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PHORBOL REGULATION OF TOPOISOMERASES I AND II IN HUMAN LEUKEMIA CELLS

STUDIES IN AN ADDITIONAL CELL PAIR SENSITIVE OR RESISTANT TO PHORBOL-INDUCED DIFFERENTIATION

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Abstract—We previously reported (Zwelling *et al.*, *Cancer Res* **50**: 7116–7122, 1990) that etoposide-induced DNA cleavage and mRNA coding for topoisomerase II are reduced in HL-60 cells induced to differentiate by phorbol ester. Reduction of etoposide-induced cleavage and topoisomerase II message did not occur in the derived cell line 1E3 (which is resistant to phorbol-induced differentiation), implying that topoisomerase II activity may be related to the state of cell differentiation. We have extended these studies using a new phorbol sensitive/resistant cell pair, S (sensitive) and PET (phorbol ester tolerant). Phorbol ester exposure not only reduced etoposide-induced DNA cleavage and topoisomerase II mRNA in S cells but also decreased the amount of immunoreactive topoisomerase II enzyme in whole S cells. However, immunoreactive topoisomerase II extracted from the nuclei of phorbol-treated S cells was not reduced compared with that from the nuclei of untreated S cells. This suggests that topoisomerase II contained in nuclear extracts is not always representative of the total cellular enzyme. Dramatic decreases in the amount, activity, or gene expression of topoisomerase II were not observed after phorbol treatment of the resistant PET cells; this is consistent with the potential involvement of topoisomerase II in monocytoid differentiation. Levels of topoisomerase I enzyme and mRNA fell in both S and PET cells after phorbol treatment; therefore, the genes for topoisomerases I and II did not appear to be regulated coordinately.

Key words: topoisomerases, differentiation, gene regulation, phorbol esters, leukemia

Phorbol esters such as phorbol-12-myristate-13-acetate (PMA)§ induce monocytoid differentiation in cultured human leukemia cells (e.g. HL-60) [1, 2], but the exact mechanism by which this occurs is not known. Previous studies in this laboratory have shown that PMA-induced monocytoid differentiation of HL-60 cells was associated with decreased etoposide-induced, topoisomerase II-mediated DNA cleavage [3]. This suggested a role for topoisomerase II in leukemia cell differentiation.

Topoisomerase II is an enzyme responsible for double-strand DNA cleavage and strand passage [4, 5], activities that are necessary for chromosomal segregation [6]. Inhibitors of this enzyme (such as etoposide or amsacrine) prevent topoisomerase II from religating DNA [7].

We investigated the possible association between topoisomerase II activity and monocytoid differentiation [8] using phorbol-responsive HL-60 cells and the derived cell line 1E3, which does not differentiate in the presence of phorbol esters [9]. As predicted, etoposide-induced DNA cleavage was reduced to a greater extent in HL-60 cells than in 1E3 cells when both were pretreated with phorbol ester [8]. In addition, phorbol exposure modestly decreased the amount and activity of topoisomerase II enzyme extracted from isolated nuclei of HL-60 cells without producing a comparable change in 1E3 cells [8]. Despite its modest effect on extractable topoisomerase II, phorbol ester treatment dramatically decreased the amount of HL-60 topoisomerase II mRNA. Once again, phorbol treatment of 1E3 cells did not effect a corresponding decrease [8]. This suggested that the gene for topoisomerase II could be regulated directly or indirectly by phorbol esters and could be a part of the biochemical cascade mediating phorbol-induced differentiation [8].

In the current study, we utilized another phorbol-sensitive and -resistant cell pair, HL-60 S (sensitive) and PET (phorbol ester tolerant), to investigate further the role of topoisomerase II in phorbol-induced cell differentiation. Confirming our experiments in HL-60 and 1E3 cells, we found that phorbol ester treatment decreased etoposide-induced DNA cleavage and topoisomerase II mRNA levels in the S cells but not in the PET cells. Examining *whole cells*, rather than nuclear extracts, for associated

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§ Abbreviations: PMA, phorbol-12-myristate-13-acetate; S, phorbol ester sensitive HL-60 cells; PET, phorbol ester tolerant HL-60 cells; DMSO, dimethyl sulfoxide; kDNA, kinetoplast DNA; PBS, phosphate-buffered saline; PMSF, phenylmethyl sulfonyl fluoride; SDS, sodium dodecyl sulfate; and MOPS, 4-morpholinepropane sulfonic acid.

alterations in immunoreactive topoisomerase II, we found concordance between this measure and etoposide-induced DNA cleavage in whole cells. We inferred that results using nuclear extracts may not be accurate reflections of topoisomerase II in intact cells. Finally, we monitored topoisomerase I mRNA and enzyme levels as well as topoisomerase I activity in S and PET cells before and after PMA treatment to investigate whether the topoisomerase I gene or its product is influenced by phorbol esters or regulated in tandem with the topoisomerase II gene.

MATERIALS AND METHODS

Cells

The HL-60 S cells and PET cells used in these experiments were provided by Dr. Donald Macfarlane, University of Iowa [10]. These cells were grown in Iscove's modified Dulbecco's medium (JRH Biochemicals, Lenexa, KS) with 10% fetal bovine serum at 37° in 5% CO_2 and did not adhere to the culture flask in the absence of phorbol esters. The doubling time for both HL-60 S cells and PET cells was approximately 16 hr. L1210 murine leukemia cells grown in RPMI medium (Gibco, Grand Island, NY) were utilized as internal standard cells for alkaline elution experiments. All cells were Mycoplasma free (American Type Culture Collection, Rockville, MD).

Drugs

PMA was obtained from the LC Services Corp. (Woburn, MA) and constituted as a 10 mM stock solution in 100% dimethyl sulfoxide (DMSO). Amsacrine (NSC249992) was from the National Cancer Institute, and etoposide was a gift from Drs. Byron Long and James H. Keller of the Bristol-Myers Squibb Co. (Wallingford, CT). Amsacrine (10 mM) and etoposide (10 mM) were dissolved in 100% DMSO and stored at −20°. When cells were treated with drugs dissolved in DMSO, the final concentration of DMSO in the medium was always 0.1%. Topotecan, obtained from SmithKline Beecham (King of Prussia, PA), was prepared in deionized water at a concentration of 10 mM and stored at −20°.

DNA substrates

Kinetoplast DNA (kDNA) utilized in the decatenation assay was isolated from *Crithidia fasciculata* grown in [*methyl*-^{3}H]thymidine (NEN-Dupont, Boston, MA) as previously described [11–13]. The radiolabeled kDNA was isolated from Sarkosyl extracts using cesium chloride–ethidium bromide centrifugation.

Covalently closed, supercoiled SV40 DNA was purchased from Bethesda Research Laboratories (Gaithersburg, MD). SV40 DNA was linearized with *Eco*RI and 3′ end-labeled with [^{32}P]ATP (Amersham, Arlington Heights, IL) [14] for use in assessing DNA–protein complexes via the sodium dodecyl sulfate (SDS)–KCl precipitation method described below [14, 15].

Studies with intact cells

Soft agar colony formation assays were performed by the method of Chu and Fisher [16] after cells were treated for 1 hr with amsacrine, etoposide, or Topotecan. S cells and PET cells that adhered to the flask after PMA treatment were removed by incubation with phosphate-buffered saline (PBS)/0.02% tetrasodium EDTA at 37° for 5–10 min after exposure to drug, before soft agar cloning. For all other assays, adherent cells were removed from the flasks by gentle scraping.

Drug-induced DNA cleavage was quantified after a 1-hr treatment utilizing alkaline elution [17]. HL-60 S cells and PET cells were incubated with 0.05 μCi/mL [2-^{14}C]thymidine (NEN-Dupont) for 48 hr to radiolabel their DNA and then incubated for 24 hr in fresh, non-radioactive medium to allow radiolabeled replication intermediates to be ligated into full-length DNA. L1210 cells utilized as an internal standard were labeled with 0.1 μCi/mL of [*methyl*-^{3}H]thymidine overnight and incubated in isotope-free medium for at least 2 hr before use.

Cell-cycle analysis was performed via flow cytometry using an EPIC 742 instrument. Cells were treated with propidium iodide and 0.05% Nonidet P-40 with 100 U/mL RNase before analysis.

Assays of the expression of Mol on the surface of cells were performed as described previously, using flow cytometry [18].

Studies with nuclear extracts

Nuclei were isolated as previously described [19, 20], and extracts were made using 0.35 M NaCl nucleus buffer [21] including the following protease and phosphatase inhibitors: 50 μg/mL leupeptin, 10 μg/mL pepstatin, 10 μg/mL chymostatin, 10 μg/mL antipain, 10 μg/mL aprotinin (all from Boehringer-Mannheim, Indianapolis, IN), 5 mM glucose 1-phosphate, 5 mM AMP, and 1 mM phenylmethyl sulfonyl fluoride (PMSF) (all from Sigma, St. Louis, MO) [8].

The following experiments were performed on the products of two independent extractions of nuclei from each cell line. Results using nuclear extracts from the independent extractions were comparable. The data presented are from experiments using one of these extracts.

Assessment of topoisomerase I and II in nuclear extracts

Relaxation. This assay was utilized to determine the activity of topoisomerase I in nuclear extracts. Approximately 0.20 μg of supercoiled SV40 DNA was used as a substrate. Reactions occurred at 37° for 30 min in a buffer containing 10 mM Tris (pH 7.4), 50 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, and 0.1 mM EDTA. The reaction buffer contained no ATP, thereby precluding topoisomerase II-induced relaxation [22, 23]. The reactions were terminated by the addition of SDS to 1% and proteinase K to 100 μg/mL. Reaction products were separated on a 1% agarose gel in a Tris–borate buffer system (pH 8) in the presence of 0.5 μg/mL ethidium bromide. Gels were photographed under ultraviolet light. Photographic negatives were densitometrically scanned to determine the amount of Form I° (relaxed) DNA. Topoisomerase I activity in a nuclear extract

is expressed as the amount of protein required to relax 50% of the supercoiled SV40 substrate.

Decatenation. This assay was utilized to determine the activity of topoisomerase II in nuclear extracts. Approximately 0.22 μg of [^{3}H]kDNA was used as a substrate. Reactions occurred at 37° for 30 min under these conditions: 50 mM Tris–HCl, 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 reactions were terminated by the addition of SDS to 1% followed by the addition of proteinase K to 100 μg/mL. Reaction products were separated on a 1% agarose gel in a Tris–borate buffer system (pH 8) in the presence of 0.5 μg/mL ethidium bromide. Catenated DNA does not enter the gel, but decatenated DNA minicircles do. The DNA in the gel was visualized under ultraviolet light and excised. The amount of DNA that migrated into the gel and the amount that remained in the well were quantified using liquid scintillation spectroscopy as described previously [14]. The topoisomerase II activity in a nuclear extract is expressed as the amount of protein required to decatenate 50% of the substitute kDNA.

Drug-induced DNA–protein cross-linking. This assay was used to compare drug sensitivities among nuclear extracts. The substrate was uniquely 3′ end-labeled [^{32}P]SV40 DNA. Drug-induced protein cross-linking of this DNA was quantified by SDS-KCl precipitation [14, 15] after a 30-min incubation at 37° of drug, nuclear extract (equivalent to the amount required to decatenate 50% of kDNA substrate, as above) and 10 ng of 3′ end-labeled DNA in the following buffer: 10 mM Tris (pH 7.4), 50 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/mL bovine serum albumin, and 1 mM ATP.

Drug-induced DNA cleavage by topoisomerase I. Supercoiled SV40 DNA (0.2 μg) was used as a substrate. Equally active (determined by topoisomerase I relaxation, as described above) amounts of nuclear extracts were used. Reactions occurred at 37° for 30 min in the same buffer used for the DNA relaxation assay plus either Topotecan or vehicle. The reactions were terminated by the addition of SDS and proteinase K, and reaction products were run on a 1% agarose gel as described above, with 0.5 μg/mL ethidium present in the running buffer. Gels were photographed under ultraviolet light. Photographic negatives were scanned densitometrically to determine the amount of form II (nicked) DNA generated in the absence and presence of drugs.

Immunoblotting

Both nuclear extracts and whole cells were utilized for immunoblotting. Whole cells were prepared according to the method of Kaufmann *et al.* [24, 25]. Briefly, 5–10 million cells were solubilized by sonication in 1 mL of buffer containing 6 M guanidine hydrochloride, 250 mM Tris–HCl (pH 8.5), 10 mM EDTA, 1% 2-mercaptoethanol, and 1 mM PMSF. Cell solutions were treated with iodoacetamide (27.75 mg/sample) and dialyzed against 4 M urea and then against 0.1% SDS before lyophilization. Prior to being loaded onto a polyacrylamide gel, lyophilized cells were resuspended in 4 M urea, 2% SDS, 62.5 mM Tris–HCl (pH 6.8) and 1 mM EDTA. The lysate from 2 million cells was loaded into each lane of the polyacrylamide gel.

Nuclear extracts or cells were run on 7.5% polyacrylamide gels in a Tris–glycine buffer, and the proteins separated on the gel were electrophoretically transferred to nitrocellulose. The nitrocellulose filters were blocked with 3% bovine serum albumin and incubated with topoisomerase I or II antibody (provided by Dr. Leroy Liu, Johns Hopkins University) at a 1:500 dilution. The antibody complex was detected with ^{125}I-labeled Protein A (Amersham). Independent immunoblots were performed on two groups of PMA- or vehicle-treated cells and on two sets of nuclear extracts derived from PMA- or vehicle-treated cells. The results of one set of blots for whole cells and for nuclear extracts are shown in Figs. 1 and 2.

Northern and dot blotting

Cellular RNA from S cells and PET cells (either untreated or incubated with 10 nM PMA for 48 hr) was harvested in 4 M guanidinium isothiocyanate and separated by ultracentrifugation through a 5.7 M CsCl cushion [26]. RNA (2 μg) from each cell type was denatured and run on 1% agarose/2.2 M formaldehyde gels in 0.1 M 4-morpholinepropane sulfonic acid (MOPS)/5 mM EDTA buffer. The gels were stained with ethidium bromide to check RNA integrity and consistency of loading before transfer to Nytran (Schleicher & Schuell) [27]. The blots were hybridized to the human topoisomerase II cDNA probe, ZII69 (a gift from Dr. Leroy Liu) under standard conditions [27]. Probes utilized in northern and dot blotting (below) were labeled using the Amersham Multiprime Labeling System.

Dot blotting was performed on concentrations of RNA ranging from 5.5 to 0.6875 μg. Filters were hybridized, as above, to the following probes: ZII69, c-*myc* (Oncor, Gaithersburg, MD), topoisomerase I (provided by Dr. Leroy Liu), and β-actin (provided by Dr. Grady Saunders).

RESULTS

Cellular experiments

After 24 hr in the presence of 10 nM PMA, 85.7 ± 9.2% (N = 7) of S cells and 35.2 ± 6.5% (N = 7) of PET cells adhered to the plastic culture flask. However, 48 hr after phorbol addition, 80.9 ± 10.7% (N = 12) of S cells and 13.5 ± 7.5% (N = 12) of PET cells remained adherent. Subsequent experiments were performed after 48 hr of PMA treatment in order to clearly distinguish between phorbol effects on the two cell lines.

Flow cytometry studies with the Mo1 antibody, which recognizes cells of human mononuclear lineage [8, 9], indicated that after incubation with PMA, the majority of adherent HL-60/S cells treated with PMA had begun monocytoid differentiation (the fraction of S cells staining positive for Mo1 increased from 10.7 to 79.4%). In contrast, but as expected, the majority of PET cells were not induced to differentiate by PMA. Adherent PET cells (fraction positive for Mo1 was 43.6%) appeared to be

Table 1. Effect of PMA pretreatment on drug-induced, topoisomerase I- or II-mediated DNA cleavage in HL-60 S and PET cells

	Per cent DNA cleavage relative to non-phorbol-treated cells		
Cell line	Etoposide	Amsacrine	Topotecan
S_{AD}	14, 18	33, 32	146 ± 29* (N = 10)
PET_{SN}	73, 79	98, 125	141 ± 81 (N = 10)
PET_{AD}	46, 52	83	129 ± 45 (N = 7)

Cells were pretreated with 10 mM PMA or vehicle for 48 hr. PMA was removed from the culture medium before incubation with drug for 1 hr, as indicated above. Following exposure of cells to 1 or 5 μM etoposide, 0.1 or 0.5 μM amsacrine, or 0.05 to 2 μM Topotecan, DNA cleavage was assessed by alkaline elution. Data are presented as the per cent DNA cleavage of PMA-treated cells relative to non-phorbol-treated controls when both were incubated with identical concentrations of etoposide, amsacrine, or Topotecan. N = number of independent determinations. If $N > 3$, data are averaged ±1 SD. If $N < 3$, data are presented as individual points. SN = supernatant cells; AD = adherent cells. % Cell adherence for etoposide and amsacrine experiments: S = 89, 91, PET = 9, 7. % Cell adherence for Topotecan experiments: S = 82 ± 13, PET = 8 ± 2. *$P < 0.01$, using the Wilcoxan Rank Sum test or *t*-test, that Topotecan-induced DNA cleavage in S_{AD} cells was significantly higher than in non-phorbol-treated S cells (where cleavage = 100%). Topotecan-induced DNA cleavage was not significantly higher in PMA-treated PET cells (either SN or AD) than in PET cells not treated with PMA (where cleavage = 100%), using either the Wilcoxan Rank Sum test or *t*-test.

Table 2. Effect of phorbol ester treatment on the cell cycle of HL-60 S and PET cells

	Phases of the cell cycle		
Cells	G_0/G_1	S	$G_2 + M$
S veh	38.1	47.8	14.1
S_{AD} + PMA	82.4	7.7	9.9
PET veh	36.5	50.9	12.6
PET_{SN} + PMA	60.1	35.5	4.3
PET_{AD} + PMA	68.8	30.9	0.3

HL-60 S and PET cells were incubated for 48 hr in the presence of 10 nM PMA or vehicle (DMSO equal to 0.1%) before cell-cycle analysis was performed utilizing flow cytometry of propidium iodide-stained cells. Cell adherence + PMA: S = 77%, PET = 7%. Veh = vehicle; AD = adherent cells; SN = supernatant cells.

somewhat more differentiated than the supernatant PET cells and the untreated PET cells (fractions positive for Mol were 28.7 and 2.1%, respectively).

PMA pretreatment decreased etoposide-induced DNA cleavage in S cells (Table 1). The reduction of etoposide-induced DNA cleavage was not as pronounced in supernatant or adherent PET cells following PMA treatment (Table 1), although cleavage reduction in adherent PET cells was greater than that in the supernatant population.

The amount of amsacrine-induced DNA cleavage was also lower in phorbol-treated S cells than in controls (Table 1). However, amsacrine-induced cleavage in PMA-treated S cells was not reduced to the same extent as was etoposide-induced cleavage (Table 1). PMA treatment did not change the suceptibility of supernatant PET cells to amsacrine-induced DNA cleavage and only modestly decreased cleavage in adherent PET cells exposed to amsacrine (Table 1).

Treatment with PMA significantly increased Topotecan-induced DNA cleavage in S cells, although Topotecan-induced DNA cleavage in PMA-treated PET cells (either supernatant or adherent) was not significantly different from that in PET cells not pretreated with the phorbol ester (Table 1).

Drug-induced DNA cleavage has been shown to be lower in S-phase cells than in cells in other phases of the cell cycle [28, 29]. After 48 hr of PMA treatment, the percentage of HL-60/S cells in S phase declined as the percentage of the cell population in G_0/G_1 increased (Table 2), so reductions in drug-induced DNA cleavage could not be explained by recruitment of cells into S phase. PMA treatment decreased the percentage of PET cells (both supernatant and adherent populations) in S phase after 48 hr, with a concomitant increase of cells in G_0/G_1. This was not as great a change from control as was observed in phorbol-treated S cells (Table 2).

Immunoblotting

Whole cell immunoblotting demonstrated that the amount of immunoreactive topoisomerase II in HL-60 S cells fell at least 6-fold after 48 hr of exposure to PMA (Fig. 1), consistent with the PMA-induced decrease in etoposide-induced cleavage (Table 1). PMA treatment did not appear to change the topoisomerase II content of supernatant PET cells and only slightly lowered topoisomerase II in adherent PET cells. PMA treatment lowered the amount of immunoreactive topoisomerase I in both S cells and PET cells, although this decrease was small in the majority of the PET cell population that do not adhere (Fig. 2).

The immunoblot for topoisomerase II performed on nuclear extracts contrasted greatly with the blot performed using whole cells in the case of HL-60 S cells (Fig. 1). After PMA treatment, S cells contained slightly more extractable topoisomerase II, quite unlike the drop in topoisomerase II seen with whole cell preparations. As seen previously in HL-60 nuclear extracts [8, 21], there appeared to be two forms of topoisomerase II in the S cell and untreated PET cell extracts with molecular masses of 194 and 172 kDa.

Low levels of immunoreactive topoisomerase I were detected in nuclear extracts from S cells, treated with PMA or vehicle. PET cell nuclear extracts appeared to have more of the enzyme than extracts derived from S cells, and this amount seemed to increase following PMA treatment (Fig. 2).

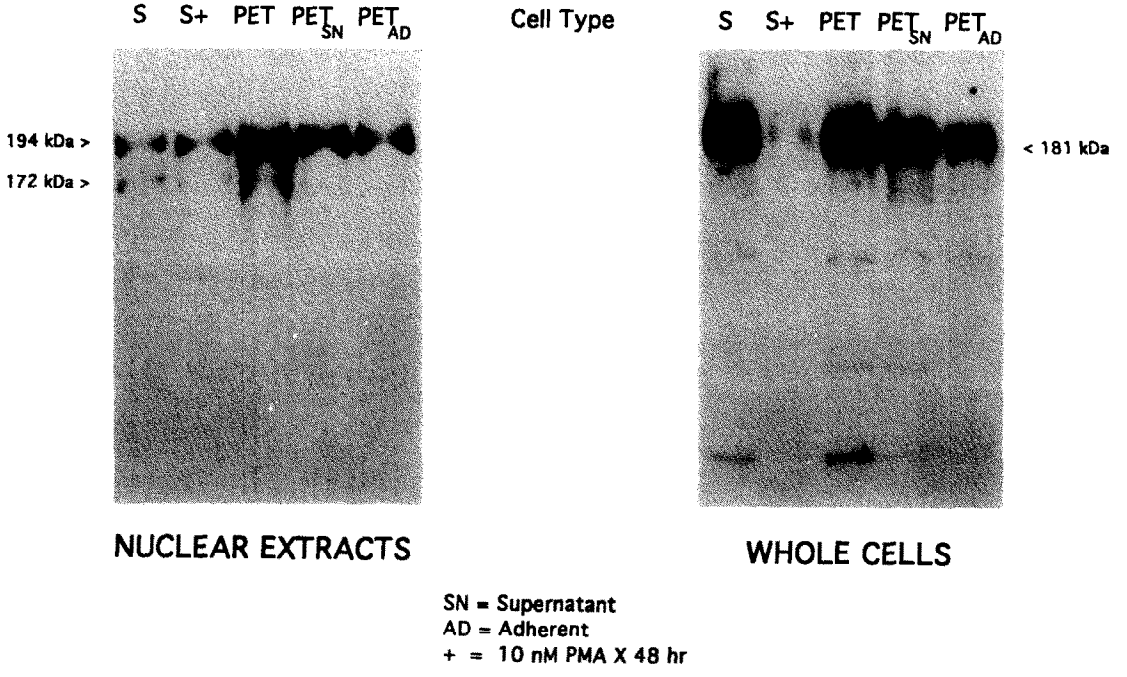

Fig. 1. Topoisomerase II immunoblot of whole cells or nuclear extracts from HL-60 S and PET cells exposed to 10 nM PMA or vehicle for 48 hr. HL-60 S and PET cells were incubated with 10 nM PMA or vehicle for 48 hr before harvest and lysis or nuclear isolation and extraction as described in Materials and Methods. Whole cells (2×10^6) or 10 μg of nuclear extract protein were loaded onto each lane of an SDS–polyacrylamide gel. Electrophoresis, transfer to nitrocellulose, and immunoblotting with anti-human topoisomerase II antibody are detailed in Materials and Methods. Molecular mass of the bands was estimated by comparison with standards. Radioactivity of the bands was determined by using a gamma counter and was as follows: nuclear extracts: S = 110 cpm, S+ = 379 cpm, PET = 661 cpm, PET_{SN} = 321 cpm, PET_{AD} = 380 cpm. AD = adherent PMA-treated PET cells; SN = supernatant PMA-treated PET cells; whole cells: S = 1021 cpm, S+ = 170 cpm, PET = 513 cpm, PET_{SN} = 682 cpm, and PET_{AD} = 356 cpm.

Experiments with nuclear extracts

The ability to decatenate kDNA (a measure of topoisomerase II activity) differed less than 2-fold between any two of the five nuclear extracts (Table 3). The sensitivity of topoisomerase II in nuclear extracts from PMA-treated or untreated S cells to drug-induced DNA–protein cross-linking appeared similar (Fig. 3, A and B). Each extract precipitated 3′ ^{32}P end-labeled SV40 DNA in a concentration-responsive fashion in the presence of either amsacrine or etoposide (Fig. 3, A and B). Topoisomerase II in extracts from untreated PET cells or phorbol-treated PET supernatant cells also precipitated 3′ ^{32}P end-labeled SV40 DNA in the presence of either drug; however, enzyme in the extract from the untreated PET cells appeared to stabilize more DNA–protein cross-links than enzyme in the other extracts, particularly in the presence of etoposide. Surprisingly, topoisomerase II in the extract from PET adherent cells (open triangles) was resistant to both drugs (Fig. 3).

Relaxation of SV40 DNA in the absence of ATP (indicative of topoisomerase I catalytic activity) was similar in nuclear extracts derived from S cells regardless of PMA treatment (Table 3). Topoisomerase I catalytic activity appeared to be lower in extracts from PMA-treated PET cells (particularly the supernatant cells) than in extracts from untreated PET cells (Table 3). Topotecan-induced topoisomerase I mediated single-stranded DNA cleavage (generation of form II DNA from form I) was significantly greater in extracts from PMA-treated S cells than untreated S cells, but was similar in PET cells regardless of PMA treatment (Fig. 4).

Northern and dot blotting

Northern blotting using the human topoisomerase II cDNA probe ZII69 (Fig. 5) showed that PMA treatment decreased dramatically the amount of topoisomerase II message in S cells. Only a slight decrease in PET cell topoisomerase II message was observed after PMA exposure. Dot blotting demonstrated that the amount of mRNA coding for topoisomerase I in *both* S and PET cells appeared to be lower after PMA treatment (Fig. 5). Dot blot experiments (Fig. 5) also illustrated the decrease of topoismerase II mRNA in S cells following PMA and demonstrated a PMA-induced down-regulation of c-*myc* mRNA in S cells that did not occur in PET cells. PMA treatment did not affect expression of the gene for β-actin, a constitutive cell protein, in S cells or PET cells.

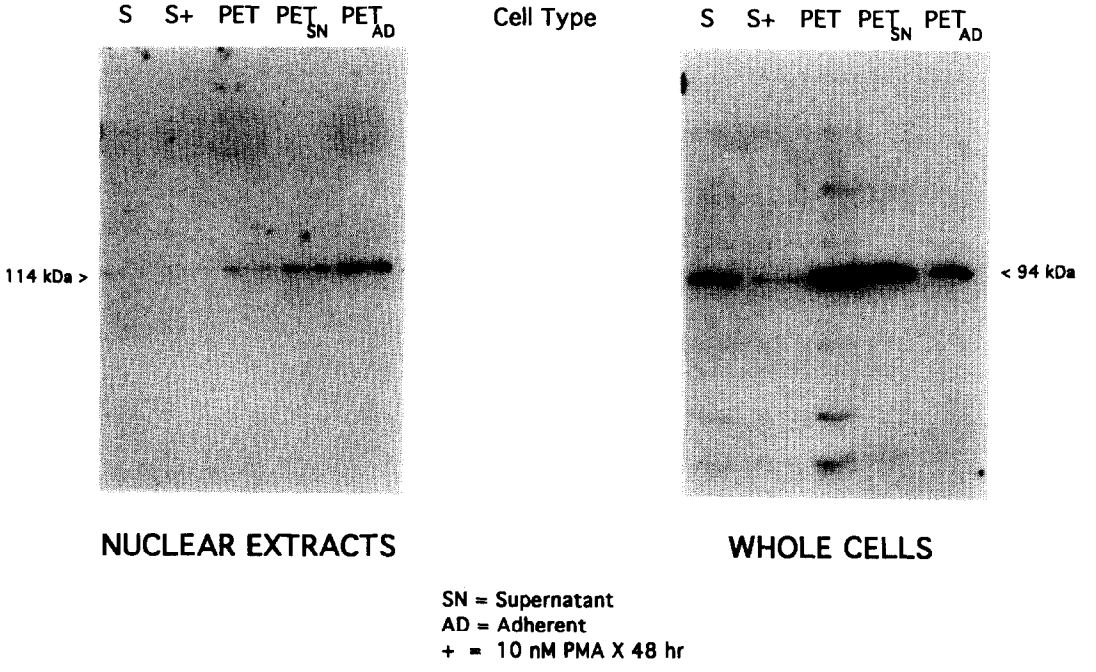

Fig. 2. Topoisomerase I immunoblot of whole cells or nuclear extracts from HL-60 S and PET cells exposed to 10 nM PMA or vehicle for 48 hr. Whole cells (2×10^6) or 15 μg of nuclear extract protein were loaded into each lane of the gels. Anti-human topoisomerase I antibody was used for immunoblotting. See legend of Fig. 1 for further experimental details. Radioactivity of the bands was determined by using a gamma counter and was as follows: nuclear extracts: S = 3 cpm, S+ = 16 cpm, PET = 18 cpm, PET_{SN} = 30 cpm, PET_{AD} = 49 cpm; whole cells: S = 131 cpm, S+ = 38 cpm, PET = 375 cpm, PET_{SN} = 203 cpm and PET_{AD} = 98 cpm.

Table 3. Effect of cellular PMA pretreatment on 0.35 M NaCl topoisomerase I- and II-containing nuclear extracts derived from HL-60 S and PET cells

Cells	Protein concentration (ng/mL)	"$Decat_{50}$" (ng)	"$Relax_{50}$" (ng)
S	94	266 ± 20	2.4, 1.4
S_{AD} + PMA	97	227 ± 48	1.8, 3.3
PET	232	160 ± 30	0.92, 0.81
PET_{SN} + PMA	472	307 ± 47	4, 3.2
PET_{AD} + PMA	112	228 ± 75	1.5, 1.9

HL-60 S and PET cells were treated with 10 nM PMA for 48 hr before harvest, isolation, and extraction of nuclei as outlined in Materials and Methods. Adherence: S = 62%, PET = 7%. The protein concentration in each extract is given, along with the "$Decat_{50}$" value, the amount of protein needed to decatenate 50% of the catenated DNA substrate (indicative of topoisomerase II activity), and the "$Relax_{50}$" value, the amount of extracted protein needed to relax 50% of supercoiled SV40 DNA substrate (in the absence of ATP, indicative of topoisomerase I activity). For details of the assays, see Materials and Methods. The "$Decat_{50}$" values are presented as means ± 1 SD of three experiments. AD = adherent cells; SN = supernatant cells.

Cell survival studies

Soft agar colony formation assays showed that a 48-hr treatment with 10 nM PMA protected HL-60 S cells from the cytotoxic effects of a 1-hr incubation with 20 μM etoposide (Fig. 6). PMA pretreatment was less effective at protecting PET cells from etoposide cytotoxicity. This contrasts with previous data presented for HL-60 and phorbol-tolerant 1E3 cells [8], where etoposide cytotoxicity was reduced to a similar level in both cell lines after incubation with phorbol ester. PMA pretreatment offered only slight protection from amsacrine toxicity in S cells and PET cells (data not shown). The cytotoxicity of 2 μM Topotecan was unaltered in S cells and PET cells after PMA treatment (data not shown).

DISCUSSION

The current studies using S and PET cells demonstrated that many of the observations made in our earlier studies comparing phorbol-treated HL-60 and 1E3 cells [8] were not unique to that cell pair. Etoposide-induced DNA cleavage in PMA-treated S and HL-60 cells was lowered to a much greater extent than in PMA-treated PET or 1E3 cells [8]. Etoposide cytotoxicity was also lower in PMA-treated S cells than in S cells that were not pretreated with PMA. Phorbol treatment did not lower etoposide cytotoxicity in PET cells. This was

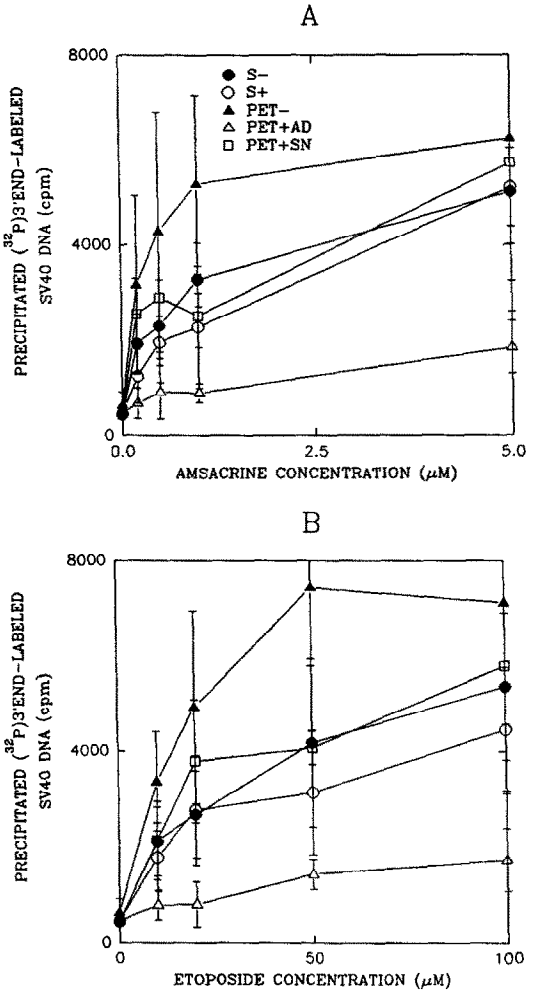

Fig. 3. Drug sensitivities of 0.35 M NaCl nuclear extracts from HL-60 S and PET cells pretreated with 10 nM PMA or vehicle. Nuclear extracts, derived as explained above, were incubated for 1 hr at 37° with the concentrations of amsacrine (A) or etoposide (B) shown in the figure. Equally active amounts (as determined by decatenation) of nuclear extracts were used. The substrate used in this assay was linear SV40 DNA that had been 3' end-labeled with [^{32}P]ATP. Reaction conditions are given in Materials and Methods. Data points represent the means ± 1 SD of three experiments.

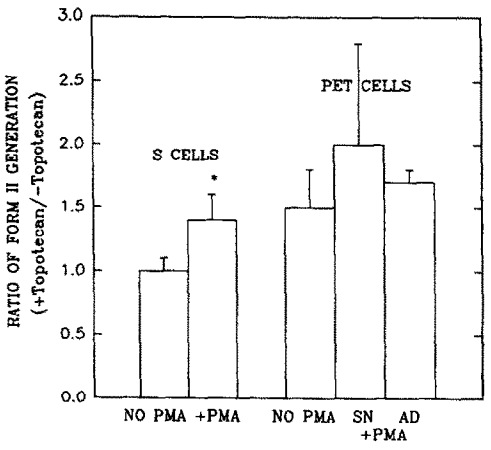

Fig. 4. Topotecan-induced cleavage of supercoiled DNA by 0.35 M NaCl nuclear extracts from HL-60 S and PET cells pretreated with 10 nM PMA or vehicle. Nuclear extracts of the cell types shown in the figure were incubated with supercoiled SV40 DNA in the presence or absence of 100 μM Topotecan under the conditions described in Materials and Methods. Equally active amounts (as determined by DNA relaxation) of the nuclear extracts were used. Reactions were stopped with SDS and digested with proteinase K to eliminate DNA–protein cross-links before being run on a 1% agarose gel using a TBE buffer system with ethidium bromide. Gels were photographed under UV light and the negatives scanned to determine the amount of form II (nicked) DNA. Three separate assays were performed on each extract, and data are presented as the ratio of form II DNA generated in the presence and absence of Topotecan ± 1 SD. Key: (*) P = 0.02, using a paired, two-tailed *t*-test, that Topotecan-induced cleavage of supercoiled SV40 DNA was significantly greater with nuclear extract from PMA-treated S cells than with nuclear extract from vehicle-treated S cells.

consistent with the etoposide cleavage data. In contrast, PMA lowered etoposide cytotoxicity in 1E3 cells as well as in HL-60, although drug-induced DNA cleavage was reduced *only* in the HL-60 cells [8]. This suggests that the effect of PMA treatment on etoposide-induced DNA cleavage may be independent of its effect on etoposide cytotoxicity.

Amsacrine-induced DNA cleavage in PMA-treated S cells was not lowered to the same extent as cleavage mediated by etoposide. The more modest influence of PMA treatment on amsacrine-induced DNA cleavage was also consistent with our previous study of HL-60 and 1E3 cells [8]. Our previous explanation [3, 8] for this is that amsacrine is able to overcome the protective effect of phorbol esters because of its intercalation of DNA (etoposide is not a DNA intercalator). Phorbol esters may have some direct or indirect action on chromatin, and intercalation may partially overcome that action.

In both S and HL-60 (8) cells (but in neither PET nor 1E3 (8) cells), mRNA coding for the topoisomerase II enzyme was decreased dramatically after PMA treatment, consistent with the drop in etoposide-induced DNA cleavage observed in the PMA-treated cells. However, immunoreactive topoisomerase II was not lower in nuclear extracts from PMA-treated S cells than in extracts from untreated cells.

Because the S cell nuclear extract data and mRNA data did not reconcile, we decided to use a whole cell immunoblotting technique to quantify immunoreactive topoisomerase II in whole S and PET cells. Whole cell immunoblotting revealed a large drop in immunoreactive topoisomerase II in PMA-treated S cells that was not observed in the PET cells. These data appeared consistent with the levels of topoisomerase II mRNA in the S and PET cells following PMA treatment. This observation suggests that the topoisomerase II contained in

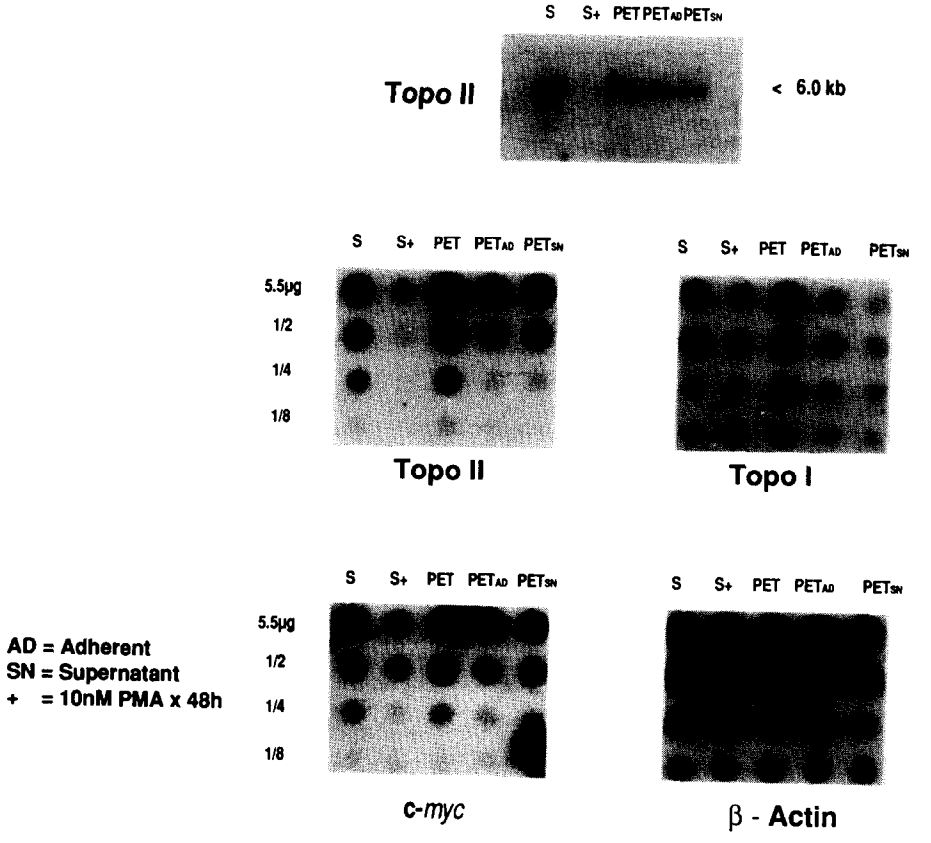

Fig. 5. Modulation of topoisomerase II RNA levels in HL-60 S and PET cells treated with 10 nM PMA or vehicle. Top: northern blot using the ZII69 human topoisomerase II probe. Each lane contained 2 µg of total cellular RNA. Below: dot blots using probes for topoisomerases II and I (center row), and c-*myc* and β-actin (bottom row). Concentrations of RNA in the blots ranged from 5.5 to 0.6875 µg, descending by half for each row.

nuclear extracts is not necessarily representative of the total enzyme contained in the cell. The unextracted topoisomerase II may be tightly associated with the nuclear matrix or compartmentalized differently from the readily extracted enzyme [30, 31].

The decrease in immunoreactive topoisomerase II and topoisomerase II mRNA observed in S cells demonstrates that the gene for this enzyme may be down-regulated directly or indirectly by PMA. This down-regulation does not occur in PET or 1E3 [8] cells, so it is consistent with the involvement of topoisomerase II in monocytoid differentiation. Data from the laboratories of other investigators also indicate a possible relationship between reductions in the amount or activity of topoisomerase II and cell differentiation. Gorsky *et al.* [32] reported a 30% drop in topoisomerase II activity in HL-60 cells after 12–24 hr of PMA exposure, when the beginning of cell differentiation could be observed. According to Kaufmann *et al.* [25], topoisomerase II levels in proliferating HL-60 and normal bone marrow progenitor cells were higher than those in either mature granulocytes or HL-60 cells induced toward granulocytic differentiation by DMSO.

The genes for topoisomerases I and II appear to be independently regulated. In whole S and PET cells, the amount of immunoreactive topoisomerase I fell slightly after 48 hr of PMA treatment, as did the amount of mRNA coding for the enzyme. These data did not correlate negatively or positively with the topoisomerase II mRNA or enzyme data in either cell line. The *activity* of topoisomerase I may have been increased in the S cells because Topotecan-induced DNA cleavage (as measured in whole cells by alkaline elution) was significantly greater after S cells were exposed to PMA. PMA-induced perturbations in topoisomerase I gene expression or activity have been observed by other investigators. Hwong and associates [33] reported that topoisomerase I mRNA levels rose within 2–4 hr in human skin fibroblasts in response to PMA. Over a similar time course, Gorsky *et al.* [32] reported an increase in topoisomerase I activity but not in cellular enzyme content.

In summary, we have described another HL-60 cell pair in which one line will and one line will not differentiate in response to phorbol ester exposure. Like the HL-60/1E3 cell pair [8], the S/PET cell pair exhibited a differential response to etoposide

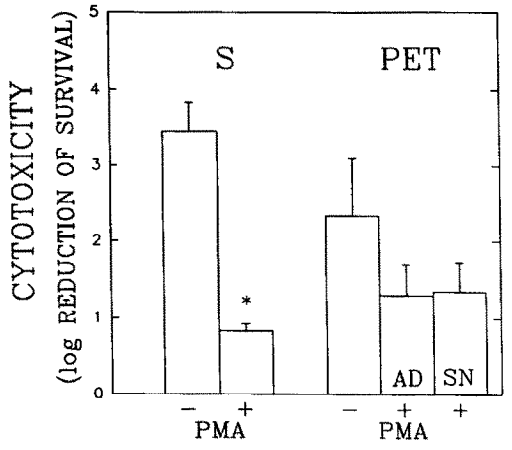

Fig. 6. Effect of PMA pretreatment on colony-forming ability of HL-60 S and PET cells exposed to etoposide. Cells were pretreated for 48 hr with 10 nM PMA or vehicle. PMA was removed and cells were treated for 1 hr with 20 μM etoposide. After removal of drug, cells were cloned in soft agar. Data are presented as the log reduction of cell survival for etoposide-treated cells vs cells not treated with etoposide. Data are the means ± SD of three experiments. Key: (*) P = 0.008 that etoposide cytotoxicity was significantly less in PMA-treated S cells than in S cells not treated with PMA when data were analyzed using a paired, two-tailed *t*-test. Mean cloning efficiency with no drug treatment ± 1 SD: S− = 0.94 ± 0.15, S+PMA = 0.25 ± 0.25, PET− = 0.93 ± 0.43, PET_{SN} = 0.37 ± 0.23 and PET_{AD} = 0.53 ± 0.23. Mean % cell adherence ± 1 SD after PMA: S = 79.1 ± 10.6 and PET = 17.2 ± 7.9.

treatment following phorbol exposure; this response suggests the involvement of topoisomerase II in the monocytoid differentiation cascade initiated by phorbol esters. The PMA-induced down-regulation of S cell mRNA (also observed previously in HL-60 cells [8]) implies that the regulation of topoisomerase II is at the transcriptional level, indicating that the gene coding for a major drug target is down-regulated as cells differentiate. The precise mechanism by which phorbols effect such changes is under study. These mechanisms could explain the differential cytotoxic action of topoisomerase II-reactive anticancer drugs on malignant tissue that is the basis of the therapeutic index underlying the clinical utility of these agents. The need for utilizing whole cell immunoblotting in order to expose the drop in immunoreactive topoisomerase II that occurs in cells after phorbol treatment indicates that topoisomerase II contained in nuclear extracts is not necessarily representative of enzyme contained in whole cells. Finally, the gene for topoisomerase I appears to be regulated independently from the gene for topoisomerase II.

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REFERENCES

1. Rovera G, O'Brien TG and Diamond L, Induction of differentiation in human promyelocytic leukemia cells by tumor promotors. *Science* **204**: 868–870, 1979.
2. Huberman E and Callahan MF, Induction of terminal differentiation in human promyelocytic leukemia cells by tumor promoting agents. *Proc Natl Acad Sci USA* **76**: 1293–1297, 1979.
3. Zwelling LA, Chan D, Hinds M, Mayes J, Silberman LE and Blick M, Effect of phorbol ester treatment on drug-induced, topoisomerase II-mediated DNA cleavage in human leukemia cells. *Cancer Res* **48**: 6625–6633, 1988.
4. Wang J, DNA topoisomerases. *Annu Rev Biochem* **54**: 665–697, 1985.
5. Liu LF, DNA topoisomerases–Enzymes that catalyse the breaking and rejoining of DNA. *CRC Crit Rev Biochem* **15**: 1–24, 1983.
6. Uemura T and Yaganida M, Mitotic spindle pulls but fails to separate chromosomes in type II DNA topoisomerase mutants: Uncoordinated mitosis. *EMBO J*: 1003–1010, 1986.
7. Osheroff N, Effect of antineoplastic agents on the DNA cleavage/religation reaction of eukaryotic topoisomerase II: Inhibition of DNA religation by etoposide. *Biochemistry* **28**: 6157–6160, 1989.
8. Zwelling LA, Hinds M, Chan D, Altshuler E, Mayes J and Zipf TF, Phorbol ester effects on topoisomerase II activity and gene expression in HL-60 human leukemia cells with different proclivities toward monocytoid differentiation. *Cancer Res* **50**: 7116–7122, 1990.
9. Leftwich JA, Carlson P, Adleman B and Hall RE, HL-60-1E3, a novel phorbol diester-resistant HL-60 cell line. *Cancer Res* **47**: 1319–1324, 1987.
10. Macfarlane DE, Gailani D and Vann K, A phorbol ester tolerant (PET) variant of HL-60 promyelocytes. *Br J Haematol* **68**: 291–302, 1988.
11. Englund PT, The replication of kinetoplast DNA networks in *Crithidia fasciculata*. *Cell* **14**: 157–168, 1978.
12. Simpson AM and Simpson L, Labeling of *Crithidia fasciculata* DNA with [^{3}H]thymidine. *J Protozool* **21**: 379–382, 1974.
13. Simpson AM and Simpson L, Isolation and characterization of kinetoplast DNA networks and minicircles from *Crithidia fasciculata*. *J Protozool* **21**: 774–781, 1974.
14. Bakic M, Chan D, Anderson BS, Beran M, Silberman L, Estey E, Ricketts L and Zwelling LA, Effect of 1-β-D-arabinofuranosylcytosine (ara-C) on nuclear topoisomerase II activity and on the DNA cleavage and cytotoxicity produced by 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (*m*-AMSA) and etoposide in *m*-AMSA-sensitive and -resistant human leukemia cells. *Biochem Pharmacol* **36**: 4067–4077, 1987.
15. Liu LF, Rowe TC, Yang L, Tewey KM and Chen GL, Cleavage of DNA by mammalian topoisomerase II. *J Biol Chem* **258**: 15365–15370, 1983.
16. Chu M-Y and Fisher GA, The incorporation of ^{3}H-cytosine arabinoside and its effect on murine leukemic cells (L5178Y). *Biochem Pharmacol* **17**: 753–767, 1968.
17. Kohn KW, Ewig RAG, Erikson LC and Zwelling LA, Measurement of DNA strand breaks and crosslinks by

alkaline elution. In: *DNA Repair, A Laboratory Manual of Research Procedures* (Eds. Friedberg EC and Hanawalt PC), pp. 379–401. Marcel Dekker, New York, 1981.
18. Zipf TF, Lauzon GJ and Longnecker BM, A monoclonal antibody detecting a 39,000 m.w. molecule that is present on B lymphocytes and chronic lymphocytic leukemia cells but is rare on acute lymphocytic leukemia blasts. *J Immunol* **131**: 3064–3072, 1983.
19. Pommier Y, Kerrigan D, Schwartz R and Zwelling LA, The formation and resealing of DNA strand breaks in isolated L1210 cell nuclei. *Biochem Biophys Res Commun* **107**: 576–583, 1982.
20. Minford J, Pommier Y, Kohn KW, Kerrigan D, Mattern M, Michaels S, Schwartz R and Zwelling LA, Isolation of intercalator-dependent protein-linked DNA strand cleavage activity from cell nuclei and identification as topoisomerase II. *Biochemistry* **25**: 9–16, 1986.
21. Zwelling LA, Hinds M, Chan D, Mayes J, Sie KL, Parker E, Silberman L, Radcliffe A, Beran M and Blick M, Characterization of an amsacrine-resistant line of human leukemia cells. Evidence for a drug-resistant form of topoisomerase II. *J Biol Chem* **264**: 16411–16420, 1989.
22. Osheroff N, Shelton ER and Brutlag DL, DNA topoisomerase II from *Drosophila melanogaster*: Relaxation of supercoiled DNA. *J Biol Chem* **258**: 9536–9543, 1983.
23. Osheroff N, Eukaryotic topoisomerase II characterization of enzyme turnover. *J Biol Chem* **261**: 9944–9950, 1986.
24. Kaufmann SH, Okre S, Wikstrom AC, Gustafsson JA and Shaper JH, Binding of the glucocorticoid receptor to the rat liver nuclear matrix. *J Biol Chem* **261**: 11962–11967, 1986.
25. Kaufmann SH, McLaughlin SJ, Kastan MB, Liu LF, Karp JE and Burke PJ, Topoisomerase II levels during granulocytic maturation *in vitro* and *in vivo*. *Cancer Res* **51**: 3534–3543, 1991.
26. Chirgwin JM, Pryzbyla HE, MacDonald RJ and Rutter WJ, Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**: 5294–5299, 1984.
27. Sambrook J, Fritsch EF and Maniatis T, *Molecular Cloning: A Laboratory Manual*, pp. 7.43–7.52. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989.
28. Estey E, Adlakha C, Hittelman WN and Zwelling LA, Cell cycle stage dependent variations in drug-induced topoisomerase II mediated DNA cleavage and cytotoxicity. *Biochemistry* **26**: 4338–4344, 1987.
29. Chow KC and Ross WE, Topoisomerase-specific drug sensitivity in relation to cell cycle progression. *Mol Cell Biol* **7**: 3119–3123, 1987.
30. Fernandes DJ, Danks MK and Beck WT, Decreased nuclear matrix DNA topoisomerase II in human leukemia cells resistant to VM-26 and m-AMSA. *Biochemistry* **29**: 4235–4241, 1990.
31. Kauffman SH and Shaper JH, Association of topoisomerase II with the hepatoma cell nuclear matrix: The role of intermolecular disulfide bond formation. *Exp Cell Res* **192**: 511–523, 1991.
32. Gorsky LD, Cross SM and Morin MJ, Rapid increase in the activity of DNA topoisomerase I, but not DNA topoisomerase II, in HL-60 promyelocytic leukemia cells treated with a phorbol diester. *Cancer Commun* **1**: 83–92, 1989.
33. Hwong CL, Chen MS and Hwang J, Phorbol ester transiently increases topoisomerase I mRNA levels in human skin fibroblasts. *J Biol Chem* **264**: 14923–14926, 1989.