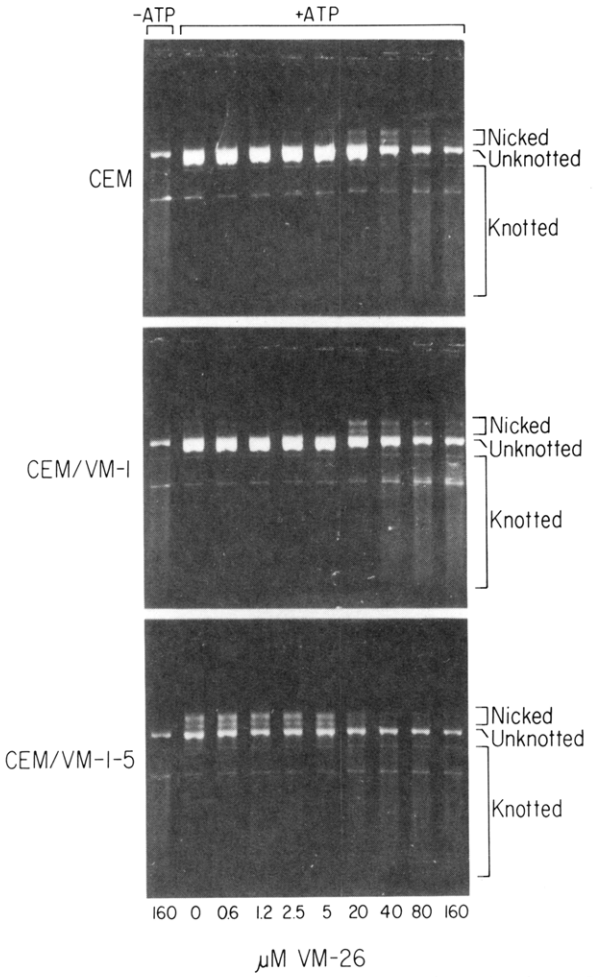

FIGURE 4: Effect of VM-26 on unknotting and nicking by DNA topoisomerase II activity in 1.0 M extracts of CEM, CEM/VM-1, and CEM/VM-1-5 nuclei. Each lane contains the reaction products after incubation of 0.5 μg of protein, 0.75 μg of P4 DNA, and the indicated concentration of VM-26. The first lane shows the negative control with no ATP; all other reactions were done in the presence of 1 mM ATP. The second lane shown is a positive control with no VM-26, i.e., maximum enzyme activity under these conditions. See Experimental Procedures for the details of the method.

Therefore, we addressed the question of drug effect on sensitive vs resistant cell extracts in another way (Figure 5). For each cell line, we used the lowest amount of nuclear protein of both 0.35 and 1.0 M salt extracts that would unknot 0.75 μg P4 DNA (CEM, 0.25 μg; CEM-VM-1, 0.5 μg; CEM-VM-1-5, 1.0 μg), thereby comparing the effect of VM-26 on equal enzyme activities or, in the case of CEM/VM-1-5, as nearly equal as possible. We stopped these reactions with EDTA–proteinase K because under these conditions the enzyme–DNA complex is not stable and DNA cleavage is not evident (Sander & Hsieh, 1983). By use of EDTA to stop the reactions, the formation of linear DNA does not obscure the effect of drug on the strand passing activity of the enzyme. Because of the EDTA stop, however, there appears also to be partial reversal of nicking, and the bands of nicked DNA are not as clear as they are in Figure 4. Figure 5 shows the effect of 5–80 μM VM-26 on enzyme activity from both 0.35 and 1.0 M NaCl extracts. With the 0.35 M NaCl preparations, topoisomerase II activity from CEM cells was inhibited by 5 μM VM-26; less unknotted DNA and more nicked DNA were evident. Unreacted substrate was visible at 10–20 μM VM-26. Using CEM/VM-1 extract, less knotted and more nicked DNA were seen at 20 μM VM-26 (vs 5 μM for CEM), and unreacted substrate was not evident until the VM-26 con-

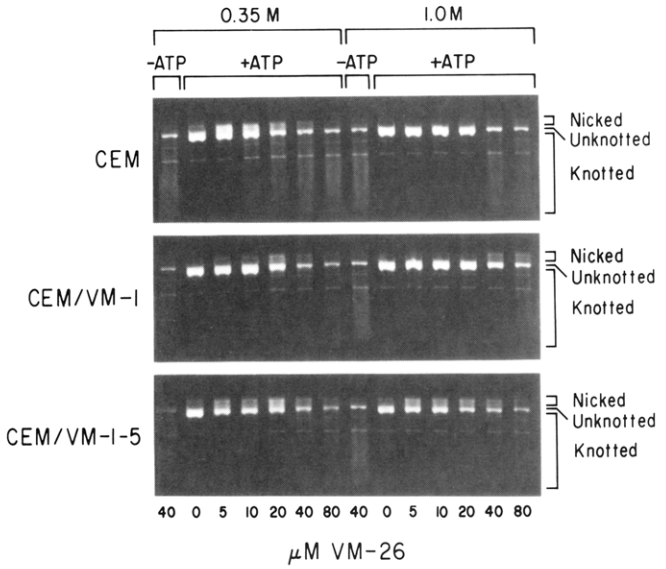

FIGURE 5: Comparison of inhibition of unknotting activity by VM-26 in 0.35 and 1.0 M NaCl nuclear extracts of CEM, CEM/VM-1, and CEM-VM-1-5 cells. Reactions were done as in Figure 2, but to equalize for DNA topoisomerase II activity in the absence of drug, the following protein concentrations were used: 0.25 μg of CEM extract, 0.5 μg of CEM/VM-1 extract, and 1.0 μg of CEM/VM-1-5 extract. The reactions were stopped with EDTA and proteinase K so that the effect of VM-26 on unknotting of P4 DNA (vs cleavage) could be seen more clearly. See Experimental Procedures for details of the method.

centration was ≈40 μM. The effect of VM-26 on enzyme activity from CEM/VM-1-5 cells was comparable to that of the other two cell lines in that increasing concentrations of VM-26 decreased unknotted DNA and first increased and then decreased nicked DNA. In contrast to the extracts from the CEM and CEM/VM-1 lines, the changes in CEM/VM-1-5 enzyme activity were gradual and occurred over the entire range of VM-26. Neither the enzyme activity from the CEM/VM-1 line nor that from the CEM/VM-1-5 line was completely inhibited by 80 μM VM-26.

With the 1.0 M enzyme preparations, inhibition of DNA topoisomerase II activity was seen at higher VM-26 concentrations than with the 0.35 M preparations. This is consistent with the Western blot data showing more enzyme present in the 1.0 M salt extracts. At 40 μM VM-26, the enzyme activity from CEM cells was almost completely inhibited. Both nicking and unknotting of DNA still occurred in the CEM/VM-1 extracts at this concentration of VM-26. The CEM/VM-1-5 results were not comparable to the other two lines. Formation of unknotted DNA was almost completely inhibited by 5 μM VM-26, but some nicked DNA could still be detected at 80 μM VM-26. Comparison of Figures 4 and 5 shows clearly that the effect of any given concentration of VM-26 is dependent on the amount and source of the topoisomerase II activity. We conclude that the nicking and unknotting activities of DNA topoisomerase II in nuclear extracts from resistant cells are less sensitive to the effects of VM-26 than are extracts from the drug-sensitive cells.

Effect of VM-26 on DNA Cleavage. To evaluate the effect of drug on the ability of the enzyme to cleave DNA, we quantitated the protein–DNA complexes stabilized by VM-26, using ^{32}P-labeled pBR322 DNA and the method of Liu et al. (1983). As can be seen in Figure 6A, increasing concentrations of VM-26 from 50 to 200 μM caused a progressive increase in the amount of cleavage mediated by the extract from CEM cells in the absence of ATP. With nuclear extracts from CEM/VM-1 cells, the increase in drug-mediated cleavage was

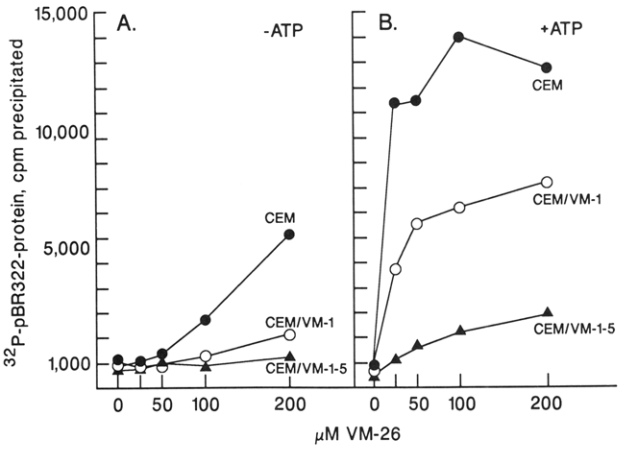

FIGURE 6: Effect of VM-26 on DNA cleavage by DNA topoisomerase II in nuclear prepreparations from CEM, CEM-VM-1, and CEM-VM-1-5 cells. 3′ end labeled [^{32}P]pBR322 (0.1 μg) was incubated for 30 min with 0.75 μg of nuclear protein from each of the three cell lines. The amount of drug in each reaction was as indicated. Details of the procedure are found in Liu et al. (1983). The reaction mix for the assays shown in panel A contained no ATP. The amount of ATP added to the reactions in panel B was 1 mM. Each point represents the average of duplicate samples, with variability of <5%. Because the specific activity of the substrate decreased with time, experiments done on different days cannot be averaged, and so a representative experiment is shown. The experiments shown in panels A and B were done one day apart, with the same DNA and enzyme preparations. Each experiment was done at least three times.

about one-third of that seen with the CEM preparation at 200 μM VM-26. Little or no effect of VM-26 on cleavable complex formation was seen with the extracts from CEM/VM-1-5 cells.

When the experiment was repeated with 1 mM ATP in the reaction mixture, qualitatively similar results were obtained (Figure 6B). However, more counts were precipitated, and the differences among the cell lines were even more apparent. At the higher concentrations of VM-26 the response was no longer linear, suggesting that the maximum binding of protein to DNA under these conditions had occurred. If lower protein/DNA ratios (e.g., 5/1) were used, no plateau was seen (data not shown). Also, note that in the absence of drug the number of stable complexes formed from each of the cell lines was approximately equal (800–1100 cpm). However, the plateau in "activity" (that is, saturation of binding that could result in stable complexes and, therefore, cleavage) with the nuclear preparations from the resistant cells is lower than in the sensitive cells. At 200 μM VM-26, the counts precipitated ranged from ≈13 000 to ≈8000 to ≈3000 in the CEM, CEM/VM-1, and CEM/VM-1-5 extracts, respectively. Fewer binding sites formed stable complexes. ATP alone caused no change in cleavage in the absence of drug (compare 0 μM VM-26 in parts A and B of Figure 6). An inverse relationship was seen between degree of primary resistance and effect of VM-26 on cleavable complex formation. This correlation could also be seen with mAMSA (data not shown).

Discussion

Our data describe two VM-26-resistant human leukemic cell lines that are altered in both catalytic activity of and DNA cleavage by DNA topoisomerase II. Nuclear extracts from these cell lines are resistant to VM-26-mediated inhibition of strand passing and effect on DNA cleavage in proportion to their resistance. Our study also provides a comparison of rates of catenation by DNA topoisomerase II from VM-26-sensitive vs -resistant cells. The cross-resistance pattern, the alterations in DNA topoisomerase II activity, and the differential effect

of VM-26 among the three cell lines support the hypothesis that at-MDR is mediated by an alteration of the topoisomerase II protein or in its regulation.[2]

Four other cell lines that appear to have an at-MDR phenotype have been reported. In three of these [DC3F/9-OHE (Pommier et al., 1986), HL-60/AMSA (Estey et al., 1987), and Vpm^R-5 (Glisson et al., 1986)], little change was seen in decatenation of kDNA by topoisomerase II or the effect of drug on decatenation, but changes were shown in the effect of drugs on DNA cleavage. The fourth cell line, P388/A20 (Per et al., 1987), was shown to have a 2.3-fold decrease in topoisomerase activity as measured by the P4 unknotting assay. The DC3F/9-OHE line (Charcosset et al., 1988) did show a 3.5-fold decrease in the rate of decatenation compared to the drug-sensitive parent line. While these four resistant cell lines have not been compared directly with ours, the similarity among them seems to be a change in the ability of drug to stabilize enzyme–DNA complexes [or, with the P388/A20 line, a decrease in protein-associated DNA single strand breaks (Per et al., 1987)]. The difference among the cell lines is that several of them have been shown to be altered in catalytic activity (P388/A20, CEM/VM-1, CEM/VM-1-5) or rate of catalytic activity (DC3F/9-OHE, CEM/VM-1-5), while others have not (HL60/AMSA, Vpm^R-5). Different assays for catalytic activity may simply be more or less sensitive or, more likely, not comparable because of the different kinds of DNA, enzyme preparations, and reaction conditions used.

We have shown in our resistant cell lines changes not only in strand passing activity but also in the concentrations of VM-26 that affect equal enzyme activity (Figure 5). Although we cannot yet say whether the alteration is in the enzyme itself or in a factor regulating its activity, we note several consistent changes in the activities of the nuclear extracts from our resistant cells. The first is that the protein concentration range over which complete unknotting activity (Figure 2) or maximum catenating activity of pBR322 DNA (not shown) occurs is more restricted with extracts from the resistant cells compared to the sensitive cells. The second is the differences among the preparations in their responses to VM-26. Catalytic activity (Figure 5) and DNA cleavage (Figure 6) are less sensitive to modulation by VM-26 in resistant cell extracts compared to those from sensitive cells. Western blots (Figure 1) show that the differences among the lines are not likely to be due to differences in amounts of topoisomerase II extracted. The difference in rate of catenation by the enzymes suggests differences in the rates of association between topoisomerase II and DNA among the three cell lines. Moreover, if differences in catalytic activity are due to nuclear components other than topoisomerase II itself, the less specific catenating assay might be more likely to distinguish among the cell lines. Both catenating and unknotting assays showed differences between extracts from the sensitive and resistant cells.

The appearance of nicked DNA in the P4 assays in Figures 2, 4, and 5 is noteworthy. The DNA in these bands is associated with protein that is removed by proteinase K treatment, and the formation of the bands is ATP dependent and is affected in a dose-dependent manner by VM-26 (Figures 4 and 5). This suggests that the formation of these topoisomers of DNA is mediated by topoisomerase II. Similar results have been reported by Osheroff (1986). The appearance of this type of DNA, seen over a greater range of protein concentration with the extracts from the resistant cells (Figure 2), could represent a defect in the ability of the enzyme either to cleave the second strand of a double-stranded DNA or to religate the second strand of double-stranded DNA that it has previously cleaved. These data are consistent with the proposal by Zechiedrich et al. (1988) of a sequential mechanism for cleavage and religation of each strand of double-stranded DNA by topoisomerase II. Either defect would result in a nicked form of DNA. Inhibition of religation has been reported by others (Osheroff & Gale, 1988; Chen et al., 1984). A less likely alternative is that an unidentified nicking enzyme, sensitive to VM-26 and covalently bound to DNA, could alter the DNA conformation, making it unsuitable for interaction with topoisomerase II. The apparent inconsistency in the effect of VM-26 of first increasing and then decreasing the formation of nicked DNA (Figures 3 and 4) may be a reflection of the overall mechanism of action of the drug. For example, while it is not known whether the increase in cleavage is due to drug stimulation of cleavage or to inhibition of religation or both, it is clear that less circular DNA remains intact. We speculate that at low VM-26 concentrations single-stranded cleavage (nicked DNA) occurs but at higher drug concentrations the equilibrium shifts toward double-stranded cleavage.

Alterations in the binding of topoisomerase II to DNA could also account for the differences in enzyme activities among the three cell lines. The results of the DNA cleavage assays (Figure 6) support this hypothesis. It is likely that an equilibrium exists between free enzyme, enzyme bound to DNA, and cleaved DNA. We postulate that in the presence of VM-26 the equilibrium is shifted to favor cleaved DNA. It is clear from Figure 6 that in the presence of VM-26 less cleaved DNA is formed by nuclear extracts from both resistant lines. It is possible that less DNA–enzyme binding occurs, thereby limiting the number of complexes available for stabilization and cleavage in the presence of VM-26. Since the drug targets (i.e., the number of bound enzymes) are effectively lowered in the extracts from resistant cells, the effect of the VM-26 is diminished. An alternative postulate is that equal amounts of enzyme from each of the three cell lines bind to DNA, but because of differences in the nature of this binding, VM-26 preferentially increases the stability of the complexes formed with the enzyme from the VM-sensitive cells; consequently, more cleavage is seen with the CEM extracts.

Clearly, the number of stable complexes is influenced both by VM-26 alone and, to an even greater extent, by the combination of VM-26 and ATP (cf. parts A and B of Figure 6). It is of interest that although the catalytic activity of DNA topoisomerase II is ATP dependent (Figures 2–5), ATP alone does not alter enzyme–DNA binding/DNA cleavage equilibrium in the absence of VM-26 (cf. 0 μM VM-26, parts A and B of Figure 6). We do not know whether the decreased binding seen with VM-26 with the extracts from the resistant cells is due to an alteration in the enzyme itself or to a change in a postulated modulator of the enzyme (Glisson et al., 1986; Pommier et al., 1986). What is clear is that fewer enzyme–

[2] With reference to the terminology used in this paper to describe the at-MDR phenotype of the VM-26-resistant lines, we suggest the following. It is becoming increasingly apparent that more than one mechanism for MDR exist. Prior to submission of the manuscript, we felt that we had insufficient data to state that "atypical" MDR, or at-MDR, was associated with altered DNA topoisomerase II activity. We now feel that we can state this with some certainty and suggest that at-MDR be an abbreviation to denote resistance to multiple natural product drugs associated with *a*ltered *t*opoisomerase II activity. Abbreviations for the other types of MDR could also be chosen so that they describe a particular mechanism of resistance. With this system, "classic" MDR (Danks et al., 1987; Moscow & Cowan, 1988; Beck et al., 1987, 1988) could be adequately and meaningfully abbreviated Pgp-MDR, i.e., that form associated with P-glycoprotein overexpression and decreased accumulation of drug. As other mechanisms for MDR are identified, suitable abbreviations could be easily designated.

DNA interactions can be converted to stable complexes under the conditions used. We note that maximum binding is probably not necessary for strand passing activity since catenation of pBR322 readily occurs at extract/DNA ratios (Figure 3; protein:DNA::5:1) lower than those required to saturate binding sites that can form stable complexes (Figure 6B; protein:DNA::7.5:1). Studies are currently under way in our laboratories to determine the basis for the observed alterations in catalytic activity and DNA cleavage.

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Registry No. VM-26, 29767-20-2; DNA topoisomerase, 80449-01-0.

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