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FURTHER CHARACTERIZATION OF AN AMSACRINE-RESISTANT LINE OF HL-60 HUMAN LEUKEMIA CELLS AND ITS TOPOISOMERASE II

EFFECTS OF ATP CONCENTRATION, ANION CONCENTRATION, AND THE THREE-DIMENSIONAL STRUCTURE OF THE DNA TARGET

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Abstract—The characterization of type II topoisomerases from amsacrine-sensitive (HL-60) and amsacrine-resistant (HL-60/AMSA) human leukemia cells was extended. The intercalator resistance and etoposide sensitivity of the HL-60/AMSA cells themselves were confirmed, and the stability of this pharmacologic phenotype over many hundreds of cell generations was demonstrated. Prolonging exposure of HL-60/AMSA cells to amsacrine did not alter their sensitivity relative to that of HL-60 cells. Improved methods of immunoblotting allowed clear demonstration that the topoisomerase II *within these cells* exhibited sensitivity and resistance characteristics that mirrored those of the cells and the isolated enzymes themselves. Additional biochemical characterization of the type II topoisomerases indicated that both enzymes relaxed supercoiled DNA in a distributive fashion and that the ATP concentrations at which optimal catalytic activity of the two enzymes was exhibited were identical. The enzymes differed, however, in their activity optima in buffers of various type and ionic strength. Furthermore, the inability of the HL-60/AMSA enzyme to exhibit enhanced DNA cleavage in the presence of amsacrine could be overcome if the DNA target molecule contained a bend cloned into its polylinker region. By contrast, a bend in a DNA plasmid containing no polylinker was resistant to amsacrine-enhanced cleavage in the presence of HL-60/AMSA topoisomerase II, as was a plasmid containing a polylinker with no bend. This suggests that an unusual DNA conformation (a bend) in a specific DNA context (a polylinker) may be a favored site for topoisomerase II action. It also suggests a mechanism by which the sites and extent of topoisomerase II activity can be controlled in cells.

Topoisomerase II is an enzyme essential for cellular division [1]. It is also a major target for a variety of active antineoplastic drugs, of which most, but not all, are DNA intercalating agents [2]. The precise biochemical mechanism by which the drugs inhibit the enzyme is not clear, but it appears to reside in the ability of the drug to inhibit the religation of the DNA cleavage produced by topoisomerase II as it performs its essential task of passing double strands of DNA through enzyme-induced gaps in other DNA double strands [3]. The signature of this drug-induced inhibition is the production of protein-associated DNA breaks. These breaks represent stabilization of the topoisomerase II–DNA complex by the drugs [4].

Drug-resistant forms of topoisomerase II have been isolated [5–8], and the genes for these aberrant forms of the enzyme have been cloned [9–12] from mammalian cell lines exhibiting resistance to topoisomerase II-reactive drugs. One such line is the HL-60/AMSA line of Beran and Andersson [13]. Our laboratory has characterized the topoisomerase II from these cells [8, 14] as well as the gene coding for it [9]. This report completes that characterization.

HL-60/AMSA topoisomerase II differs from the resistant enzyme described by Danks *et al.* [15] in that the former *does not* appear to differ from that purified from its sensitive parent line (HL-60) in the magnitude of its requirement for ATP to support strand passage. Furthermore, the resistance of HL-60/AMSA to drug-induced stabilization in a complex with DNA is limited to topoisomerase II-reactive DNA intercalators and does not include the topoisomerase II-reactive nonintercalator etoposide [14]. The intercalator-resistant enzyme displays a different spectrum of activities in salt solutions selected to reproduce the internal environment of cells. The use of specific, unusual DNA forms as targets of the enzyme in biochemical assays can overcome the inability of amsacrine (an intercalator) to induce cleavage by HL-60/AMSA topoisomerase II. As a result of this finding, we offer a testable hypothesis to explain the resistance of HL-60/AMSA topoisomerase II to intercalators as well as a more encompassing theory on how intercalator, topoisomerase II, and DNA collaborate to initiate processes that ultimately lead to the demise of the cell.

MATERIALS AND METHODS

Cellular studies

Cells. HL-60 and HL-60/AMSA human leukemia

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cells were a gift from Drs. M. Beran and B. Andersson, Department of Hematology, The University of Texas M.D. Anderson Cancer Center, Houston, TX [13]. The cells were grown in Iscove's modified Dulbecco's medium, obtained from JRH Biosciences (Lenexa, KS), and 10% fetal bovine serum at 37° in 5% CO_2 as previously described [8]. All cells were free of Mycoplasma (as screened by the American Type Culture Collection, Rockville, MD).

DNA. Kinetoplast DNA (kDNA*) was isolated from *Crithidia fasciculata* by incubating kDNA-containing trypanosomes with [*methyl*-^{3}H]thymidine and separating the DNA from Sarkosyl lysates using cesium chloride-ethidium bromide density centrifugation [8]. Supercoiled SV40 DNA was purchased from Gibco-BRL Life Technologies, Inc. (Gaithersburg, MD). In sodium dodecyl sulfate (SDS)-KCl precipitation assays, SV40 DNA was linearized with *Eco*RI prior to 3′ end-labeling with [^{32}P]ATP (see below).

pSP65 was obtained from Promega Biotec (Madison, WI); pPK201/CAT was a gift from Dr. Paul Englund, The Johns Hopkins School of Medicine, and pBluescript II S/K$^+$ was a gift from Dr. Ji Chen, The University of Texas M.D. Anderson Cancer Center. pSP65 and pBluescript II S/K$^+$, which contain multiple cloning sites (MCS), were linearized with *Ava*II prior to 3′ end-labeling with [^{32}P]ATP. ^{32}P-Labeled pBluescript II S/K$^+$ was further restricted with *Bgl*I to remove one of the 3′ ends. pPK201/CAT, a pSP65-derived plasmid with a 219 bp containing a 211 bp *C. fasciculata* bend inserted into the *Bam*HI site of its MCS, was restricted with the *Ava*II isoschizomer, *Sin*I. The DNA insert of pPK201/CAT bends because of 18 runs of 4–6 adenine residues [16].

pBR322 was purchased from the Stratagene Co. (La Jolla, CA). pBR322 bent plasmid was produced by separating the 219 bp insert from pPK201/CAT and ligating it into the *Bam*HI site of pBR322 followed by transformation into Epicurean coli XL-1 Blue (Stratagene). Colonies containing the bent insert were identified by filter hybridization using a ^{32}P-labeled insert as a probe. Additionally, positive colonies were tested for the presence of the insert by examining the electrophoretic mobility of the linearized plasmid in polyacrylamide gels [16]. [^{32}P]-ATP and [^{32}P]TTP were used to 3′ end-label pBR322 and pBR322 bent DNA *Nde*I restriction fragments. Digestion of the fragments with *Afl*III produced DNA with only one labeled 3′ end.

Drugs. Amsacrine [4′-(9-acridinylamino)methanesulfon-*m*-anisidide] was obtained from the National Cancer Institute. Etoposide was a gift from Drs. Byron Long or James H. Keller of the Bristol-Myers Co. Both drugs were solubilized in dimethyl sulfoxide (DMSO).

Soft agar colony formation. The soft agar method of Chu and Fisher [17] was employed to assess the stability of the resistance exhibited by HL-60/AMSA cells to amsacrine. HL-60 and HL-60/AMSA cells from various passage generations were treated with DMSO, amsacrine, or etoposide for 1 hr at 37° before being washed free of drug.

Immunoblotting-band depletion. A modification of the method of Kaufmann *et al.* [18] was used to immunologically identify topoisomerase II that was cross-linked to DNA from HL-60 and HL-60/AMSA cells. Cells (5×10^5) from each cell line were incubated with various concentrations of amsacrine or etoposide for 30 min at 37°, followed by sonication with 40 bursts at 60% power (Heat Systems-Ultrasonics, Inc. sonicator) in alkylation buffer (6 M guanidine-HCl, 250 mM Tris (pH 8.5), 10 mM Na_2 EDTA) with 1% 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride added. The reactions were allowed to reduce overnight, after which 100 μL of 1.5 M iodoacetamide in alkylation buffer was added to each 1.02-mL sample and incubated for 1 hr at room temperature; then, 10 μL of 2-mercaptoethanol was added. Each sample was dialyzed once for 90 min against 4 M urea, 50 mM Tris, pH 7.4; four times for 90 min each against 4 M urea; three times for 90 min each against 0.1% SDS; and then was lyophilized for storage. Samples were solubilized in SDS sample buffer [4 M urea, 2% SDS, 62.5 mM Tris–HCl (pH 6.8), 1 mM Na_2 EDTA] and electrophoresed in a 7.5% polyacrylamide gel.

Immunoblotting was otherwise performed as previously described [8] using a 1:500 dilution of anti-human topoisomerase II polyclonal antibody obtained from Dr. Leroy Liu, the Johns Hopkins School of Medicine. ^{125}I-Labeled protein A (Amersham Corp., Arlington Heights, IL) was utilized to detect the antibody complex. Bovine serum albumin (3%) was used to block nonspecific binding. Nitrocellulose strips containing the ^{125}I-labeled topoisomerase II–antibody complex were quantified with a gamma counter.

SDS-KCl precipitation assay. Cells (4×10^5) from each cell line were radiolabeled with [^{3}H]thymidine and [^{14}C]leucine and then incubated at 37° with DMSO or with various concentrations of etoposide for 1 hr. DNA–protein complexes were precipitated from HL-60 and HL-60/AMSA cells using a modification of previously reported methods (see Ref. 8). The method of Hsiang and Liu [19] was employed to examine the rate of topoisomerase II-mediated DNA religation. Prior to lysis, the cells were incubated for 0–40 min at 65°.

Biochemical studies

Topoisomerase II purification. Topoisomerase II was purified from HL-60 or HL-60/AMSA twice (preparations 1 and 2). Cells ($9.6–14.8 \times 10^9$) were used to isolate nuclei in nucleus buffer [150 mM NaCl, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetra-acetic acid (EGTA), 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, pH 6.4] [20] for each purification. These nuclei were extracted with nucleus buffer containing 0.35 M NaCl for 30 min, and then centrifuged at 100,000 *g*. The supernatants were saved for further purification by sequential column chromatography.

* Abbreviations: kDNA, kinetoplast DNA; MCS, multiple cloning site(s); DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; FSP, fraction of strands passed; and F kDNA, fraction of kDNA remaining in the well of a gel.

Table 1. Stability of the sensitivity or resistance of HL-60 and HL-60/AMSA to amsacrine or etoposide

Passage number	HL-60		HL-60/AMSA		Ratio of slopes*
		Amsacrine			
	1-log†	1 μM‡	1-log†	50 μM‡	
132	0.91	0.08	130.6	0.42	147
160	1.02	0.12	131.0	0.41	141
> 400	0.81	0.05	118.0	0.38	145
> 500	0.96	0.09	77.1	0.21	68
		Etoposide			
	1-log†	10 μM‡	1-log†	20 μM‡	
132	6.5	0.03	17.8	0.04	3.5
160	14.9	0.1	27.6	0.13	2
> 400	6.3	0.02	31.6	0.13	4.8
> 500	9.2	0.07	23.5	0.07	3.1

* Ratio of the slope of the survival curve of HL-60 divided by the slope of the survival curve of HL-60/AMSA using three concentrations of each agent. This is taken as the magnitude of the drug resistance of HL-60/AMSA.

† The amount of drug (μM) that produced a 1-log reduction in survival following a 1-hr exposure at 37°.

‡ The colony-forming efficiency produced by the indicated concentration of amsacrine or etoposide.

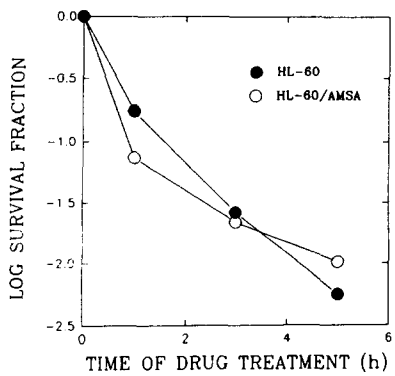


Fig. 1. Reduction in colony-forming ability produced by prolonged exposure of HL-60 or HL-60/AMSA cells to amsacrine. Exponentially growing cells were treated with either 1 μM (HL-60) or 50 μM (HL-60/AMSA) amsacrine for 1, 3, or 5 hr, and the reduction in survival fraction was quantified using soft agar colony-formation assays (see Materials and Methods).

Topoisomerase II was purified from nuclear extracts by the method of Drake *et al.* [7], as described by Sullivan *et al.* [6]. Each extract was passed through hydroxylapatite, and proteins were eluted using an increasing salt gradient. Fractions that contained topoisomerase II were identified by decatenation of kDNA using gel electrophoresis (see below). Topoisomerase II from the active hydroxylapatite fractions was passed through and eluted from phenyl-Sepharose by a decreasing salt gradient. Then, an increasing salt gradient was used with either Q-Separose (Preparation 1) or Mono Q (Preparation 2) for final purification. Topoisomerase II purified from HL-60 and HL-60/AMSA cells was used for the five biochemical assays described below.

Decatenation of kinetoplast DNA. Approximately 0.22 μg of ^{3}H-labeled kDNA was used to assess topoisomerase II activity [21, 22]. The standard reaction buffer contained 50 mM Tris, 85 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM Na_2EDTA, 0.03 mg/mL bovine serum albumin, and 1 mM ATP, pH 7.6.

The effect of anion type and concentration on topoisomerase II activity was evaluated using standard decatenation buffer substituted with the indicated concentrations of potassium glutamate [23].

The responsiveness of topoisomerase II to changes in ATP concentration was determined using a standard buffer containing differing amounts of ATP. The reactants were incubated for 30 min at 37°, after which SDS and proteinase K were added to final concentrations of 1% and 100 μg/mL, respectively. The reaction products were resolved by 1% agarose gel electrophoresis in 89 mM Tris-borate buffer containing 0.5 μg/mL ethidium bromide. Ultraviolet light was utilized for DNA visualization. Catenated DNA (fraction in well) and decatenated DNA (fraction that entered gel) were excised, melted in scintillation vials, and quantified by liquid scintillation spectroscopy. One unit of topoisomerase II was defined as the amount of enzyme required to decatenate 50% of 0.22 μg of kDNA in 30 min at 37°. The fraction of strands passed (FSP) was calculated as follows:

$$FSP = \frac{F\,kDNA\,(no\ protein) - F\,kDNA\,(protein)}{F\,kDNA\,(no\ protein)}$$

where F kDNA is the fraction of kDNA remaining in the well in the presence (protein) or absence (no protein) of topoisomerase II.

ATP-dependent DNA relaxation. Topoisomerase II was incubated with 0.2 μg of closed circular, supercoiled SV40 DNA in a relaxation buffer containing 10 mM Tris, 50 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM Na_2EDTA, and 1 mM ATP, pH 7.4, for 0–30 min at 37°. Reactions were terminated with 1% SDS and 100 μg/mL proteinase K. Reaction products were separated on 1% agarose gels. DNA was visualized with ultraviolet light following staining with 0.5 μg/mL ethidium bromide.

Drug-induced DNA cleavage. SV40 DNA (0.2 μg) was used as a substrate for cleavage by topoisomerase II. Reactions were performed in relaxation buffer (see above) in the absence and in the presence of 1 mM ATP for 30 min at 37° with DMSO, amsacrine, or etoposide. Reactions were stopped with SDS and proteinase K and the products separated by agarose gel electrophoresis as previously described [8].

A modification of the method of Hsiang and Liu [19] was used to assess the rate of reversibility of DNA–topoisomerase II complexes in purified systems. Topoisomerase II was incubated with SV40 DNA in the presence of DMSO or 100 μM etoposide for 30 min at 37° as described above. Prior to termination of the reactions with SDS and proteinase K, reactants were incubated at 65° for 0–120 sec. Then the reaction products were analyzed using agarose gels containing 0.5 μg/mL ethidium bromide. DNA was visualized with ultraviolet light and photographed with Polaroid type 55 film. DNA bands were quantified by scanning densitometry.

Drug-induced DNA–protein cross-linking. The SDS-KCl precipitation method [21] was used to quantify topoisomerase II–DNA complexes utilizing uniquely 3′ end-labeled [^{32}P]SV40 DNA. Results were expressed as the counts per min (cpm) of [^{32}P]-DNA precipitated in the presence of drug minus that precipitated in the absence of drug. As in the DNA cleavage assays, reaction products of SDS-KCl DNA–protein complex reversal studies were shifted to 65° for 0–120 sec before SDS denaturation to quantify DNA–protein complex reversal rates.

DNA conformation. To determine the role of DNA conformation on the ability of HL-60 or HL-60/AMSA topoisomerase II to induce the formation of protein-associated DNA cleavage, purified enzyme from each cell line was incubated with a minimum of 10,000 cpm of ^{32}P-labeled pSP65 (non-bent) or pPK201/CAT (bent) linearized fragments (see above) for 30 min at 37° in standard relaxation buffer. The reactions were terminated with 1% SDS, and KCl was added to a final concentration of 80 mM. Each sample was heated to 65° for 10 min, precipitated at 4° for 10 min, and spun at 12,000 *g* for 10 min. After discarding the supernatant, each pellet was resuspended in 50 μL of 0.5 mg/mL proteinase K and incubated at room temperature for 30 min. This was followed by the addition of another 5 μL of 5 mg/mL proteinase K. The samples were allowed to incubate overnight at 37°. Reaction products were separated on 1% agarose gels at 40 V. The gels were subsequently fixed in three changes of 300 mL methanol and dried onto plastic film; autoradiography was then performed.

To distinguish the influence of bent DNA from that of the surrounding MCS on topoisomerase II-mediated DNA cleavage, ^{32}P-labeled DNA fragments from pBluescript II S/K$^+$ (containing MCS without a DNA bend), pBR322 (no MCS and no bend), and pBR322 bent DNA (no MCS but containing a bend) were also incubated with topoisomerase II and the cleavage was visualized as described above.

RESULTS AND DISCUSSION

Cellular studies

The resistance exhibited by HL-60/AMSA cells to amsacrine appeared to be stable over a number of cell passages (Table 1). Compared with this cell line's small degree of resistance to etoposide, its resistance to amsacrine ranged from approximately 30- (passage greater than 400) to 70-fold (passage 160). Prolonging the exposure of HL-60/AMSA cells to amsacrine did not appear to decrease their resistance to this drug (Fig. 1). Resistance actually increased from 21- to 89-fold when incubation time was lengthened from 1 to 5 hr.

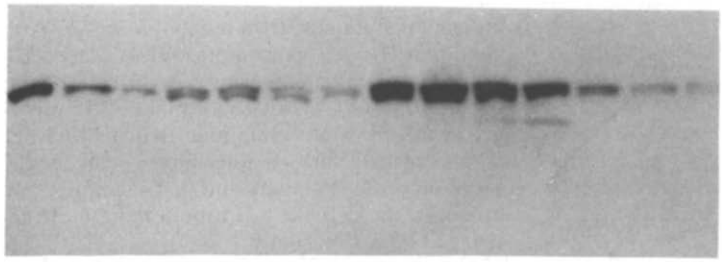
Immunoblotting-band depletion can be used to identify the participation of topoisomerase II in the production of drug-induced, DNA–protein complexes *in cells* and to quantify this effect. In cells containing drug-sensitive topoisomerase II, drug treatment followed by detergent lysis causes the enzyme to covalently bind to the cellular DNA, which is too large to enter an SDS–PAGE system. The DNA thus depletes the topoisomerase II from the gel and prevents it from being detected when the gel is incubated with monospecific antihuman topoisomerase II antibody [8]. Using the novel immunoblotting technique of Kaufmann *et al.* ([18] and Materials and Methods), we detected three bands of 164, 161, and 151 kDa (Fig. 2). Previous data had suggested that these bands are probably proteolytic breakdown products of the 170-kDa form of topoisomerase II [8].

Fewer DNA–protein cross-links were formed in HL-60/AMSA cells treated with amsacrine than in HL-60 cells treated with amsacrine, as little band depletion was noted in lanes containing lysates from amsacrine-treated HL-60/AMSA cells. In contrast, etoposide *did* produce band depletion in HL-60/AMSA, as expected. Thus, our previous findings [8] were confirmed using this newer assay.

Biochemical studies

DNA strand passage. Distributive relaxation of supercoiled DNA has been associated with the p170 form of topoisomerase II from P388 cells [7]. When supercoiled DNA is relaxed in a distributive (as opposed to a processive) manner, topoisomers of intermediate superhelicity are formed [7]. ATP-dependent relaxation of supercoiled SV40 DNA by HL-60 or HL-60/AMSA topoisomerase II appeared to be distributive (Fig. 3). In both cell lines, formation of topoisomers occurred by 2 min of incubation at 37°, with increased accumulation of intermediate forms over 30 min of incubation.

ATP is required for the catalytic activity of topoisomerase II and enhances enzyme-induced DNA cleavage [8, 24]. ATP (0.03 to 1 mM) enhanced decatenation of [^{3}H]kDNA by both topoisomerases (Fig. 4). No difference was found between the two



D	A			V			D	A			V			Treatment
	1_0	5_0	10_0	5_0	10_0	20_0		1_0	5_0	10_0	5_0	10_0	20_0	Drug Concentration (μM)
60_3	36_2	16_3	35_1	35_9	23_8	19_0	70_5	97_5	78_4	67_8	32_6	20_2	19_0	cpm
100	60	27	58	60	39	32	100	138	111	96	46	29	27	% Control
HL-60							HL-60/AMSA							

D = DMSO vehicle control
A = Amsacrine
V = Etoposide

Fig. 2. Immunoblotting-band depletion using the method of Kaufmann *et al.* [18]. HL-60 or HL-60/AMSA cells (5×10^5) were incubated with the indicated concentrations of amsacrine (A) or etoposide (V) for 30 min at 37° or with DMSO vehicle control (D). Samples were prepared as described in Materials and Methods before electrophoresis in a 7.5% SDS–PAGE system and transfer to nitrocellulose. Immunoblotting was performed using an anti-human topoisomerase II polyclonal antibody. The numbers to the right of the gel indicate the migration distance of markers of those molecular weights. The cpm numbers were obtained by gamma counting of the nitrocellulose strips containing ^{125}I-labeled protein A and anti-topoisomerase II antibody.

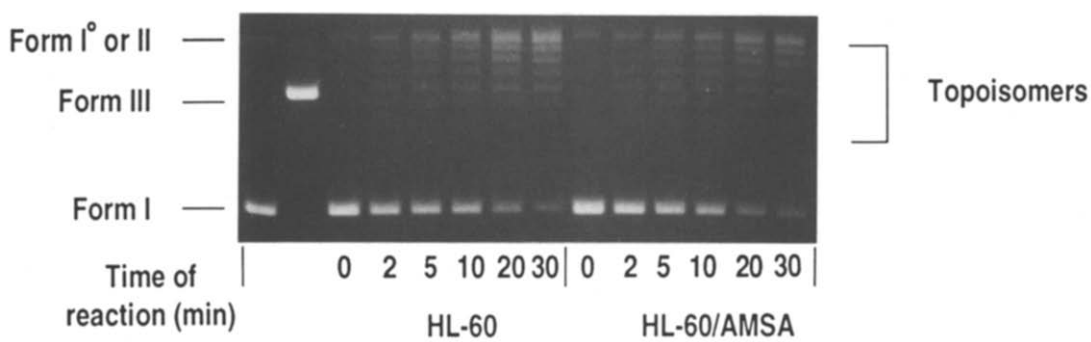


Fig. 3. ATP-dependent relaxation of DNA by purified topoisomerase II from HL-60 and HL-60/AMSA cells. Approximately equal amounts of topoisomerase II activity (100 units) from each cell line were incubated with 0.2 μg closed circular SV40 DNA in a buffer containing 1 mM ATP for the various times as indicated in the figure at 37°. Reactions were terminated with 1% SDS and 100 μg/mL proteinase K. Reaction products were resolved using an agarose gel electrophoresis system as described in Materials and Methods. Form I is closed circular, supercoiled DNA. Form I° is relaxed DNA. Form II is nicked DNA. Form III is linearized DNA.

enzymes in their response to increasing ATP concentrations. This contrasts with the results obtained using type II topoisomerases from another sensitive/resistant cell pair in which the ATP responsiveness of the resistant enzyme was diminished [15].

Further characterization of HL-60/AMSA topoisomerase II purified using the method of Drake *et al.* [7] was performed. The effect of potassium chloride and potassium glutamate concentration on the strand-passing ability of the enzymes from HL-60 and HL-60/AMSA was quantified as we had

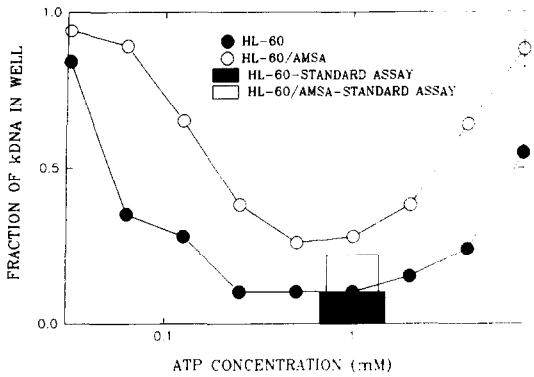


Fig. 4. Effect of ATP concentration on the catalytic activity of topoisomerase II from HL-60 and HL-60/AMSA cells. kDNA (0.22 μg) was incubated with a quantity of topoisomerase II that would result in approximately 90% of the kDNA remaining catenated during a 30-min incubation at 37° in decatenation buffer containing 0.03 mM ATP. All other reactions were performed using the same buffer except that the ATP concentration varied from 0.03 to 8 mM. Data points represent means of the fraction of kDNA that remained as catenated starting material for two experiments. The fraction of catenated kDNA remaining using topoisomerase II under standard 1 mM ATP conditions is indicated by the bars (solid = HL-60; open = HL-60/AMSA).

previously done with topoisomerases purified by a different method [8, 23]. As in our previous work, results revealed a difference between the type II enzymes (Fig. 5). Topoisomerase II from HL-60/AMSA cells produced greater decatenation in potassium glutamate than did that from HL-60 cells. Note that HL-60 topoisomerase II was *less* active in potassium glutamate than in KCl at all concentrations but that HL-60/AMSA topoisomerase II was *more* active in potassium glutamate than in KCl. These data suggest that anion fluxes can influence topoisomerase II activity and that such fluxes would influence the activities of topoisomerases from the two cell lines differently.

Drug-induced DNA–topoisomerase II complex stabilization. Previously, using the method of Shelton *et al.* [25] as modified by Osheroff [24] to purify topoisomerase II, we demonstrated that HL-60 topoisomerase II differs from HL-60/AMSA topoisomerase II in its ability to form a stable complex with DNA in the presence of amsacrine [8]. Thus, resistance of HL-60/AMSA cells to amsacrine appeared to result from the presence of a resistant form of topoisomerase II. Because HL-60/AMSA cells and HL-60/AMSA topoisomerase II were relatively sensitive to etoposide, we could examine the reversal of the effects of this drug in both cell lines and in isolated biochemical systems. The rate of etoposide-induced topoisomerase II-mediated DNA complex reversal was comparable in intact cells ([18] and Materials and Methods). Furthermore, reversal of complexes produced in a biochemical system using purified enzyme, DNA and etoposide also revealed no differences in the reversal rates using HL-60 versus HL-60/AMSA topoisomerase II (data not shown). These results suggest that the resistance of HL-60/AMSA to intercalators is *not* due to some intrinsic property of HL-60/AMSA topoisomerase II to religate more rapidly than HL-60 topoisomerase II does.

DNA conformation. Previous studies have examined various intercalating and non-intercalating drugs

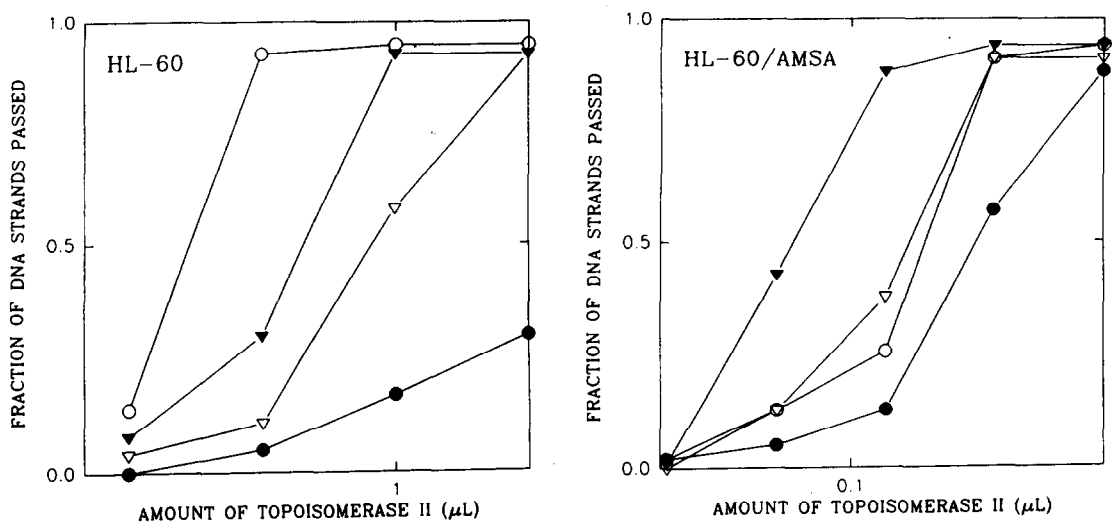


Fig. 5. Effect of anion type and concentration on the catalytic activity of topoisomerase II purified from HL-60 or HL-60/AMSA cells. kDNA (0.22 μg) was incubated in decatenation buffer containing 85 mM KCl (○) or potassium glutamate at 110 mM (●), 150 mM (▽), or 175 mM (▼) for 30 min at 37° with purified topoisomerase II as described in Materials and Methods. Approximately equal topoisomerase II decatenating activity in 85 mM KCl was used per reaction.

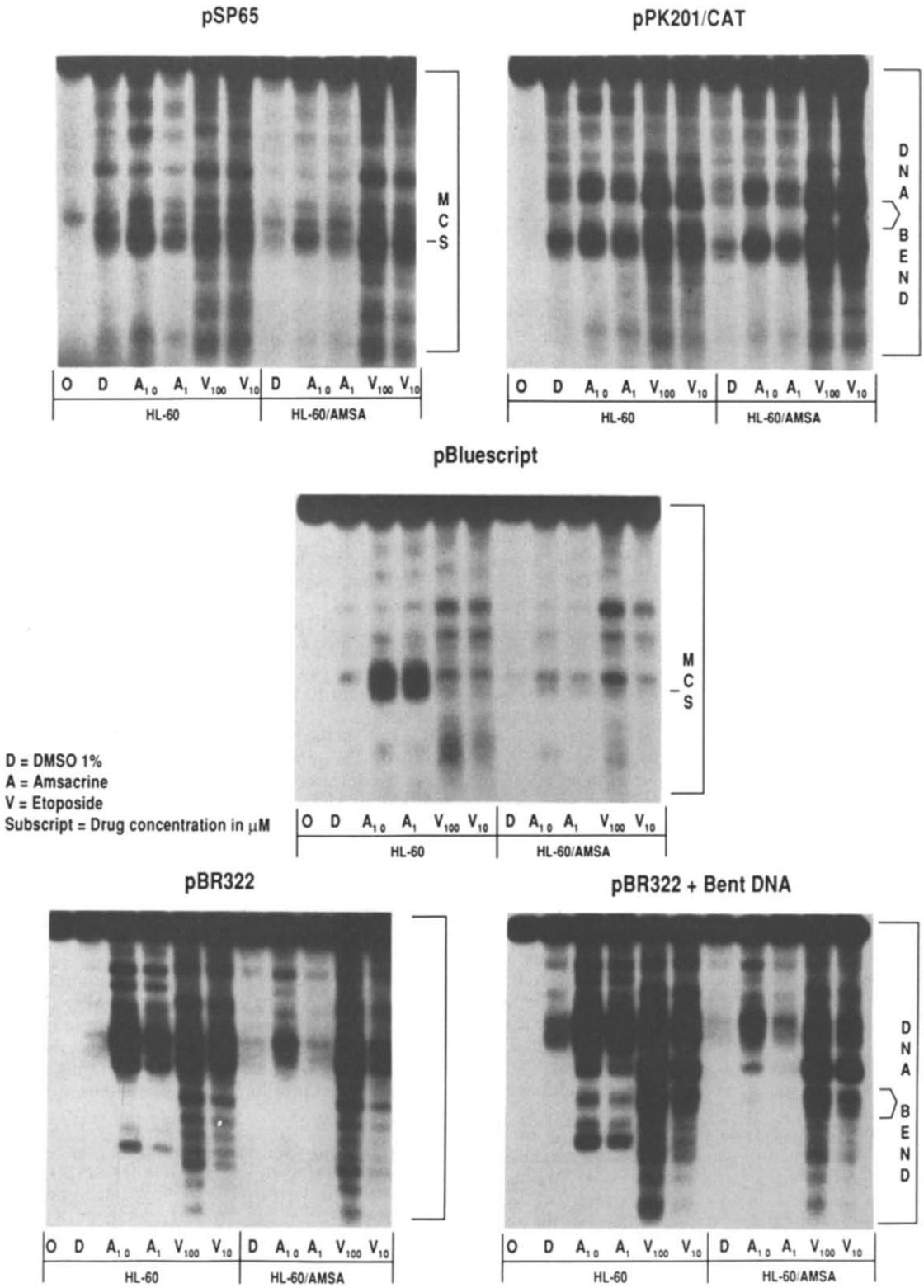


Fig. 6. Effect of DNA conformation on the production of drug-induced DNA cleavage. Purified topoisomerase II from HL-60 and HL-60/AMSA cells was incubated with uniquely 3′ ^{32}P-labeled pSP65 (top left), pPK201/CAT (top right), pBluescript (middle), pBR322 (bottom left), or pBR322 + bent DNA (bottom right) (see Materials and Methods) linearized DNA for 30 min at 37° in standard relaxation buffer, as described in Materials and Methods in the presence of DMSO (D), amsacrine (A), or etoposide (V) at the concentrations indicated by the subscripts. The amount of enzyme used in each experiment was that which would produce approximately equal DNA–protein cross-linking in the presence of 100 μM etoposide in SDS-KCl protein precipitation assays. 0 is no enzyme.

and various cell line sources of topoisomerase II in order to identify critical determinants of topoisomerase II-mediated DNA cleavage. The third component of the cleavable complex, DNA, is rarely more than an indicator molecule and at most is used to study the sequence specificity of individual drug action (see, for example, Refs. 26 and 27). But topoisomerase II alters DNA *three*-dimensional structure. While sequence recognition may play a role in the actions of the enzyme, recognition of larger three-dimensional structural determinants may be of equal or greater importance to the site and degree of topoisomerase II action.

Bent DNA has been shown to be a favored target of topoisomerase II action. The enzyme binds at the junction of the bent and unbent DNA [28]. Furthermore the DNA $\gamma\delta$ resolvase that produces recombination via DNA bending also promotes intercalation [29]. Thus, topoisomerase II not only favors bent DNA but if it bends DNA itself, as several DNA-binding proteins like the resolvase do [30], it also could promote DNA binding of the very drugs that inhibit its function (i.e. intercalating agents).

We have begun to study whether DNA bending plays a role in topoisomerase II-reactive drug action. Initially we wondered whether bent DNA could overcome the inability of HL-60/AMSA topoisomerase II to cleave DNA in the presence of amsacrine. To determine whether the presence of a bent DNA structure can facilitate the stable formation of amsacrine-induced DNA cleavage (relative to etoposide-induced DNA cleavage) using HL-60/AMSA topoisomerase II, linearized ^{32}P-labeled pSP65, pPK201/CAT, pBluescript II S/K$^+$, pBR322, and pBR322 bent DNA fragments (see Materials and Methods) were incubated with DMSO, amsacrine, or etoposide (Fig. 6). HL-60 topoisomerase II produced an increase in DNA cleavage for all DNA fragments exposed to amsacrine or etoposide compared with the DMSO controls. However, a large increase in cleavage was apparent in HL-60/AMSA topoisomerase II reactions containing both concentrations of amsacrine *only* when the DNA substrate contained the 211 bp kDNA bent fragment in the MCS of pPK201/CAT. Incubation with pBR322 bent DNA, which contained the same kDNA bent insert (but not in an MCS), failed to produce the same result.

This strongly indicated that DNA is not a passive participant in drug-induced, topoisomerase II-mediated DNA cleavage but rather that its three-dimensional structure can influence the magnitude of this reaction. Three-dimensional structural changes can be secondary to DNA sequence as is the case for the kDNA bend used here, or they can be induced by proteins, which themselves can produce DNA bending.

Topoisomerase II itself could induce DNA bending (like the DNA resolvase described above) and could thus become an unwitting participant in its own demise if the sequence of events leading to drug-induced DNA cleavage was: (1) topoisomerase II-DNA binding, (2) topoisomerase II-induced DNA bending, (3) bending-enhanced drug intercalation, and (4) cleavable complex formation. Hypothesizing further, HL-60/AMSA topoisomerase II could be deficient in the second of these steps as we know that HL-60/AMSA topoisomerase II can bind to and cleave DNA (steps 1 and 4; Figs. 3–6). Thus, HL-60/AMSA topoisomerase II would not promote intercalation and would remain resistant to the actions of intercalating agents unless the DNA was bent by some other factor (e.g. intrinsic curvature; [30]). As etoposide is not an intercalator, this cascade may be of no relevance to the actions of this drug on DNA, a theory supported by the relative sensitivity of HL-60/AMSA topoisomerase II and the cells in which it resides to actions of this class of drug.

But why does the DNA bend have to be within the MCS for HL-60/AMSA topoisomerase II to cleave DNA in the presence of amsacrine? Again, we can only speculate, but the pattern of nicking produced by *C. fasciculata* nicking enzyme appears to be randomly distributed along the DNA molecule except for increased nicking in a 440 bp region adjacent to the bent kDNA of the pSP65 derivative, pPK201/CAT, and a 750 bp region near the kDNA minicircle of pSAL8, a pBR322 derivative [31]. This suggests that the increased nicking seen in these regions may result from structural changes caused by the bent segment on the surrounding DNA sequences [31]. Thus, the bend, its effects on surrounding DNA, or the susceptibility of the surrounding DNA to be influenced by the presence of the bend can alter the susceptibility of the DNA segment to topoisomerase II-mediated DNA cleavage.

Regardless, the present work extends our previous work by examining further biochemical and molecular determinants of topoisomerase II-reactive drug action. The work with the bent DNA provides an alternative to the sequence-based models of site selectivity of topoisomerase and topoisomerase-reactive drug action [27]. We are currently testing whether topoisomerase II itself can bend DNA and whether the HL-60/AMSA form of the enzyme differs from that purified from the HL-60 cells in this regard.

The ability of DNA three-dimensional structure to influence the actions of enzymes that alter DNA topology makes intrinsic sense. Alterations in DNA three-dimensional conformation within cells, due to sequence- or protein-induced curvature, may be a mechanism for controlling the actions of these critical enzymes.

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