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ACTIVITY OF TWO NOVEL ANTHRACENE-9,10-DIONES AGAINST HUMAN LEUKEMIA CELLS CONTAINING INTERCALATOR-SENSITIVE OR -RESISTANT FORMS OF TOPOISOMERASE II

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Abstract—We have examined the activities of two novel azaanthracene-9,10-diones (aza), 1-aza and 2-aza, in HL-60 human leukemia cell lines containing type II topoisomerases with different sensitivities to inhibition by other intercalating agents. The sensitive line, HL-60, was sensitive to 2-aza but not to 1-aza, whereas the resistant HL-60/AMSA was sensitive to neither agent. Measurements of 1- and 2-aza-induced, topoisomerase II-mediated DNA cross-linking in the cells revealed patterns of resistance and sensitivity that paralleled the results in the cytotoxicity assays. However, measurements of drug-induced topoisomerase II-mediated DNA cross-linking using purified HL-60 and HL-60/AMSA topoisomerase II indicated that *both* agents could stabilize a covalent complex between DNA and the HL-60 enzyme. HL-60/AMSA topoisomerase II resisted stabilization by either agent. This suggests that the resistance of HL-60 cells to 1-aza is *not* due to the inability of this drug to inhibit topoisomerase II but rather to another, undefined mechanism.

Identifying the precise determinants of the cytotoxicity of an antineoplastic drug requires detailed knowledge of the structure of the drug, its target, and the cellular processes interrupted by the drug–target interaction. We have been studying a series of compounds that target the vital intracellular enzyme topoisomerase II [1–3]. These studies have been aided by using drugs [4], topoisomerases [5], and DNAs of various structures.

We recently described some of the structural determinants of the cytotoxic actions of intercalating amsacrine analogs [4]. This was done using various amsacrine derivatives and both intercalator-sensitive and intercalator-resistant forms of topoisomerase II from human leukemia cells [4, 5]. In the present work, we explored the determinants of the cytotoxic actions of 1-azaanthracene-9,10-dione (1-aza§) and 2-azaanthracene-9,10-dione (2-aza) (Fig. 1), newly synthesized derivatives of the topoisomerase II-reactive intercalator mitoxantrone, using the same sensitive/resistant cell pair. Unlike the previous work, in which most biochemical findings would have been predicted from results of cellular studies, these new findings revealed an unexpected discrepancy between cellular and biochemical results that suggests a mechanism of cellular anthracendione resistance unrelated to topoisomerase II.

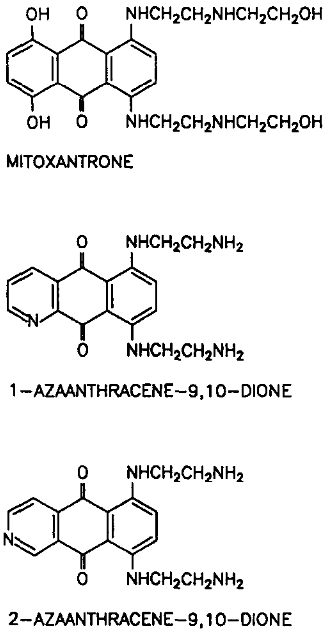

Fig. 1. Structures of anthracenedione analogs.

MATERIALS AND METHODS

Cells and radioactive labeling. H-60 and HL-60/AMSA cells were provided initially by Drs. M. Beran and B. Andersson of the Department of Hematology, The University of Texas M.D.

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§ Abbreviations: 1-aza, 1-azaanthracene-9,10-dione; 2-aza, 2-azaanthracene-9,10-dione; SDS, sodium dodecyl sulfate; SV40, simian virus 40; and kDNA, kinetoplast DNA.

Anderson Cancer Center. Initial characterization of the lines and of the genesis of HL-60/AMSA has been reported elsewhere [6]. The cells were propagated in our laboratory in Iscove's Modified Dulbecco's medium (JRH Biosciences, Lenexa, KS) and 10% fetal bovine serum at 37° in 5% CO_2 as previously described [4]. The resistance of HL-60/AMSA was stable and could be maintained without exposure to amsacrine. The doubling times of the cells were similar (approximately 20 hr) [7]. All cells were Mycoplasma free (American Type Culture Collection, Rockville, MD). Mouse leukemia L1210 cells served as internal standard cells in alkaline elution experiments (see below).

HL-60 or HL-60/AMSA cells were radiolabeled with 0.03 μCi/mL of [2-^{14}C]thymidine (New England Nuclear, Boston, MA) for 48 hr to label their cellular DNA for alkaline elution experiments (see below). Another group of cells was labeled with 0.6 μCi/mL of [*methyl*-^{3}H]thymidine and 0.2 μCi/mL of [^{14}C]-leucine for 24 hr for sodium dodecyl sulfate (SDS)–KCl precipitation experiments (see below). L1210 cells were labeled with 0.1 μCi/mL [*methyl*-^{3}H]-thymidine and served as the internal standard in alkaline elution experiments [7, 8]. Cells were incubated for at least 1 hr in radiolabel-free medium prior to treatment with any drug.

Drugs. Mitoxantrone (NSC 279836) was obtained from the National Cancer Institute and constituted as a stock solution in 0.02 N HCl. The azaanthracene-9,10-diones were synthesized at the Vermont Cancer Center. (Details of these syntheses are included in a series of manuscripts currently being submitted for publication.) They were constituted as 5×10^{-3} M stock solutions in water.

DNA. Kinetoplast DNA (kDNA) was isolated from the trypanosome *Crithidia fasciculata* as previously described [7]. The kDNA was radiolabeled with [*methyl*-^{3}H]thymidine by incubating the trypanosomes with the label prior to isolating kDNA from Sarkosyl extracts using cesium chloride-ethidium bromide density centrifugation.

Covalently closed, supercoiled simian virus 40 (SV40) DNA and calf thymus topoisomerase I were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Topoisomerase II from HL-60 and HL-60/AMSA was purified using a modification of the method of Drake and co-workers [9–11] as previously described [4].

Studies with intact cells. Soft agar colony formation assays were performed as previously described using the method of Chu and Fisher [12]. DNA alkaline elution by the method of Kohn was performed as previously described [8].

The SDS–KCl precipitation assay of DNA–protein complexes was as described by Trask and co-workers [13, 14] and as modified by Liu *et al.* [15, 16] and by Denstman *et al.* [17]. Cells (4×10^5) with their DNA and protein radiolabeled (see above) were treated with various concentrations of drugs for 1 hr at 37° as described above. Data are expressed as the ratio of [^{3}H]DNA to [^{14}C]protein. The protein serves as an internal measure of the exact number of cells used for any given experimental condition.

Biochemical assays. Decatenation was used to quantify amounts of purified topoisomerase II [4, 5, 7, 18]. This has been described in detail in several previous publications. In experiments quantifying the inhibition of decatenation (i.e. strand passage) by the various topoisomerase II-reactive drugs, calculations were as follows:

$$\mathrm{FSP} = \frac{(\mathrm{F\ kDNA\ in\ well})^{\mathrm{No\,protein}} - (\mathrm{F\ kDNA\ in\ well})^{+\,\mathrm{Protein}}}{(\mathrm{F\ kDNA\ in\ well})^{\mathrm{No\,protein}}}$$

where F kDNA is the fraction of kDNA in the well of the agarose gel and FSP is the fraction of strand passage for each experimental condition. Then, to calculate the inhibition of strand passage produced by the drugs, the following formula was used:

$$\mathrm{Inhibition} = \frac{(\mathrm{FSP})^{\mathrm{No\,drug}} - (\mathrm{FSP})^{+\,\mathrm{Drug}}}{(\mathrm{FSP})^{\mathrm{No\,drug}}}$$

Drug-induced DNA–protein cross-linking was quantified via the precipitation of uniquely 3′ end-labeled [^{32}P]SV40 DNA using the SDS–KCl precipitation method [7, 15]. Results are expressed as the cpm precipitated in the presence of drug minus that precipitated in the absence of drug.

Intercalation and the inhibition of type I topoisomerase-mediated DNA relaxation were measured as previously described [4] after techniques developed by Pommier *et al.* [19].

RESULTS

The 2-aza analog was more cytotoxic to HL-60 than the 1-aza analog (Fig. 2). Neither agent was particularly cytotoxic to HL-60/AMSA. HL-60/AMSA was resistant to mitoxantrone as well, although mitoxantrone is more cytotoxic to HL-60 than either of the new analogs [18]. The results of the cytotoxicity assays were paralleled by results of measurements of drug-induced DNA–protein cross-linking by the SDS–KCl method (Fig. 3) or the alkaline elution method (Fig. 4, see Ref. 8). The DNA–protein cross-linking produced by 2-aza in HL-60 was accompanied by an approximately equal frequency of DNA cleavage (ratio of DNA breaks to cross-links was 1.13 ± 0.36 for $N = 4$, data not shown), as expected for the DNA effects of topoisomerase II-directed agents [8].

The data in Fig. 5 confirm the intercalative capacity of the new analogs. When topoisomerase I acted upon supercoiled DNA, the supercoils were relaxed and the DNA migrated as form I^0 (lane marked 0 in Fig. 5, top). If that DNA contained intercalated drug, the resultant structure following drug removal and the inactivation of the enzyme by SDS and proteinase K would be different from the structure when the topoisomerase acted on DNA in the absence of intercalated drug (Fig. 5, top, lanes containing drug, and Refs. 4 and 19).

The 2-aza analog was a better intercalator than the 1-aza analog [topoisomerase I could overcome the drug effect at a higher concentration of 1-aza (5 μM) than of 2-aza (2 μM)]. Additionally, both analogs were capable of inhibiting topoisomerase I-mediated relaxation of closed circular, supercoiled DNA (Fig. 5, bottom, see legend and Refs. 4 and 19) as concentrations of drug producing comparable

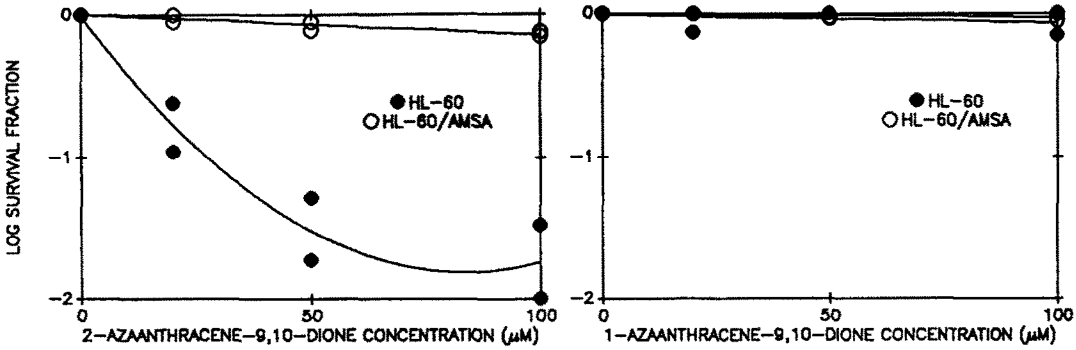

Fig. 2. Cytotoxicity of 2-aza (left) and 1-aza (right) against the human leukemia cell lines HL-60 and HL-60/AMSA. Exponentially growing cells were exposed to various drug concentrations for 1 hr at 37° followed by soft agar colony formation, as previously described [12]. The results from two independent experiments are shown.

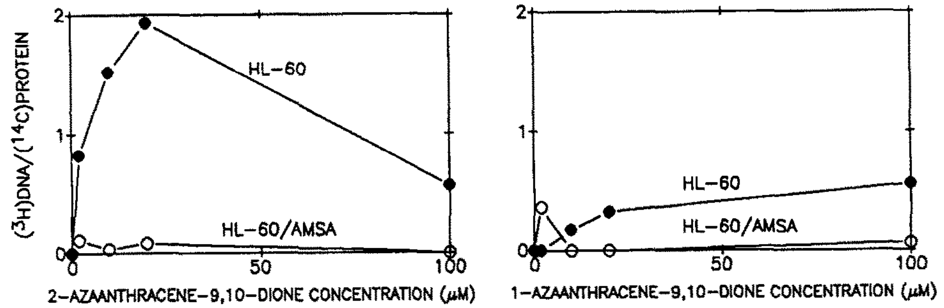

Fig. 3. Quantification of SDS–KCl precipitable DNA–protein complexes from HL-60 and HL-60/AMSA cells following their exposure to various concentrations of 2-aza (left) or 1-aza (right). Drug treatments were for 1 hr at 37°. Data from this single experiment are expressed as the ratio of precipitable radiolabeled DNA to precipitable radiolabeled protein (see Materials and Methods and Refs. 13–18).

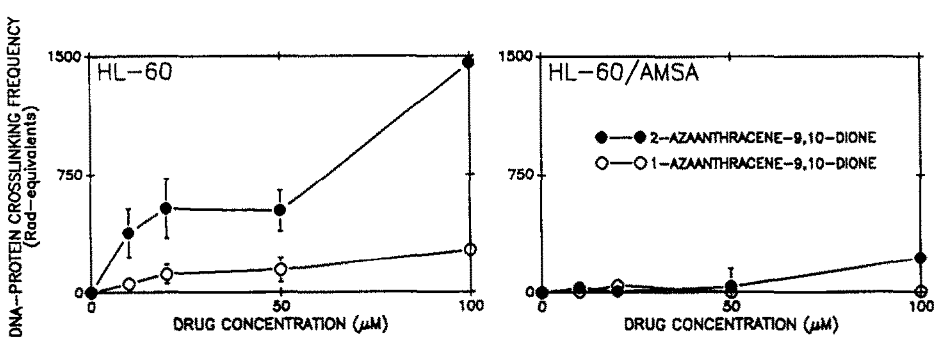

Fig. 4. Quantification of 2-aza- or 1-aza-induced DNA–protein cross-links in HL-60 or HL-60/AMSA cells using the alkaline elution method [8]. Cell treatments were for 1 hr at 37°. Values are means ± 1 SD for at least three independent experiments. If fewer than three experiments were performed, individual values are shown.

intercalation effects using 2 units of topoisomerase I (Fig. 5, top) also reduced the amount of topoisomerase I-mediated strand passage at 1 and 0.75 units of topoisomerase I (Fig. 5, bottom) without changing the structure (gel migration patterns) of the resultant DNA.

Topoisomerase II is the more likely intracellular target of these drugs and their more commonly

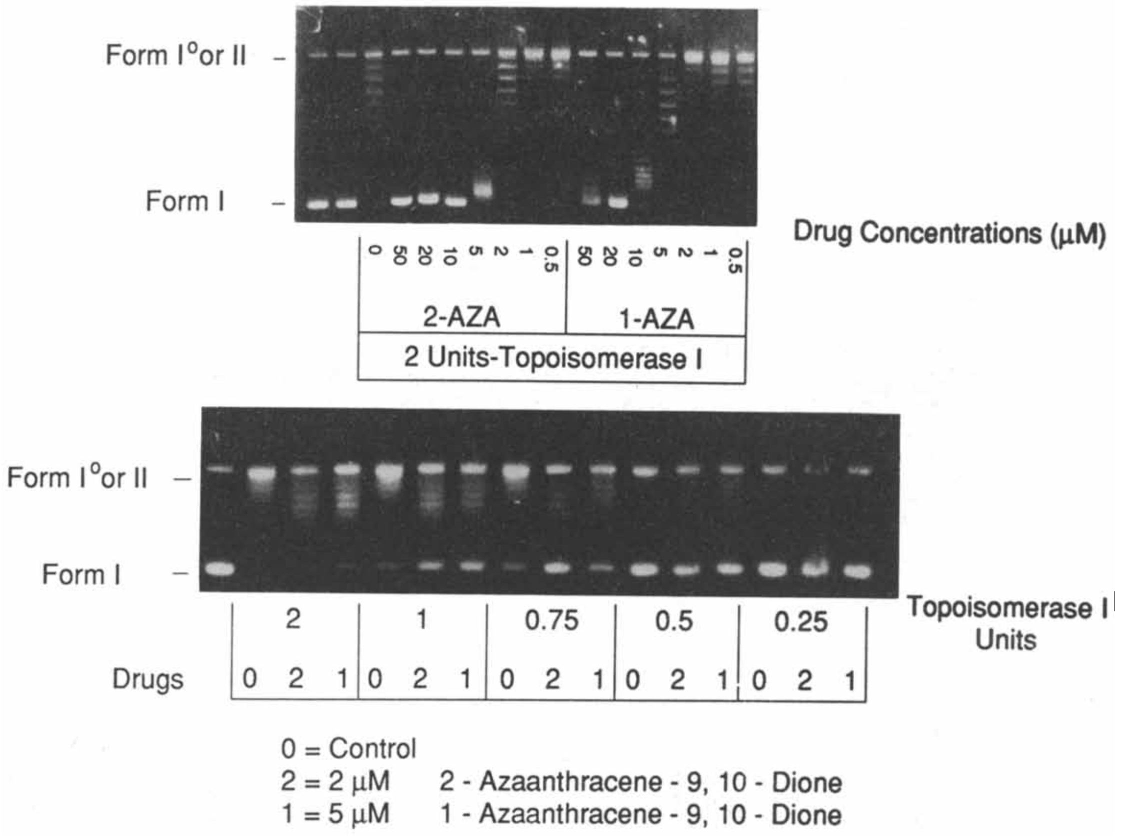

Fig. 5. DNA intercalation by 2-aza and 1-aza. Top, various concentrations of the two drugs were incubated with 0.2 µg of covalently closed, supercoiled SV40 DNA for 30 min at 37° and 2 units of topoisomerase I. The reaction was stopped with SDS and proteinase K, and the reaction products separated on agarose gels (see Materials and Methods and Ref. 4). The location of form I (supercoiled), form I° (relaxed) or II (nicked) DNA is identified to the left of the figure. The ladder patterns between forms I and II are topoisomers of SV40 DNA produced by the topoisomerase I acting on DNA containing intercalated drug [19]. Bottom, utilizing concentrations of 2-aza or 1-aza producing comparable effects on relaxation by 2 units of topoisomerase I (top), we then examined the effects of these drugs on DNA relaxation by lesser amounts of topoisomerase I as per Pommier *et al.* [19]. The greater amount of form I DNA in the drug-treated lanes containing 1 and 0.75 units of topoisomerase I without altering the topoisomer pattern indicates that the drugs block topoisomerase I relaxation as well as intercalate. (The unmarked lanes contain SV40 starting material only, as a control.)

employed analog mitoxantrone [18]. Mitoxantrone (Fig. 6) and *both* azaanthracene-9,10-diones (Fig. 7) stimulated the formation of a stable complex between purified HL-60 topoisomerase II and SV40 DNA. However, none of the three agents could overcome the resistance of HL-60/AMSA topoisomerase II to intercalator-induced complex formation. The biphasic curves observed with all three drugs were reminiscent of observations we have made previously with mitoxantrone (and other intercalators) using nuclear extracts as a source of topoisomerase II [18].

Finally, we examined the abilities of 2-aza and 1-aza to interfere with the abilities of HL-60 and HL-60/AMSA topoisomerase II to pass DNA double strands (Fig. 8). For the most part, 2-aza was the more potent of the two drugs. However, unlike the production of topoisomerase II–DNA complex formation, strand passage blockade using HL-60/AMSA topoisomerase was drug sensitive.

In summary, while neither new intercalating agent could overcome the resistance of HL-60/AMSA cells, the 2-aza analog (but not the 1-aza analog) was cytotoxic to intercalator-sensitive HL-60 cells. In contrast, biochemical assays revealed that the ability of HL-60 topoisomerase II to be stabilized in a complex with DNA was sensitive to both agents. HL-60/AMSA topoisomerase II was sensitive to stabilization by neither agent. However, both enzymes were sensitive to both drugs when the ability of the drugs to inhibit topoisomerase II-mediated DNA strand passage was measured.

DISCUSSION

In previous work, we used computer simulations

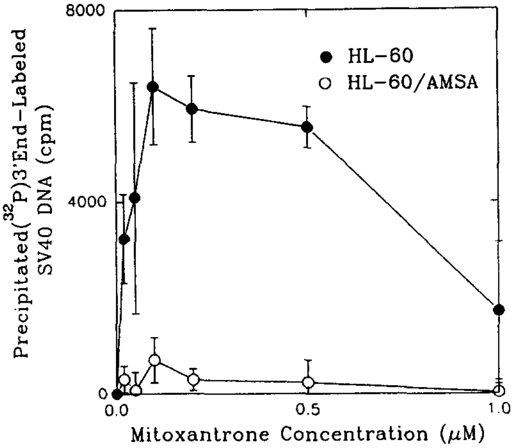

Fig. 6. Drug-induced, topoisomerase II-mediated DNA–protein cross-linking by mitoxantrone. The SDS–KCl method (see Materials and Methods) was used to quantify the ability of various concentrations of mitoxantrone to stabilize a complex between SV40 DNA and purified HL-60 or HL-60/AMSA topoisomerase II. Incubations were for 30 min at 37°. The amounts of topoisomerase II used were 1- (for HL-60) or 5- (for HL-60/AMSA) times that required to decatenate 50% of the kDNA used in our standard assay of topoisomerase II activity (see Ref. 4 and Materials and Methods). Values are the means of three independent experiments ± 1 SD. If fewer than three experiments were performed, individual values are shown.

of intercalated drug structures to hypothesize why a particular amsacrine analog was ineffective as a cytolytic agent and as an inhibitor of topoisomerase II [4]. In the work presented here, results of cellular studies (Figs. 2–4) suggested that noncytotoxic 1-aza, like noncytotoxic *o*-AMSA, would be incapable of inhibiting topoisomerase II. However, the data in Fig. 7 demonstrate that both 2-aza *and* 1-aza can stabilize the covalent interaction between HL-60 topoisomerase II and DNA. This suggests that the reason 1-aza was an ineffective cytotoxic agent (Fig. 2) even against intercalator-sensitive cells was not because of its inability to inhibit topoisomerase II. Rather, it is likely that 1-aza cannot reach intracellular targets like topoisomerases because it does not penetrate cells as readily as the 2-aza analog.

Like mitoxantrone (Fig. 6) and a number of other topoisomerase II-reactive DNA intercalating agents [18], 1- and 2-aza produced biphasic concentration–response curves when drug-induced DNA–protein cross-linking was quantified (Figs. 3 and 7). We recently demonstrated that drugs producing this type of effect are probably inhibiting topoisomerase II "at a position in its cycle prior to the step at which cleavable complex even forms" [18]. Despite this inhibitory effect of higher concentrations of these drugs and the associated absence of cleavable complex formation, the cytotoxicity of the drugs was not decreased [18], suggesting that the *inhibition* of topoisomerization may itself be cytotoxic even in the absence of the production of cleavable complexes. As 2-aza cytotoxicity did not decrease (Fig. 2) even at concentrations at which cross-linking declined (Figs. 3 and 7), the inhibition of topoisomerase II by 2-aza in HL-60 cells may account for the cytotoxicity of the drug at elevated concentrations.

We were surprised by two findings in this work. The first was the activity of 1-aza in biochemical systems despite its inactivity in cellular systems. This has been discussed above. The second was the activity of 1- and 2-aza to inhibit both HL-60 *and* HL-60/AMSA topoisomerase II-mediated strand passage (Fig. 8). However, we recently noted a similar phenomenon with analogs of amsacrine. Amsacrine itself was rather ineffective as an inhibitor of HL-60/AMSA strand passage, but two amsacrine

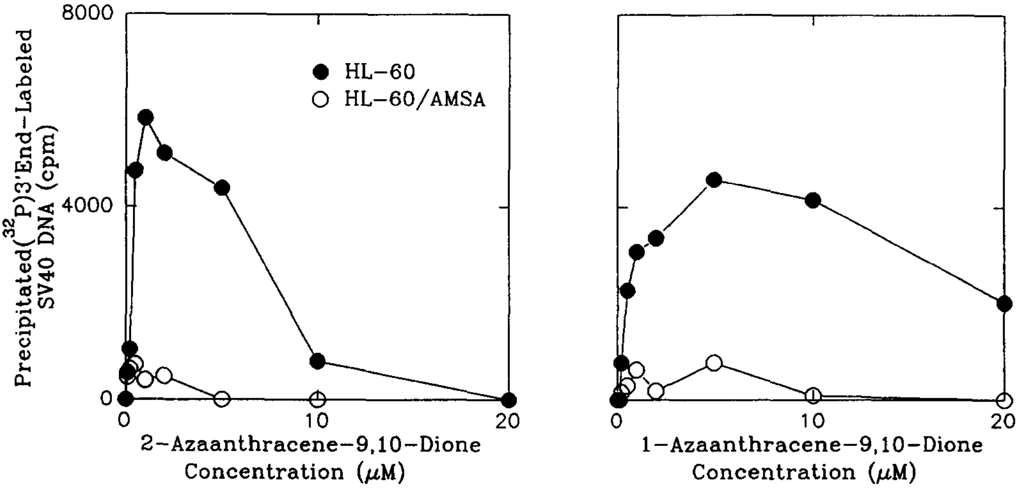

Fig. 7. Drug-induced, topoisomerase II-mediated DNA–protein cross-linking by 2-aza (left) or 1-aza (right). Experiments were performed exactly as those whose results are shown in Fig. 6. Data shown are the means of two independent experiments.

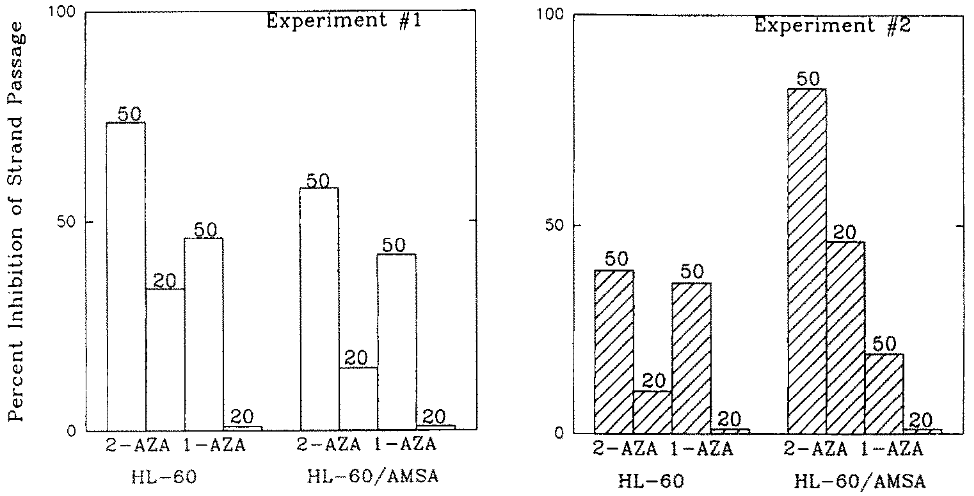

Fig. 8. Drug-induced inhibition of topoisomerase II-mediated DNA strand passage. Either 50 or 20 μM 2-aza or 1-aza was incubated with 3- (Experiment No. 1) or 1.5- (Experiment No. 2) times the amount of purified topoisomerase II needed to decatenate 50% of the kDNA used in our standard assay (see Materials and Methods). The fraction of strand passage produced by these amounts of topoisomerase II were: Experiment No. 1, HL-60 0.92, HL-60/AMSA 0.93; Experiment No. 2, HL-60 0.9, HL-60/AMSA 0.78. Data are expressed as the per cent inhibition of strand passage as calculated using the formulae given in Materials and Methods.

analogs were inhibitory [4, 5]. Several important points need to be made regarding the relationship of strand-passage inhibition to cytotoxicity. First, the amount of drug needed to inhibit strand passage was greater than that needed to stabilize topoisomerase II–DNA complexes (compared Figs. 7 and 8 and Ref. 4) and in some cases was greater than that needed to produce cytotoxicity [4, 18]. Second, drugs have been described that can inhibit strand passage without producing any cleavable complex formation [20]. Third, though inhibition of strand passage can undoubtedly be cytotoxic [18], the correlation between cytotoxicity and cleavable complex formation is better, particularly when the necessity for large, extremely toxic concentrations of drug to inhibit strand passage is considered. Thus, although topoisomerase II-directed agents can inhibit the enzyme at different steps in its topoisomerization cycle [18, 21], the stabilization of the cleavable complex is still the most likely initiator of the pathway to cell death.

The anthracenedione analogs resemble the amsacrine analogs [4] in that a very small change in structure can render an analog inactive. This suggests that key drug–target interactions have fastidious biochemical requirements if cytotoxicity is to occur. However, unlike our results with the amsacrine analogs, the results here indicate a non-topoisomerase II-mediated mechanism of the cellular resistance to the "inactive" analog. Thus, a note of caution should be raised concerning the evaluation of future analogs *only* in cellular systems. Direct biochemical studies are needed before one can be sure of the mechanism by which cells resist the cytotoxic actions of any new agent. While we cannot be certain why 1-aza did not kill HL-60 cells, an uptake-mediated mechanism of resistance is hypothesized. Regardless, it appears that the reason was not the inactivity of 1-aza as an inhibitor of topoisomerase II.

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