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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*

MILORAD BAKIC, DIANA CHAN, BORJE S. ANDERSSON, MILOSLAV BERAN, LYNN SILBERMAN, ELIHU ESTEY, LAURIE RICKETTS and LEONARD A. ZWELLING†

Departments of Medical Oncology and Hematology, Division of Medicine, The University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston, TX, U.S.A.

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Abstract—The ability of a noncytotoxic dose of ara-C to modulate the amount of 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (*m*-AMSA)- or etoposide-induced topoisomerase II-mediated DNA cleavage and cytotoxicity was examined in *m*-AMSA-sensitive and -resistant HL-60 human leukemia cells. Ara-C pretreatment (0.1 μM × 48 hr) sensitized *m*-AMSA-sensitive cells to the cytotoxicity and DNA cleavage produced by both *m*-AMSA and etoposide. The actions of *m*-AMSA in the *m*-AMSA-resistant cells were affected minimally by ara-C. By contrast, ara-C enhanced etoposide-induced DNA cleavage and, to an even greater extent, etoposide-induced cytotoxicity in *m*-AMSA-resistant cells. These cells were only minimally cross-resistant to etoposide. Ara-C did not affect the cellular uptake of *m*-AMSA or etoposide, the amount of 0.35 M NaCl-extractable nuclear topoisomerase II activity from either cell line, or the ability of this enzyme activity to covalently bind to DNA in the presence of the drugs. *m*-AMSA- and etoposide-induced DNA cleavage is thought to result from drug-induced stabilization of a topoisomerase II–DNA complex. The ability of ara-C to modulate this effect and associated cytotoxicity appears to be mediated by the effects of ara-C on cellular targets other than topoisomerase II but which are important to topoisomerase II-mediated events, such as protein-associated DNA cleavage. A good candidate for such a target may be cellular chromatin.

Topoisomerase II is an intracellular target of several active antineoplastic agents including 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA‡) and etoposide (VP-16) [1, 2]. Whether intereference with the function of this enzyme explains the cytotoxic potency of these drugs remains a critical unresolved issue. Three lines of evidence support a mechanistic connection between drug-induced effects on topoisomerase II and drug-induced cytotoxicity. First, inactive analogs of active topoisomerase II-reactive agents are concomitantly ineffective when reacting with topoisomerase II in cells or in biochemical systems [3–8]. Second, mammalian cells resistant to the cytotoxic actions of topoisomerase II-reactive agents exhibit a lower frequency of drug-induced, protein-associated DNA strand cleavage when treated with these agents than do their drug-sensitive counterparts [9–12]. Protein-associated DNA strand cleavage as quantified using the filter elution technique of Kohn *et al.* [13] is a measure of drug–topoisomerase II interactions [1, 2]. Third, treating murine leukemia cells with certain antineoplastic antimetabolites including 1-β-D-arabinofuranosylcytosine (ara-C) potentiates the ability of topoisomerase II-reactive compounds to produce protein-associated DNA strand cleavage and to kill the cells in a parallel fashion [14, 15].

We have recently studied the effects of *m*-AMSA in sensitive and resistant human acute myelogenous leukemia cells in culture [9]. Topoisomerase II activity was present in the nuclei of both the sensitive HL-60 line and its *m*-AMSA-resistant daughter line, HL-60/AMSA [16]. However, only that activity from the sensitive cells could cleave DNA in response to *m*-AMSA. This result corresponded to the low frequency of *m*-AMSA-induced DNA cleavage produced in HL-60/AMSA cells or their isolated nuclei

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† To whom correspondence should be addressed: Department of Medical Oncology, MD Anderson Hospital and Tumor Institute, Box 52, 6723 Bertner Ave., Houston, TX 77030.

‡ Abbreviations: *m*-AMSA, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide; VP-16, etoposide; ara-C, 1-β-D-arabinofuranosylcytosine; SDS, sodium dodecyl sulfate; D_o, dose increment that reduces survival by a factor of 1/e; k, kinetoplast; EGTA, ethyleneglycolbis(amino-ethylether)tetra-acetate; and PMSF, phenylmethylsulfonyl fluoride.

and the relative cytotoxicity produced by *m*-AMSA in these human leukemia cells [9]. We wished to examine how ara-C would affect topoisomerase II activity, *m*-AMSA-induced DNA cleavage, and *m*-AMSA-induced cytotoxicity in human leukemia cells, the critical clinical target of both *m*-AMSA and ara-C. Particularly, could ara-C potentiate the actions of *m*-AMSA in human cells as it had in the murine system and could *m*-AMSA resistance of human leukemia be overcome by ara-C treatments? In addition, we extended these observations with ara-C and *m*-AMSA to include studies with ara-C and VP-16, a topoisomerase II-reactive drug to which HL-60/AMSA cells are only minimally resistant.

MATERIALS AND METHODS

The HL-60 cell line was originally obtained from Dr. Robert Gallo, National Cancer Institute. The *m*-AMSA-resistant subline, HL-60/AMSA, was developed by repeated short exposures of HL-60 to *m*-AMSA. The characteristics of this resistant subline have been described [17]. The doubling times of the two lines approximate one another (see Results). Both lines were maintained in a 3:1 (v/v) mixture of RPMI medium 1630 and Iscove's modified Dulbecco's medium (GIBCO, Grand Island, NY) with 10% fetal calf serum at 37°. All cells were demonstrated to be mycoplasma free (American Type Culture Collection, Rockville, MD).

Exponentially-growing cells were exposed to 0.1 μCi/ml of [2-^{14}C]thymidine (New England Nuclear, Boston, MA) for 48 hr to radiolabel their cellular DNA. Murine leukemia L1210 cells grown in RPMI medium 1630 plus 10% fetal calf serum labeled with [*methyl*-^{3}H]thymidine (0.1 μCi/ml) (New England Nuclear) served as internal standard cells for alkaline elution experiments as previously described [8, 13].

m-AMSA (NSC 249992) and ara-C (NSC 63878) were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute. *m*-AMSA was kept as a stock solution (10 mM) in 100% dimethyl sulfoxide. Etoposide was a gift from Dr. Byron Long, Bristol-Baylor Laboratories, Houston, TX. This compound was constituted as a 10 mM solution in 100% dimethyl sulfoxide and stored frozen. Ara-C (0.1 mM) was dissolved in sterile water prior to each experiment. [^{14}C]*m*-AMSA (19.6 mCi/mmol) was synthesized by SRI International, Menlo Park, CA, and was obtained through the Chemical Resources Section, National Cancer Institute. This, too, was dissolved as a stock solution in dimethyl sulfoxide. [^{3}H]Etoposide (480 mCi/mmol) in methanol was obtained from Moravek Biochemical (Brea, CA).

After radiolabeling of HL-60 or HL-60/AMSA, cells were resuspended in radiolabel-free medium for 24 hr, and then they were either treated or not treated with ara-C (usually 0.1 μM for 48 hr). Next, cells were resuspended in drug-free medium and then treated with *m*-AMSA or etoposide. An identical protocol with unlabeled cells was used in studies of *m*-AMSA or etoposide uptake or drug effects on colony-forming ability (see below).

DNA cleavage and DNA–protein cross-link formation were quantified as previously described [8, 9] using the alkaline elution method of Kohn *et al.* [13]. Results of experiments with *m*-AMSA or etoposide are expressed as rad-equivalents, the amount of X-irradiation that produces an effect comparable to that of the *m*-AMSA or etoposide cell treatment. All *m*-AMSA or etoposide cell treatments lasted for 60 min at 37°.

Nuclei were isolated as previously described [18] and exposed to *m*-AMSA or etoposide for 30 min at 37°. NaCl (0.35 M) extracts of cell nuclear protein were made as previously described [19]. Briefly, nuclei were suspended in nucleus buffer (150 mM NaCl, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 1 mM EGTA, 0.1 mM dithiothreitol, 0.1 mM PMSF, pH 6.4), and a sufficient quantity of 2 M NaCl was then added so as to bring the NaCl concentration to 0.35 M. Nuclei were extracted on ice for 30 min and centrifuged, and the resulting supernatant fraction was assayed for topoisomerase II activity.

Topoisomerase II activity was assayed by quantifying the potency of nuclear extracts to decatenate [^{3}H]kinetoplast (k) DNA isolated from the trypanosome *Crithidia fasciculata* using cesium chloride density gradients as previously described [20]. Reactions with this DNA are for 30 min at 37° in a buffer containing 5 mM ATP, 10 mM Tris, 5 mM $MgCl_2$, 50 mM KCl, 0.5 mM EDTA, 30 μg/ml bovine serum albumin, and 0.5 mM dithiothreitol, pH 7.6. Reaction product (decatenated DNA) was separated from starting catenated DNA in two ways. In one method, a 50-μl reaction volume was centrifuged for 15 min at 12,000 *g*. The upper 40 μl contained the decatenated reaction product; the lower 10 μl contained the catenated starting material. The amount of [^{3}H]kDNA in these two portions was quantified using a liquid scintillation spectrometer. The utility and applicability of this method were confirmed by the other, more conventional method. This was agarose gel electrophoresis. The entire 50-μl reaction volume was loaded into a well of 1% agarose gel in a 89 mM Tris–borate buffer system (pH 8.0). The DNA was electrophoresed overnight at 20 V. The gel was stained with ethidium bromide (1 μg/ml) for 40 min and the DNA visualized using an ultraviolet light source. Each well and each remaining gel lane were excised and placed into a liquid scintillation vial for counting. The well agarose and that from the entire lane were processed for liquid scintillation counting by melting the agarose in a microwave oven followed by the addition of water and scintillation fluid. Well and lane [^{3}H]kDNA were quantified using a liquid scintillation spectrometer. The two assays were shown to give virtually identical results prior to their use in the experiments presented here.

Drug-stimulated linking of a protein to the 5′end of DNA is putatively a topoisomerase II specific reaction [21]. We therefore 3′end-labeled an EcoRI-digested piece of SV-40 DNA with [α-^{32}P]ATP and the Klenow fragment of DNA polymerase as modified from a previously described method [21]. This became the substrate for measurements of drug-stimulated 5′end DNA–protein cross-linking. Reactions were for 30 min at 37°. Precipitation of this protein–DNA complex utilized the alkali SDS-KCl technique of Liu *et al.* [21].

Quantification of cell-associated *m*-AMSA using [^{14}C]*m*-AMSA has been described [22]. Results are expressed as micromoles of *m*-AMSA per liter of cell water. The volume of cell water was quantified using ^3H_2O (New England Nuclear). [^{3}H]VP-16 cellular uptake was similarly quantified. The exception was that cell pellet volumes were quantified using ^3H_2O in replicate cell samples rather than in identical cell samples to those used to quantify [^{3}H]VP-16 uptake due to the identical radiolabel on ^3H_2O and [^{3}H]VP-16.

Colony formation in soft agar tubes was performed according to the method of Chu and Fisher [23] as previously described [8].

RESULTS

Effects of ara-C alone. The doubling time of HL-60 was 28.0 ± 2.3 hr (N = 6). That of HL-60/AMSA was 28.6 ± 6.3 hr (N = 6). Ara-C alone (0.1 μM × 48 hr) inhibited cell growth by approximately 50% in each cell line [fraction of control growth was 0.55 ± 0.16 for HL-60 (N = 13) and 0.39 ± 0.02 for HL-60/AMSA (N = 14)]. Colony-formation of ara-C-treated cells was 125% of control for HL-60 (N = 4) and 88% of control for HL-60/AMSA (N = 4). Despite comparable doubling times, [^{3}H]ara-C was incorporated into acid-insoluble material to a greater extent in HL-60 (0.0076 ± 0.0003 nmol/10^6 cells, N = 3) than in HL-60/AMSA (0.0049 ± 0.0003 nmol/10^6 cells, N = 3). In the experiment in which radioactive ara-C incorporation was quantified, ara-C (0.1 μM × 48 hr) reduced HL-60 cell growth to 0.50 ± 0.006 (N = 3) (fraction of control growth) and HL-60/AMSA cell growth to 0.54 ± 0.01 (N = 3) (fraction of control growth).

Colony formation. Ara-C potentiated the ability of *m*-AMSA to reduce the colony-forming ability of HL-60 cells (Fig. 1). D_o values (i.e. the dose increment that reduced survival by a factor of 1/e in the exponential portion of the curve) were 0.325 μM *m*-AMSA for HL-60 cells treated with ara-C and 0.60 μM *m*-AMSA for HL-60 cells not treated with ara-C (ratio = 1.8). In these experiments, the D_o value for HL-60/AMSA without ara-C pretreatment was approximately 7.0 μM. Thus, HL-60/AMSA was 12-fold less sensitive to *m*-AMSA than HL-60. Ara-C had no detectable effect on the cytotoxicity of *m*-AMSA in HL-60/AMSA.

HL-60 was slightly more sensitive to VP-16 than was HL-60/AMSA (Fig. 1). (The ratio of the D_o values was 1.6). The HL-60/AMSA cells were approximately 6- to 8-fold more resistant to *m*-AMSA than to VP-16 depending on the precise culture conditions used and the method of colony-formation assay employed [7, 8]. Regardless, in these experiments, HL-60/AMSA resistance to VP-16 was minimal relative to the resistance of HL-60/AMSA to *m*-AMSA.

Reductions in colony formation produced by VP-16 were enhanced markedly by ara-C pretreatment in HL-60/AMSA as compared with HL-60 (Fig. 1). In these experiments, ara-C pretreatment increased the cytotoxicity of VP-16 by a factor of 2 in HL-60, whereas the pretreatment increased the VP-16 effect by a factor of 7 in HL-60/AMSA. HL-60/AMSA cells pretreated with ara-C were actually *more* sensitive to VP-16 than similarly treated HL-60 cells. In contradistinction to the *m*-AMSA-resistance of HL-60/AMSA, the small degree of VP-16 resistance exhibited by the *m*-AMSA-resistant cells was overcome by ara-C pretreatment.

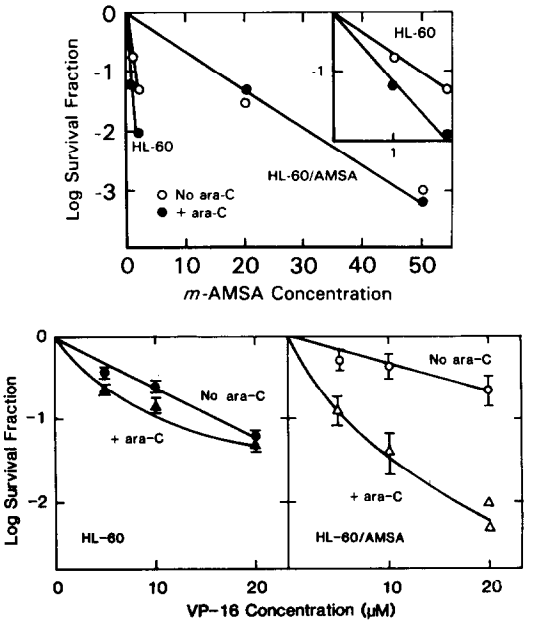

Fig. 1. (Top) Enhancement of *m*-AMSA-induced cytotoxicity by ara-C in HL-60 cells, but not in HL-60/AMSA. HL-60 or HL-60/AMSA cells were either exposed to 0.1 μM ara-C for 48 hr (●) or remained in drug-free medium for 48 hr (○). Cells were treated with various concentrations of *m*-AMSA (μM) for 60 min at 37° in the absence of ara-C, and colony-formation in soft agar was quantified as previously described for L1210 cells [see Refs. 8, 14 and 23] using the methodology of Chu and Fisher [23]. Each point is the mean of least three independent determinations with the exception of the point at 50 μM with ara-C which represents only two determinations. (Inset): HL-60 data points identical to those in the larger figure using an expanded scale to allow visualization of the effect of ara-C on *m*-AMSA-induced cytotoxicity. (Bottom) Effect of pretreatment with ara-C on the reduction of colony-forming ability produced by VP-16 in HL-60 or HL-60/AMSA. Ara-C treatment was as described previously. VP-16 treatments were for 60 min at 37°. Each point is the mean ± 1 SEM of least three dependent experiments. If fewer than three experiments were performed, individual points are shown.

[^{14}C]m-*AMSA and* [^{3}H]*VP-16 cell uptake.* The amount of cell-associated *m*-AMSA was shown to be comparable in HL-60 and HL-60/AMSA in previous work [9]. We have now found that pretreatment with ara-C had no effect on [^{14}C]*m*-AMSA uptake in either cell line (Fig. 2). The cell-associated [^{3}H]VP-16 concentration was unaffected by ara-C pretreatment (Table 1).

DNA cleavage and DNA–protein cross-linking. DNA cleavage induced by *m*-AMSA was enhanced in cells previously exposed to 0.1 μM ara-C for 48 hr (Fig. 3). Cleavage was enhanced 46% in HL-60 cells (twenty-seven determinations) ($P < 0.01$ by

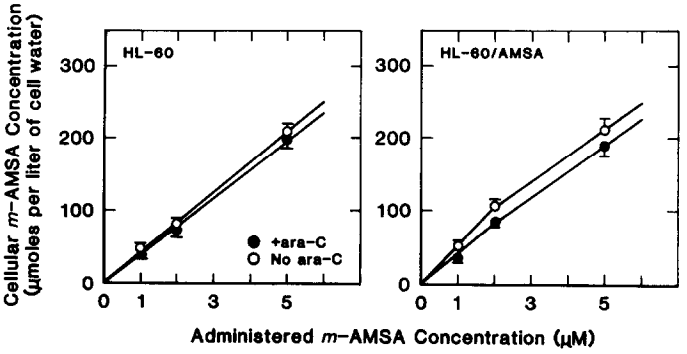

Fig. 2. Effect of ara-C on the amount of *m*-AMSA associated with human leukemia cells. HL-60 or HL-60/AMSA cells were either exposed to 0.1 μM ara-C for 48 hr (●) or remained in drug-free medium for 48 hr (○). Cells were treated for 60 min with 0.5 μM [^{14}C]*m*-AMSA plus appropriate amounts of unlabeled *m*-AMSA to obtain the concentrations indicated. Micromoles of cell-associated *m*-AMSA per liter of cell water (measured using $^{3}H_2O$) was quantified as previously described [22]. Each point is the mean ± 1SD of three independent determinations.

Wilcoxon's Rank Sum test) and 71% in HL-60/AMSA cells (forty determinations) ($P < 0.01$). Treatment with ara-C alone produced no DNA cleavage.

An increase in the ara-C treatment time to 72 hr did not further augment the effects of *m*-AMSA on DNA (data not shown). Higher doses of ara-C (up to 1.0 μM for 48 hr) did augment the effect of *m*-AMSA on DNA somewhat, but it never resulted in more than a doubling of the DNA cleavage frequency produced by *m*-AMSA alone (data not shown).

The DNA cleavage produced by *m*-AMSA in cells exposed or not exposed to ara-C was protein-concealed. That is, the cleavage was not detected in elution assays performed without sodium dodecylsulfate (SDS) and proteinase K. The DNA cleavage frequency produced by a dose of *m*-AMSA was associated with the production of an approximately equal frequency of DNA–protein cross-linking in both cell lines, regardless of whether the cells had been exposed to ara-C (Fig. 4). Thus, DNA cleavage produced by *m*-AMSA in ara-C-treated cells was qualitatively identical to that produced by *m*-AMSA in cells unexposed to ara-C.

Pretreatment with ara-C (0.1 μM for 48 hr) did enhance VP-16-induced DNA cleavage in both cell lines (Fig. 3) ($P < 0.01$ by Wilcoxon's Rank Sum test). The increase in cleavage was greater in HL-60/AMSA (an average of 71% in fourteen determinations) than in HL-60 (an average of 15% in thirteen determinations). In these experiments, VP-16-induced cleavage was slightly less in HL-60/AMSA than in HL-60, and the level of DNA cleavage produced following ara-C treatment was comparable in the two cell lines. In both cell lines, the VP-16-induced DNA cleavage was protein-associated in that, within a factor of 2, the VP-16-induced DNA–protein cross-linking frequency approximated that of the VP-16-induced DNA cleavage (Fig. 4). The rate of VP-16-induced DNA cleavage disappearance was slightly slower in HL-60/AMSA than in HL-60. However, residual DNA cleavage 60 min following VP-16 removal at 37° was 10–15% of that present immediately following drug removal in both lines (Fig. 4). No cleavage disappearance occurred in VP-16-treated cells incubated in drug-free medium at ice temperature (Fig. 5). The production by VP-16 of protein-associated DNA cleavage displaying temperature-sensitive reversibility in human leukemia cells was identical to results obtained by others using VP-16 in other mammalian cell systems [24, 25].

Table 1. Uptake of [^{3}H]VP-16 in human leukemia cells

Cell line	Cell associated [^{3}H]VP-16* (μmol/liter of cell water)	
	−ara-C	+ara-C†
HL-60	3.4 ± 0.2‡	4.2 ± 0.2
HL-60/AMSA	4.4 ± 0.7	4.4 ± 1.1

* Cells treatments were with 12 μM VP-16 for 60 min at 37° (see Materials and Methods and Ref. 13).

† Cells were treated with 0.1 μM ara-C for 48 hr after which ara-C was removed and [^{3}H]VP-16 treatment begun.

‡ Mean ± 1 SD of three independent determinations.

Isolated nuclei. Nuclei isolated from cells treated with or unexposed to ara-C were treated with *m*-AMSA, and the amount of DNA cleavage was quantified. *m*-AMSA-induced cleavage was augmented in nuclei isolated from ara-C-treated HL-60 or HL-60/AMSA (Table 2). The results mirrored those obtained for whole cells (Fig. 3). Cleavage was enhanced by 40% in HL-60 (five determinations) and by 55% in HL-60/AMSA (six determinations). The enhancement of *m*-AMSA-induced DNA cleavage by ara-C was significant ($P < 0.01$ by the Wilcoxon's Rank Sum Test). Thus, the biochemical alteration produced by ara-C that resulted in enhanced *m*-AMSA-induced DNA cleavage is localized in the cell nucleus.

VP-16 induced a lower DNA cleavage frequency in the isolated nuclei of both HL-60 and HL-60/AMSA than in VP-16-treated whole cells. However, the DNA of isolated nuclei from HL-60/AMSA displayed a much greater decrease in VP-16-induced

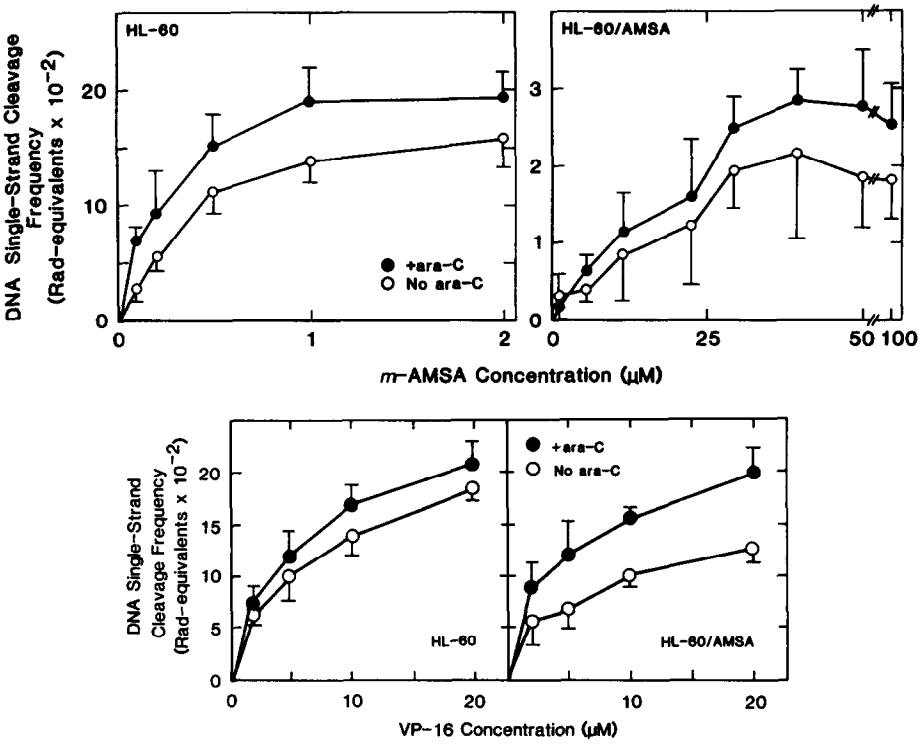

Fig. 3. (Top) Enhancement of *m*-AMSA-induced DNA cleavage by pretreating human leukemia cells with ara-C. HL-60 or HL-60/AMSA cells were either exposed to 0.1 μM ara-C for 48 hr (●) or remained in drug-free medium for 48 hr (○). Cells were treated with various concentrations of *m*-AMSA for 60 min in the absence of ara-C, and DNA single-strand cleavage was quantified using alkaline elution with proteinase. Results are expressed as rad-equivalents (see Materials and Methods and Refs. 8 and 9). Each point is the mean ± 1 SD of at least three independent determinations. Note that *m*-AMSA concentrations are lower (abscissa) and DNA cleavage frequencies higher (ordinate) on HL-60 axes than on HL-60/AMSA axes. (Bottom) Effect of pretreatment with ara-C on VP-16-induced DNA single-strand cleavage in HL-60 and HL-60/AMSA human leukemia cells. Cells either were (●) or were not (○) exposed to 0.1 μM ara-C for 48 hr prior to a 60-min treatment with various concentrations of VP-16. DNA single-strand cleavage was quantified using alkaline elution with proteinase. Results are expressed as rad-equivalents (see Materials and Methods and Refs. 8 and 13). Each point is the mean ± 1 SD of at least three independent determinations.

DNA cleavage when compared with that measured in cells than the DNA in nuclei from HL-60 (Fig. 6). This was the first example of such a marked dissociation between results in whole cells and results in isolated nuclei for topoisomerase II-reactive compounds [see Ref. 26]. Ara-C treatment of cells increased VP-16-induced DNA cleavage in nuclei isolated from the cells (Fig. 6).

Topoisomerase II activity extracted from nuclei. The topoisomerase II activity in 0.35 M NaCl extracts of HL-60 or HL-60/AMSA nuclei was quantified using [^{3}H]kDNA as a substrate (see Materials and Methods). Decatenated kDNA was separated from catenated starting material using either centrifugation (upper panels, Fig. 7) or agarose gel electrophoresis (middle and lower panels, Fig. 7). Extracts were made using nuclei from ara-C-treated or untreated cells.

The decatenating activity per μg of protein was similar in the two cell lines regardless of their prior exposure to ara-C. The amount of protein required to decatenate half of the substrate DNA was between 0.8 and 0.9 μg for HL-60 and 1.3 and 1.4 μg for HL-60/AMSA. The resistance of HL-60/AMSA DNA (cells or nuclei) to *m*-AMSA-induced DNA cleavage was not due to an absence of topoisomerase II activity. Further, the augmented DNA cleavage in ara-C-treated HL-60 cells was not due to an enhanced amount of topoisomerase II activity.

Using amounts of these extracts producing approximately 50% decatenation of substrate [^{3}H]kDNA, we used a 3'end-labeled piece of SV-40 DNA to quantify *m*-AMSA-induced 5'end DNA–protein cross-linking in each extract (Fig. 8). This assay is putatively a measure of the drug-reactivity of topoisomerase II [21]. Cross-linking was readily detectable using extracts from HL-60. Virtually none was detectable using extracts from HL-60/AMSA. Prior cell treatment with ara-C was without effect on this topoisomerase II-mediated reaction. Topoisomerase II-containing protein extracts from the nuclei of both cell lines produced equal amounts of 5'-DNA–protein cross-linking in response to VP-16 using exogenous SV-40 DNA (Fig. 8). Prior treatment of either cell line with ara-C was without effect on this result.

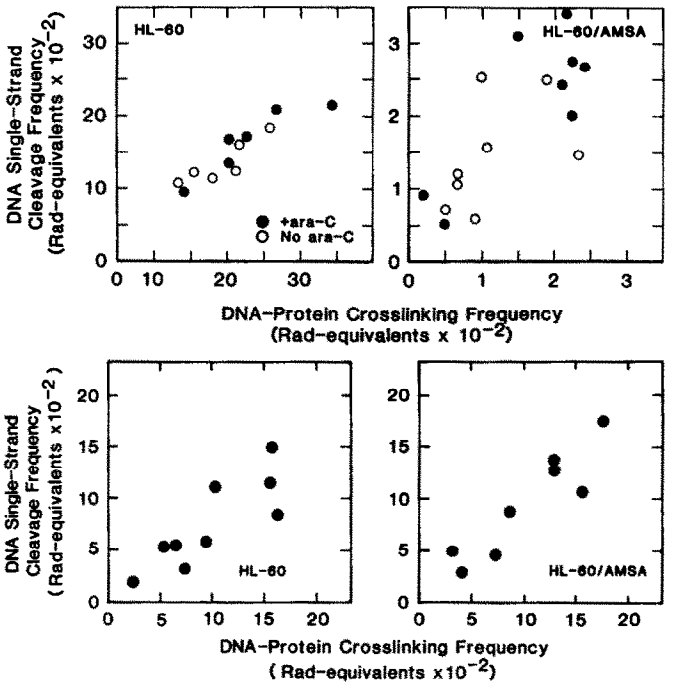

Fig. 4. (Top) Relationship between *m*-AMSA-induced DNA cleavage and *m*-AMSA-induced DNA–protein cross-linking in human leukemia cells pretreated with ara-C. HL-60 or HL-60/AMSA cells were either exposed to 0.1 μM ara-C for 48 hr (●) or remained in drug-free medium for 48 hr (○). Cells were treated with various concentrations of *m*-AMSA for 60 min in the absence of ara-C, and DNA single-strand cleavage or DNA–protein cross-linking was quantified in aliquots from a single treatment population using alkaline elution. Results are expressed as rad-equivalents (see Materials and Methods). As in Fig. 3 (top), the axes denote higher DNA cleavage and cross-link frequencies with HL-60 than with HL-60/AMSA. The mean values ± 1 SD and the median values for DNA cleavage frequency divided by DNA–protein cross-linking frequency were as follows: HL-60: 0.98 ± 0.17 and 0.92 (six determinations); HL-60 + ara-C: 0.96 ± 0.16 and 0.96 (six determinations); HL-60/AMSA: 1.42 ± 0.65 and 1.42 (eight determinations); and HL-60/AMSA + ara-C: 1.64 ± 1.10 and 1.19 (eight determinations). (Bottom) Relationship between VP-16-induced DNA cleavage and VP-16-induced DNA-protein cross-linking in HL-60 and HL-60/AMSA human leukemia cells. Cells were treated with various concentrations of VP-16 for 60 min at 37°. DNA single-strand cleavage and DNA–protein cross-linking were quantified in aliquots from a single treatment population using alkaline elution. Results are expressed as rad-equivalents (see Materials and Methods and Refs. 8 and 13). The mean values ± 1 SD and median values for DNA cleavage frequency divided by DNA–protein cross-linking frequency were: HL-60: 0.81 ± 0.24 and 0.79 (nine determinations); HL-60/AMSA: 0.94 ± 0.14 and 0.97 (eight determinations).

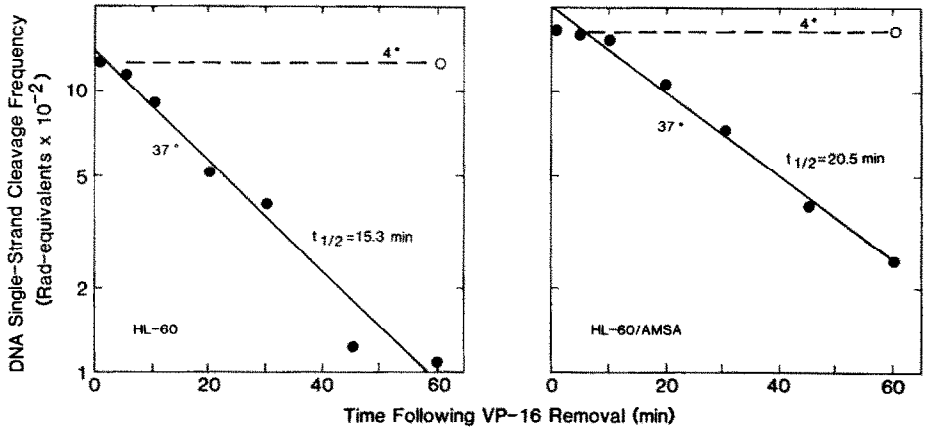

Fig. 5. Disappearance of VP-16-induced DNA single-strand cleavage in HL-60 and HL-60/AMSA human leukemia cells following drug removal. Cells were treated for 60 min at 37° with 10 μM VP-16. Drug was removed by centrifugation and resuspension of the cells in fresh, drug-free medium at either 37° (●) or 4° (○). At various times following drug removal, DNA single-strand cleavage was quantified using alkaline elution with proteinase. Results are expressed as rad-equivalents (see Materials and Methods and Refs. 8 and 13).

Table 2. Enhancement of *m*-AMSA-induced DNA cleavage in nuclei from human leukemia cells treated with ara-C

Cell line	*m*-AMSA conc* (μM)	Independent determinations (N)	DNA single-strand cleavage frequency (rad-equivalents)	
			−ara-C†	+ara-C
HL-60	1.0	1	1358	1902
	0.5	4	891 ± 288‡	1254 ± 317
HL-60/AMSA	50	3	203 ± 169	296 ± 167
	25	3	112 ± 46	192 ± 97

* All treatments with *m*-AMSA were for 30 min at 37°.
† Nuclei were isolated from cells that had (+ara-C) or had not (−ara-C) been treated with 0.1 μM ara-C for 48 hr.
‡ Data are mean ± 1 SD.

DISCUSSION

Topoisomerase II does appear to be a critical intracellular target through which the cytotoxic effects of *m*-AMSA may be mediated [1, 2]. The present results substantiate this in two ways. First, ara-C pretreatment of HL-60 cells concordantly augmented *m*-AMSA-induced cytotoxicity (Fig. 1) and *m*-AMSA-induced topoisomerase II-mediated DNA cleavage in HL-60 cells (Fig. 3). Second, the topoisomerase II activity from the *m*-AMSA-resistant HL-60/AMSA cell nuclei, while abundant (Fig. 7), was refractory to *m*-AMSA-induced 5′-DNA–protein cross-linking (Fig. 8). We therefore conclude that the cytotoxicity of *m*-AMSA in HL-60, and in L1210 [14], is somehow mediated through an interaction of the drug with topoisomerase II that can manifest itself as protein-associated DNA cleavage and is somehow amenable to augmentation by ara-C treatment (see below). This *m*-AMSA-topoisomerase II interaction was minimally demonstrable in HL-60/AMSA. In HL-60/AMSA, topoisomerase II activity was intrinsically refractory to *m*-AMSA-induced, DNA–protein complex stabilization (Fig. 8). The augmentation of *m*-AMSA-induced DNA cleavage by ara-C in HL-60/AMSA cells or nuclei was small (Fig. 3, Table 2). Thus, it is not surprising that ara-C pretreatment did not sensitize HL-60/AMSA to *m*-AMSA (Fig. 1).

Two questions are thus raised by these data. First, how might *m*-AMSA kill HL-60/AMSA (Fig. 1) if not via stabilization of a topoisomerase II–DNA complex. Second, how does ara-C augment the action of *m*-AMSA in HL-60 (and putatively L1210 [14]) if not through a quantitative (Fig. 7) or qualitative (Figs. 3 and 8, Table 2) change in the enzyme activity or in its reactivity with the drug.

m-AMSA can kill cells in at least two ways. The first is through a mechanism that has as its putative biochemical correlate the production of protein-associated DNA cleavage. It is important to note, however, that topoisomerase II cleavage sites do not exist as such within the living cell as free swivels [27, 28]. Rather, the cleavage is detectable only following protein denaturation [27]. It is presumed that drugs like *m*-AMSA stabilize a normal intermediate (cleavable complex [1, 2]) in the cycle of enzyme-mediated DNA strand passage and that this complex becomes cleaved only after chemical denaturation with detergents or alkali. Exactly how this stabilized complex (rather than true DNA helix phosphodiester backbone interruption) leads to cell death is not known. Presumably cleavable complex formation by drugs leads to a chain of other biochemical events that lead to cytotoxicity.

As HL-60/AMSA were susceptible to *m*-AMSA-induced killing (Fig. 1) despite extreme resistance to *m*-AMSA-induced DNA cleavage, HL-60/AMSA

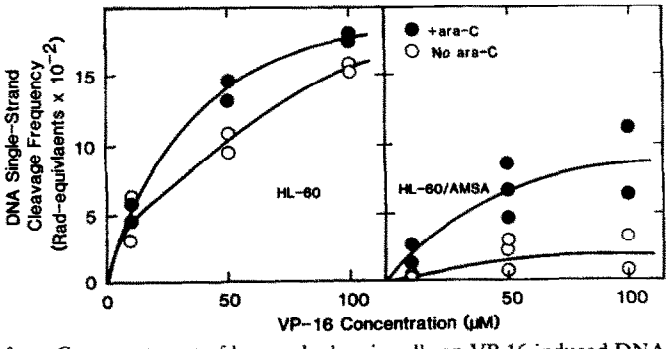

Fig. 6. Effect of ara-C pretreatment of human leukemia cells on VP-16-induced DNA cleavage in their isolated nuclei. Ara-C treatment was as described in the legend of Fig. 3. VP-16 treatment of nuclei was for 30 min at 37°. DNA cleavage measurements and quantification were as described in the legend to Fig. 3.

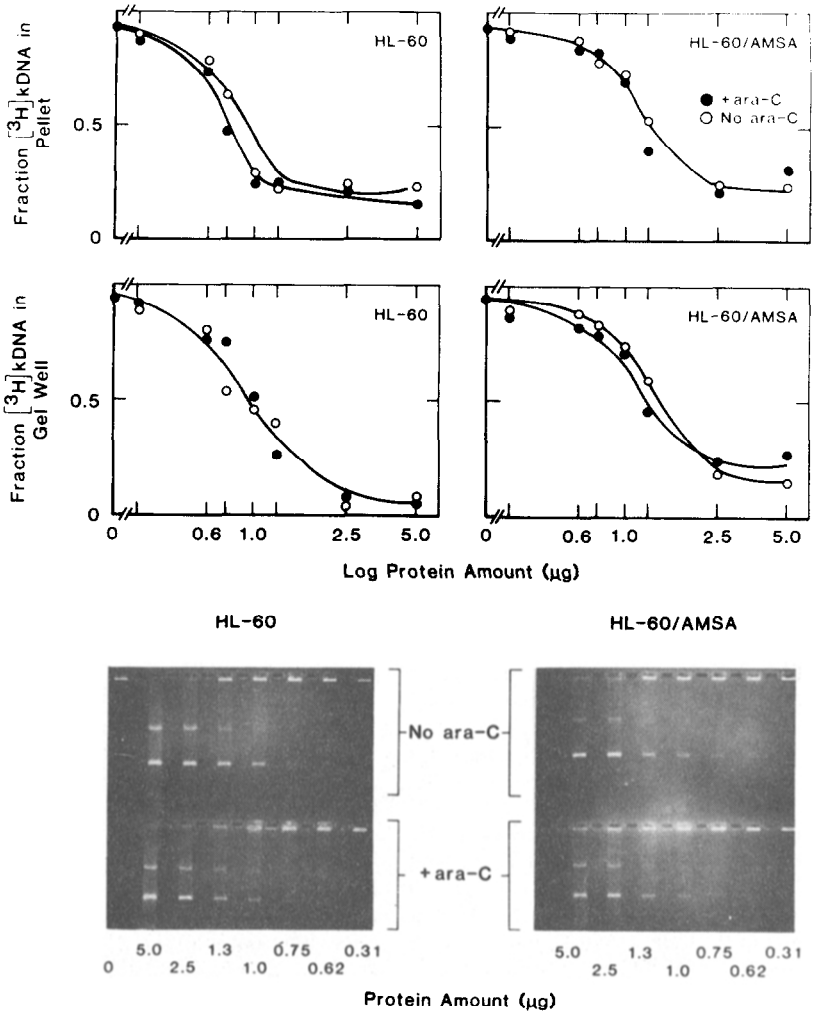

Fig. 7. Effect of ara-C treatment (0.1 μM × 48 hr) on the extractable topoisomerase II activity from HL-60 or HL-60/AMSA nuclei. The ability of 0.35 M NaCl extracts to decatenate [^{3}H]kinetoplast DNA was quantified as described in Materials and Methods. Decatenated reaction products (30 min × 37°) were separated by centrifugation at 12,000 *g* for 15 min (upper panels) or by using a 1% agarose gel electrophoresis system (middle and lower panels). Following centrifugation, the top 40 μl of a 50-μl reaction volume was removed. This contained decatenated DNA. The 10-μl pellet contained the catenated starting material. Pellet and supernatant fraction were counted using a liquid scintillation spectrometer. Results are expressed as the fraction remaining in the pellet per μg of topoisomerase II-containing protein. Replicate 50-μl reaction mixes were loaded onto agarose gels following the 30-min reaction and electrophoresed at 20 V overnight. The gels were stained with ethidium bromide (1 μg/ml) for 30 min, and DNA was visualized using an ultraviolet light box. DNA in the well (catenated starting material) was carefully excised and placed into a liquid scintillation vial. The entire remainder of the lane (decatenated DNA) was likewise processed for liquid scintillation counting. Results in the middle panels were derived from the gels in the lower panels. Key: (○) nuclear extract from cells unexposed to ara-C, and (●) nuclear extracts from cells exposed to 0.1 μM ara-C for 48 hr.

were probably killed by an alternative mechanism. Blockade of topoisomerase II-mediated strand passage (as opposed to the stabilization of a DNA–topoisomerase II complex) is one possibility as this effect is produced by *m*-AMSA [29]. An alternative mechanism for *m*-AMSA cytotoxicity in HL-60/AMSA could be free-radical-mediated DNA cleavage [30]. However, such cleavage is characteristically not protein-concealed when produced by other agents such as ionizing radiation [31]; thus this mechanism is not a likely cause of the DNA cleavage produced in HL-60/AMSA (Fig. 3). Non-DNA-related cytotoxic mechanisms are also possible. Regardless, the mechanism of *m*-AMSA-induced cytotoxicity in HL-60/AMSA was not accessible to modulation by ara-C (Fig. 1).

Ara-C modulation of *m*-AMSA-induced DNA cleavage and cytotoxicity was readily produced in HL-60 (Fig. 1) as had been the case in murine leukemia cells [14]. However, ara-C treatment did not alter the quantity of nuclear topoisomerase II activity (Fig. 7). Ara-C treatment did not quantitatively alter

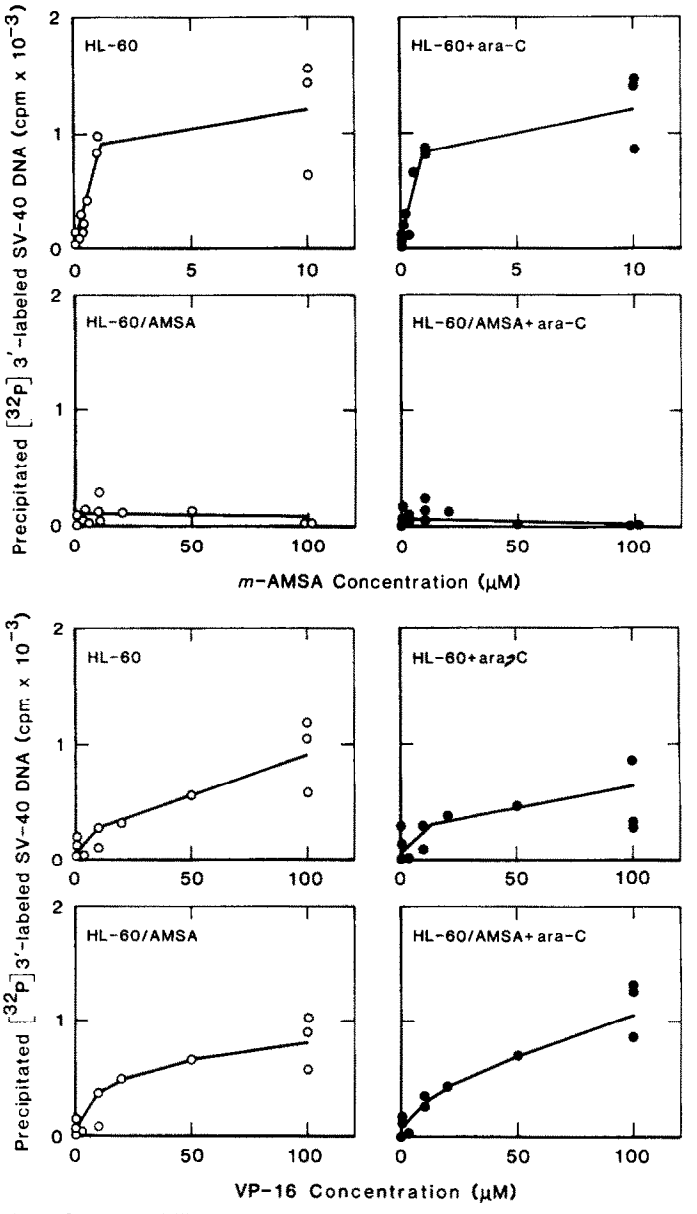

Fig. 8. Effect of ara-C on the ability of topoisomerase II-containing nuclear extracts to bind to 3′end-labeled SV-40 DNA in the presence of *m*-AMSA or VP-16. (Top) [^{32}P]3′end-labeled DNA was incubated with 0.9 μg of HL-60 nuclear extract or 1.3 μg of HL-60/AMSA nuclear extract for 30 min at 37° in the presence of various concentrations of *m*-AMSA. 5′ DNA–protein cross-linking was quantified using the alkaline SDS-KCl method of Liu *et al.* [21] as described in Materials and Methods. Extracts used in these experiments were identical to those used in experiments depicted in Fig. 7. (Bottom) [^{32}P]SV-40 was incubated with 0.9 μg of HL-60 nuclear extract or 1.3 μg of HL-60/AMSA nuclear extract for 30 min at 37° in the presence of various concentrations of VP-16. 5′DNA–protein cross-linking was quantified using the alkali SDS-KCl method of Liu *et al.* [21]. Key: (○) nuclear extract from cells unexposed to ara-C; and (●) nuclear extract from cells exposed to ara-C (0.1 μM × 48 hr).

the ability of *m*-AMSA to stabilize a topoisomerase II–DNA complex (Fig. 8). Ara-C treatment did not alter the amount of cell-associated *m*-AMSA (Fig. 2). Ara-C treatment did not qualitatively alter the DNA cleavage produced in HL-60 cells (Figs. 3 and 4). Thus, a possible target of the action of ara-C is cellular chromatin [32], the putative site of topoisomerase II action, rather than the enzyme itself. Topoisomerase activities have been modified by DNA sequence changes [33] and by changes in DNA structure exerted by non-topoisomerase DNA-binding proteins [34]. Further, topoisomerase II DNA cleavage sites within a homogeneous DNA target sequence are not identical for the different topoisomerase II-reactive drugs [35]. Thus, drug-induced topoisomerase II-mediated DNA cleavage in cells must result from a tripartite interaction between drug, enzyme, and cellular chromatin. Modulations

in the resulting amount or locale of DNA cleavage produced by a drug treatment can thus be effected through any and all components of the system.

The VP-16 results raise several additional points. Most importantly cellular resistance to one topoisomerase II-reactive compound does not necessarily indicate comparable cross-resistance to all topoisomerase II-reactive compounds. Further, the inability of a modulating drug like ara-C to overcome resistance to one topoisomerase II-reactive drug (e.g. *m*-AMSA) does not mean topoisomerase II-mediated DNA cleavage (Fig. 3) or associated cytotoxicity (Fig. 1) in the resistant cell line cannot be modulated when a different topoisomerase II-reactive drug is used (e.g. VP-16). Finally, the unusual characteristic of decreased susceptibility of the DNA of isolated HL-60/AMSA nuclei to VP-16-induced cleavage would not have been detected using *m*-AMSA, as this cell line (Fig. 3) and its topoisomerase II activity (Fig. 8) are intrinsically resistant to *m*-AMSA-induced DNA cleavage. We cannot explain this result. It certainly is not due to a selective depletion of topoisomerase II from HL-60/AMSA as opposed to HL-60 nuclei as it is from similar nuclei that the topoisomerase II-containing nuclear extracts are made (Fig. 7). Either the VP-16 reacts aberrantly with HL-60/AMSA nuclei or the topoisomerase II–drug–chromatin interaction to which we alluded above is different in HL-60/AMSA nuclei and HL-60 nuclei and leads to decreased cleavability of HL-60/AMSA nuclear chromatin.

Of great interest, the HL-60/AMSA cells were highly sensitized to VP-16 following exposure to ara-C. This indicates that the small effect of ara-C on *m*-AMSA-induced events in HL-60/AMSA was not due to the decreased incorporation of [^{3}H]ara-C in HL-60/AMSA. The magnitude of this cytotoxic sensitization was greater than anticipated from DNA cleavage measurements (Fig. 3). The discrepancy between the relatively small effect on VP-16-induced DNA cleavage produced by ara-C treatment of HL-60/AMSA (Fig. 3) and the relatively large effect on VP-16-induced cytotoxicity (Fig. 1) is not readily explicable. Once again, DNA cleavage (cleavable complex formation) may not fully explain the cytotoxic actions of these drugs [36, 37]. That HL-60/AMSA could be killed by *m*-AMSA despite extremely low levels of *m*-AMSA-induced cleavage formation adequately demonstrated this point (Fig. 1). As the cytocidal events that follow DNA cleavable-complex formation are not as yet described, ara-C could affect these events giving rise to a discrepancy between ara-C's modulation of VP-16-induced DNA cleavage and VP-16-induced cytotoxicity. These events may be even more crucial to the way *m*-AMSA and VP-16 kill sensitive cells, the way these same drugs do not kill resistant cells, and the way drugs like ara-C can modulate cytotoxicity.

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