Cross-Resistance of an Amsacrine-Resistant Human Leukemia Line to Topoisomerase II Reactive DNA Intercalating Agents. Evidence for Two Topoisomerase II Directed Drug Actions†

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ABSTRACT: HL-60/AMSA is a human leukemia cell line that is 50–100-fold more resistant than its drug-sensitive HL-60 parent line to the cytotoxic actions of the DNA intercalator amsacrine (*m*-AMSA). HL-60/AMSA topoisomerase II is also resistant to the inhibitory actions of *m*-AMSA. HL-60/AMSA cells and topoisomerase II are cross-resistant to anthracycline and ellipticine intercalators but relatively sensitive to the nonintercalating topoisomerase II reactive epipodophyllotoxin etoposide. We now demonstrate that HL-60/AMSA and its topoisomerase II are cross-resistant to the DNA intercalators mitoxantrone and amonafide, thus strongly indicating that HL-60/AMSA and its topoisomerase II are resistant to topoisomerase II reactive intercalators but not to nonintercalators. At high concentrations, mitoxantrone and amonafide were also found to inhibit their own, *m*-AMSA's, and etoposide's abilities to stabilize topoisomerase II–DNA complexes. This appears to be due to the ability of these concentrations of mitoxantrone and amonafide to inhibit topoisomerase II mediated DNA strand passage at a point in the topoisomerization cycle prior to the acquisition of the enzyme–DNA configuration that yields DNA cleavage and topoisomerase II–DNA cross-links. In addition, amonafide can inhibit the cytotoxic actions of *m*-AMSA and etoposide. Taken together, these results suggest that the cytotoxicity of *m*-AMSA and etoposide is initiated primarily by the stabilization of the topoisomerase II–DNA complex. Other topoisomerase II reactive drugs may inhibit the enzyme at other steps in the topoisomerization cycle, particularly at elevated concentrations. Under these conditions, these latter drugs may not produce protein-associated DNA cleavage while still inhibiting topoisomerase II function as well as the actions of other topoisomerase II reactive drugs.

Topoisomerase II is an enzyme that catalyzes DNA double-strand passage and is required for postsynthetic DNA chromosomal segregation (Zwelling, 1989) as well as other critical, DNA-dependent cellular processes. It is the target of a number of the most active antineoplastic drugs, including adriamycin, etoposide, and amsacrine (*m*-AMSA)[1] (Liu, 1989; Beck, 1989; Zwelling, 1989). The drugs stabilize a DNA–topoisomerase II complex, an event thought to lead to the inhibition of enzyme function and to subsequent cell death. Low amounts of the enzyme lead to little complex stabilization and relatively reduced drug-induced cytotoxicity. In addition, topoisomerase II that resists drug-induced complex stabilization has been isolated from drug-resistant mammalian cells (Drake et al., 1989a; Sullivan et al., 1989; Zwelling et al., 1989). The presence of a resistant form of topoisomerase II probably explains the resistance of the cells.

HL-60/AMSA is an example of a human cell line selected for its resistance to *m*-AMSA (Beran & Andersson, 1987). HL-60/AMSA topoisomerase II is resistant to stabilization in a complex with DNA by *m*-AMSA (Zwelling et al., 1989). HL-60/AMSA and its topoisomerase II are cross-resistant to topoisomerase II reactive drugs from the anthracycline and ellipticine families. Anthracyclines, ellipticines, and *m*-AMSA are DNA intercalating agents, that is, drugs that interdigitate between adjacent DNA base pairs and untwist the DNA helix. Most topoisomerase II reactive drugs intercalate, but the topoisomerase II reactive epipodophyllotoxins etoposide and teniposide do not. Interestingly, in contrast to most other cell lines exhibiting resistance to topoisomerase II reactive intercalators, HL-60/AMSA and its topoisomerase II are sensitive to etoposide.

The present study had the following objectives: (1) to examine whether HL-60/AMSA was cross-resistant to topoisomerase II reactive DNA intercalating agents from other chemical classes containing drugs of clinical importance; (2) to examine the sensitivity or resistance of HL-60/AMSA to the *topoisomerase I* reactive compound camptothecin; (3) to examine the sensitivity or resistance of HL-60/AMSA to actinomycin D, an intercalating agent reported to have *both* topoisomerase I (Trask & Muller, 1988) and topoisomerase II (Tewey et al., 1984a) reactive properties; and (4) to examine

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[1] Abbreviations: *m*-AMSA, amsacrine or 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide; etoposide, VP-16 or 4′-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside); teniposide, VM-26 or 4′-demethylepipodophyllotoxin 9-(4,6-*O*-2-thenylidene-β-D-glucopyranoside); mitoxantrone, 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)-amino]ethyl]amino-9,10-anthracenedione; BIDA, amonafide or benzisoquinolinedione; CPT, camptothecin; ActD, actinomycin D; SDS, sodium dodecyl sulfate; SV40, simian virus 40; kDNA, kinetoplast DNA.

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whether the cellular findings were mirrored by results of more mechanistic experiments in well-characterized biochemical systems. In the course of fulfilling these objectives, we found an unexpected self-inhibitory action of two of the topoisomerase II reactive intercalating agents. This allowed us to perform experiments the results of which lead us to hypothesize how certain topoisomerase II reactive agents can inhibit topoisomerase II and kill cells without necessarily stabilizing the topoisomerase II–DNA complex.

Experimental Procedures

Cells and Radioactive Labeling. HL-60 and HL-60/AMSA were intially provided by Drs. M. Beran and B. Andersson of the Department of Hematology, The University of Texas M. D. Anderson Cancer Center. Initial characterization of the lines and of the genesis of HL-60/AMSA has been reported elsewhere (Beran & Andersson, 1987). The cells were propagated in our laboratory in Iscove's modified Dulbecco's medium (Hazleton, Lenexa, KS) and 10% fetal calf serum at 37 °C in 5% CO_2 as previously described (Zwelling et al., 1989). The resistance of HL-60/AMSA was stable and could be maintained without exposure to *m*-AMSA. The doubling times of the cells were similar (approximately 28 h) [see Bakic et al. (1987)]. 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 and HL-60/AMSA cells were radiolabeled with 0.05 μCi/mL [2-^{14}C]thymidine (New England Nuclear, Boston, MA) for 48 h to label their cellular DNA for alkaline elution experiments (see below). These cells were labeled with 0.6 μCi/mL [*methyl*-^{3}H]thymidine and 0.2 μCi/mL [^{14}C]leucine for 24 h for SDS–KCl precipitation experiments (see below). L1210 cells were labeled with 0.1 μCi/mL [*methyl*-^{3}H]thymidine and served as an internal standard in alkaline elution experiments [see Zwelling et al. (1981) and Bakic et al. (1987)]. Cells were incubated for at least 1 h in radiolabel-free medium prior to treatment with any drug.

Drugs. *m*-AMSA [4′-(9-acridinylamino)methanesulfon-*m*-anisidide] (NSC 249992), mitoxantrone (NSC 279836), actinomycin D (NSC 3053), camptothecin sodium (NSC 100880), and camptothecin lactone (NSC 94600) were all obtained from the National Cancer Institute and were constituted as sterile stock solutions. Stocks (10^{-2} M) of *m*-AMSA and camptothecin lactone were in DMSO. Mitoxantrone (10^{-2} M) was in 0.02 N HCl. Actinomycin D (10^{-3} M) was in 95% ethanol. Camptothecin sodium (10^{-2} M) was in water. Etoposide was a gift from Dr. Byron Long or Dr. James H. Keller of Bristol-Myers Co. and was constituted as a 10^{-2} M solution in DMSO. BIDA was a gift from Dr. Robert Newman, M. D. Anderson Cancer Center, and was constituted as a 2×10^{-2} M stock solution in 0.03 N HCl.

DNA. Kinetoplast DNA (kDNA) was isolated from the trypanosome *Crithidia fasciculata* as previously described (Bakic et al., 1987). The kDNA was radiolabeled with [*methyl*-^{3}H]thymidine by incubating the trypanosomes with the label prior to kDNA isolation from Sarkosyl extracts using cesium chloride–ethidium bromide density centrifugation.

Covalently closed, supercoiled SV40 DNA was purchased from either Bethesda Research Laboratories (Gaithersburg, MD) or Lofstrand Labs Limited (Gaithersburg, MD).

Studies with Intact Cells. Soft agar colony formation assays were performed as previously described according to the methods of Chu and Fisher (1968). DNA alkaline elution by the method of Kohn was performed as previously described (Zwelling et al., 1981). In assays of the DNA–protein cross-linking produced by camptothecin and actinomycin D, SDS was used to lyse the cells as per Covey et al. (1989).

The SDS–KCl precipitation assay of DNA–protein complexes was as described by Trask and Muller (1983; Trask et al., 1984) and as modified by Liu et al. (1983), Rowe et al. (1986), and Denstman et al. (1987). Cells (4×10^{5}) with their DNA and protein radiolabeled (see above) were treated with various concentrations of topoisomerase-reactive drugs for 1 h at 37 °C (as previously described). Because the effects of topoisomerase I reactive drugs are reversible even at ice temperature (Covey et al., 1989), drug was not removed prior to cell lysis at 65 °C in a solution of 1.25% SDS, 5 mM EDTA (pH 8), and 0.4 mg/mL denatured herring sperm DNA. The lysate was passed through a 22-gauge needle 4 times and incubated at 65 °C for 10 min. The lysate was then brought to 65 mM KCl (using a stock solution of 325 mM KCl), and this mixture was rapidly vortexed for 10 s and placed at ice temperature for 10 min. A precipitate formed and was collected with a microcentrifuge. This pellet was then washed twice in 10 mM Tris (pH 8), 100 mM KCl, 1 mM EDTA, and 0.1 mg/mL denatured herring sperm DNA at 65 °C before being suspended in H_2O and then transferred to a liquid scintillation vial for determination of the radioactivity in the pellet. 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.

Topoisomerase-Containing Nuclear Extracts. Nuclei were isolated from the cell lines as previously described (Pommier et al., 1982). Extracts of these nuclei using 0.35 M NaCl were as previously described (Estey et al., 1987; Bakic et al., 1987). The buffer used was the nucleus buffer of Minford et al. (1986) (150 mM NaCl, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 1 mM EGTA, 0.1 mM dithiothreitol, and 0.1 mM PMSF, pH 6.4). Nuclei were extracted with 0.35 M NaCl for 30 min, after which they were centrifuged at 100000 *g*, and the supernatant was retained. Drug susceptibility measurements were performed using protein amounts having approximately equal decatenating activity. This allows measurements of qualitative differences among type II topoisomerases under conditions of comparable catalytic activity.

Biochemical Assays. (*A*) *Decatenation.* This assay was used to quantify the amount of topoisomerase II in nuclear extracts from the two cell lines. [^{3}H]kDNA (approximately 0.22 μg) was used as a substrate. Reactions were performed in 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. Reactions were performed for 30 min at 37 °C and were usually stopped with 1% SDS and 100 μg/mL proteinase K. However, for measurement of the various drugs' abilities to block decatenation, reactions were stopped with 10 mM Na_2EDTA and 100 μg/mL proteinase K so that no SDS-induced, topoisomerase II mediated DNA cleavage would be produced. Reaction products were separated by 1% agarose gel electrophoresis in an 89 mM Tris–borate buffer system (pH 8) containing 0.5 μg/mL ethidium bromide. Catenated kDNA does not enter the gel, but decatenated DNA circles do. The DNA was visualized under ultraviolet light without further staining, the agarose containing the decatenated and catenated DNA was separately excised, placed in liquid scintillation vials, melted in a microwave oven, and combined with scintillation fluid, and the DNA was quantified by using liquid scintillation spectroscopy. The activity of a nuclear extract is expressed as the amount of protein required to decatenate 50% of the substrate.

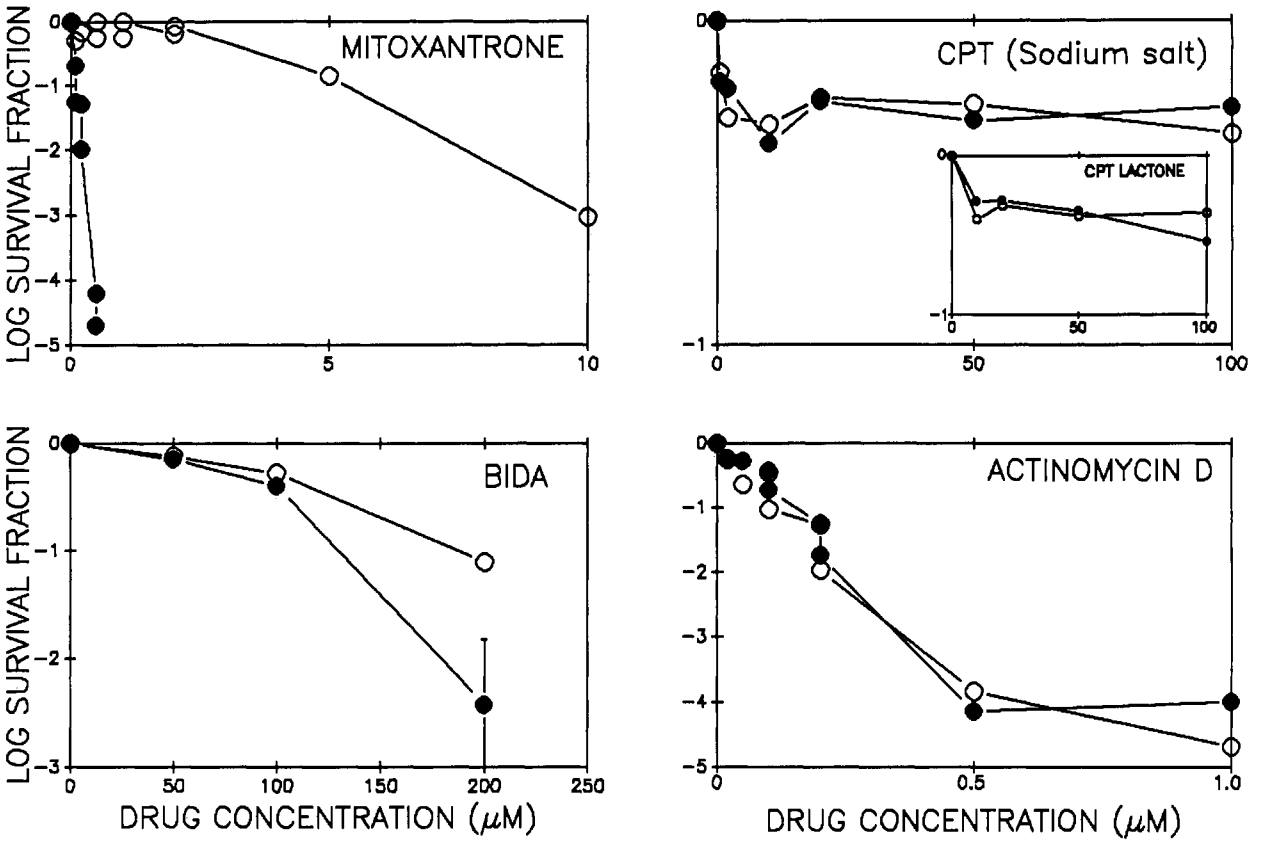

FIGURE 1: Cytotoxicity of mitoxantrone, BIDA, camptothecin, and actinomycin D. Exponentially growing HL-60 (closed circles) and HL-60/AMSA (open circles) cells were exposed to various concentrations of drug for 1 h at 37 °C followed by soft agar colony formation as previously described (Chu & Fisher, 1968). Both the sodium and lactone (inset) forms of camptothecin were employed. Mean colony-forming efficiency of untreated cells was 0.49 ± 0.12 for HL-60 and 0.46 ± 0.24 for HL-60/AMSA (n = 12). Error bars are ±1 SD for at least three independent determinations.

In experiments quantifying the inhibition of decatenation (i.e., strand passage) by the various topoisomerase II reactive drugs, calculations were as follows:

$$\text{FSP} = \frac{[F(\text{kDNA}) \text{ in well}]^{\text{no protein}} - [F(\text{kDNA}) \text{ in well}]^{+\text{ protein}}}{[F(\text{kDNA}) \text{ in well}]^{\text{no protein}}}$$

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:

$$\text{inhibition} = \frac{\text{FSP}^{\text{no drug}} - \text{FSP}^{+\text{ drug}}}{\text{FSP}^{\text{no drug}}}$$

(*B*) *Drug-Induced DNA Cleavage.* Covalently closed circular supercoiled SV40 DNA (0.2 μg) was incubated with nuclear extracts and the various agents or vehicle controls for 30 min at 37 °C in a buffer consisting of 10 mM Tris-HCl, 50 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 15 μg/mL bovine serum albumin, pH 7.4. Some reactions included 1 mM ATP. The reactions were terminated with 1% SDS and 100 μg/mL proteinase K in most cases. In some experiments, EDTA was substituted for SDS to demonstrate that DNA cleavage was SDS-dependent and, thus, probably mediated by the actions of a topoisomerase (Osheroff, 1986). The reaction products were separated on 1% agarose gels as used in the decatenation assay.

(*C*) *Drug-Induced DNA–Protein Cross-Linking.* The precipitation of uniquely 3′ end-labeled ^{32}P-SV40 DNA was quantified by the SDS–KCl precipitation method (Liu et al., 1983; Bakic et al., 1987). Results are expressed as the cpm precipitated in the presence of drug minus that precipitated in the absence of drug for experiments using nuclear extracts. Ratios were not used as there was a possibility that proteins other than topoisomerase II in the nuclear extracts contributed to the DNA precipitated.

Drug Stability and Compatibility Studies. Stock solutions (10^{-2} M) of mitoxantrone and *m*-AMSA in DMSO were diluted to a final concentration of 2×10^{-5} and 10^{-6} M, respectively, in 500 μL of the buffer used in assays of DNA cleavage (see above) in a 2.0-mL screw-capped glass vial. Control solutions of the individual drugs were also prepared. These solutions were agitated on a vortex shaker for 30 s and then shaken on a water bath at 37 °C. At selected time intervals (typically 1, 10, 30, 60, and 120 min), aliquots (20 μL) were removed and analyzed immediately by HPLC on a reverse-phase column (Waters Associates, Milford, MA; 10-μm Bondapak C-18; 15 cm × 3.9 mm i.d.). An isocratic mobile phase of acetonitrile–0.2 M ammonium acetate buffer, pH 4.0 (28:72 v/v), was used. The flow rate was maintained at 1.3 mL/min. Eluted compounds were monitored with a variable-wavelength UV detector set at 258 nm and 0.005 AUFS sensitivity and were quantitated electronically as a function of time using a Hewlett-Packard Model 3390A integrator. The retention times of mitoxantrone and *m*-AMSA were 2.27 and 4.67 min, respectively.

Results

Cellular Studies. HL-60/AMSA cells were cross-resistant to the cytotoxic actions of the topoisomerase II reactive DNA intercalators mitoxantrone and BIDA (Figure 1). Neither cell line was particularly sensitive to the topoisomerase I reactive drug camptothecin (Hsiang et al., 1985; Holm et al., 1989), but the lines did not differ in their sensitivity to this agent or to actinomycin D, an intercalator that has been re-

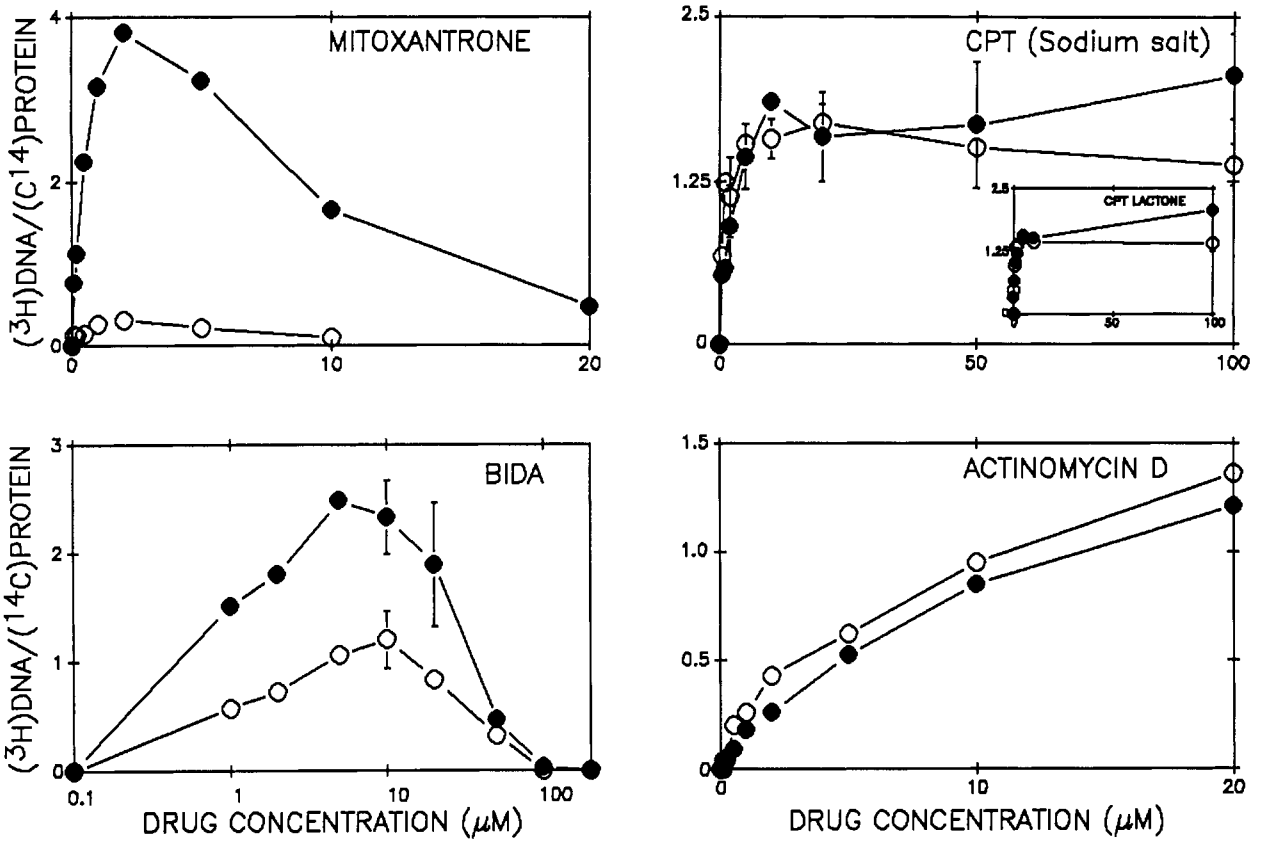

FIGURE 2: Precipitation of topoisomerase–DNA complexes directly from drug-treated HL-60 (closed circles) and HL-60/AMSA (open circles) cells using the SDS–KCl method. The DNA and protein of the cells were radiolabeled (see Experimental Procedures) prior to treatment of the cells with various concentrations of mitoxantrone, BIDA, camptothecin, or actinomycin D for 1 h at 37 °C. Data are expressed as the amount of radiolabeled DNA per radiolabeled cellular protein. Both the sodium and lactone (inset) forms of camptothecin were employed. Error bars are ±1 SD of at least three independent determinations.

ported to be *both* topoisomerase I (Trask & Muller, 1988) and topoisomerase II reactive (Tewey et al., 1984a) (see below). Quantifying drug-induced DNA–protein cross-linking using the SDS–KCl method (Figure 2) or the more sensitive alkaline elution method (data not shown) demonstrated that HL-60/AMSA was resistant not only to the cytotoxic actions of mitoxantrone and BIDA but also to the DNA–protein cross-linking produced by these two drugs. By contrast, cross-linking was not reduced in HL-60/AMSA cells treated with camptothecin or actinomycin D.

The mitoxantrone- and BIDA-induced DNA–protein cross-linking dose–response curves were biphasic even though the cytotoxicity of these drugs increased over this concentration range (Figure 1). This suggested either that the magnitude of the putative cytotoxic lesion was being underestimated for technical reasons or that drug effects other than topoisomerase–DNA cross-linking formation were contributing to the cytotoxic actions of these agents. However, we detected no non-protein-concealed DNA cleavage (alkaline elution without proteinase) that could shorten the protein-linked, radiolabeled DNA strands and thus cause an underestimation of DNA–protein cross-linking in drug-treated cells. Furthermore, DNA–protein cross-link and cleavage frequencies were within a factor of 2 of one another, and we detected little low molecular weight DNA in the lysates of drug-treated cells (data not shown). Thus, it appeared that the biphasic curves were due to a true inhibition of the drug-induced topoisomerase II–DNA interaction.

Further substantiating this is the fact that mitoxantrone and BIDA also inhibited the ability of *m*-AMSA and etoposide to produce DNA–protein cross-linking. Concurrent treatment of cells with mitoxantrone or BIDA plus *m*-AMSA or etoposide produced approximately the same frequency of DNA–protein cross-linking as that produced by mitoxantrone or BIDA alone (Table I). Furthermore, inhibition occurred rapidly (Figure 3, bottom), with half the cross-links produced by etoposide reversible within 2.3 min of the addition of BIDA. The inhibition of *m*-AMSA- and etoposide-induced cross-linking occurred over a broad range of *m*-AMSA and etoposide concentrations (data not shown).

Table I: Inhibition of *m*-AMSA- and Etoposide-Induced DNA–Protein Cross-Linking by Concurrent Treatment with Mitoxantrone or BIDA[a]

	cells			
	HL-60, expt		HL-60/AMSA, expt	
treatment and drug and concn	1	2	1	2
m-AMSA, 1 μM	2.94	3.06	ND	ND
etoposide, 100 μM	4.69	4.91	1.81	2.76
mitoxantrone, 20 μM	0.33	0.78	0.00	0.00
BIDA, 200 μM	0.00	ND	0.00	ND
BIDA, 100 μM	ND[b]	0.02	ND	0.00
m-AMSA + mitoxantrone	0.36	0.55	ND	ND
m-AMSA + BIDA, 200 μM	0.00	ND	ND	ND
m-AMSA + BIDA, 100 μM	ND	0.04	ND	ND
etoposide + mitoxantrone	0.58	1.09	0.00	0.23
etoposide + BIDA, 200 μM	0.00	ND	0.00	ND
etoposide + BIDA, 100 μM	ND	0.03	ND	0.00

[a] All numbers are the ratio of [^{3}H]DNA to [^{14}C]protein. The results of two independent experiments are shown. [b] Not determined.

The cytotoxicity of *m*-AMSA and etoposide can also be inhibited by BIDA (Table II). [Mitoxantrone is too toxic to HL-60 cells at high concentrations (see Figure 1) to allow an accurate assessment of its inhibitory actions on the cyto-

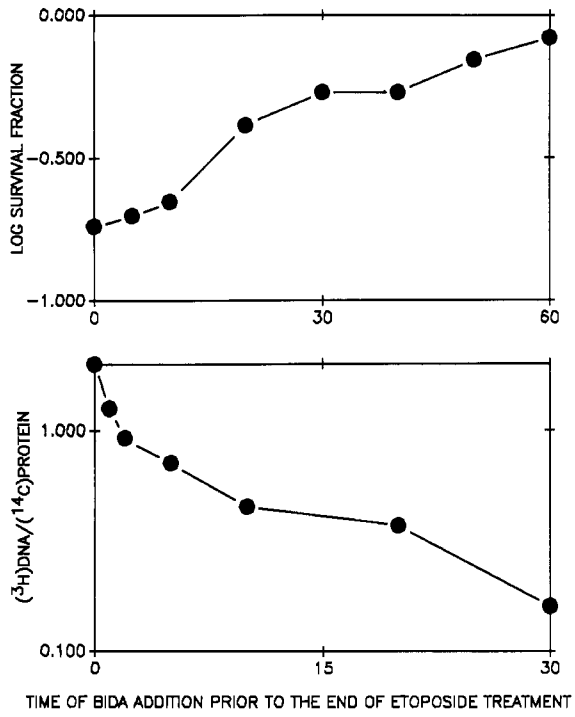

FIGURE 3: BIDA can inhibit the cytotoxicity and DNA–protein cross-linking produced by etoposide. HL-60 cells were treated with 10 μM etoposide for 1 h at 37 °C. At various times *during* this 1-h treatment, 100 μM BIDA was added for the remainder of the hour. At the end of the hour, cells were assayed for their ability to form colonies in soft agar (top) or for the presence of DNA–protein cross-linking by the SDS–KCl precipitation method (bottom). The cytotoxicity of BIDA alone for 60 min was 0.78 (log survival fraction = −0.11) while that of BIDA + etoposide was 0.83 (log survival fraction = −0.08). This latter figure is the point at 60 min in the top graph. The DNA–protein cross-linking produced by BIDA alone for 60 min was 0.05.

toxicity of other drugs.] Taken together, all of these data indicate that the cytotoxic actions of *m*-AMSA and etoposide are mechanistically linked to the production of topoisomerase II mediated DNA–protein cross-links. If the cross-links are not formed, these two drugs are not cytotoxic. The cytotoxicity of high concentrations of BIDA (and probably of mitoxantrone as well) may be due to a mechanism other than the stabilization of topoisomerase II–DNA complexes.

The HL-60/AMSA data on Table II support these points as well. Again, BIDA plus etoposide was as cytotoxic as was BIDA alone. The cytotoxic mechanism of etoposide in this line is probably the same as the cytotoxic mechanism of etoposide in HL-60, that is, stabilization of the topoisomerase II–DNA complex. However, at the high *m-AMSA* concentrations needed to reduce HL-60/AMSA colony formation, BIDA was not fully inhibitory, suggesting that the mechanism of *m*-AMSA's limited cytotoxic action in HL-60/AMSA is *not* via stabilization of the topoisomerase II–DNA complex.

Finally, the inhibition of etoposide-induced cytotoxicity by BIDA is not as rapid as is the inhibition of etoposide-induced DNA–protein cross-link production (Figure 3). The $t_{1/2}$ of this action was approximately 37 min. This suggests that it is the *duration* rather than simply the *magnitude* of etoposide-induced topoisomerase II–DNA complex stabilization that is related to cytotoxicity (Zwelling et al., 1982). That is, once topoisomerase II directed drug action has accumulated in a sufficient number of cells in a population or at a sufficient number of sites within a cell, cell death will ensue even if complex stabilization is subsequently reversed (Chatterjee et al., 1990).

Table II: Inhibition of *m*-AMSA- and Etoposide-Induced Cytotoxicity by Concurrent Treatment with BIDA[a]

	HL-60[b] cells, expt	
treatment and drug concn	1	2
m-AMSA, 1 μM	0.13	0.12
etoposide, 10 μM	0.06	0.15
BIDA, 100 μM	0.53	0.47
m-AMSA + BIDA	0.55	0.62
etoposide + BIDA	0.64	0.56
BIDA + BIDA, 200 μM		0.05

	HL-60/AMSA[c] cells, expt				
treatment and drug concn	1	2	3	4	5
m-AMSA, 50 μM			0.20	0.76	0.92
				0.64	0.94
etoposide, 20 μM	0.16	0.16	0.13		
BIDA, 100 μM	0.90	0.64	0.86	0.56	0.69
m-AMSA + BIDA			0.53	0.43	0.42
				0.41	0.48
etoposide + BIDA	0.89	0.51	0.70		
BIDA + BIDA, 200 μM		0.08			

[a] Numbers are survival fraction using the soft agar colony formation assay of Chu and Fisher (1968). [b] The results of two independent experiments are shown. [c] The results of five independent experiments are shown. Some experimental conditions in experiments 4 and 5 were done in duplicate but independently.

Biochemical Studies. *m*-AMSA, etoposide, mitoxantrone, and BIDA stimulated the production of double-stranded DNA cleavage using HL-60 nuclear extract as a source of topoisomerase II (Figure 4). Only etoposide could produce this cleavage using the HL-60/AMSA nuclear extract despite the presence of sufficient HL-60/AMSA topoisomerase II activity to produce catenation. Note that actinomycin D did not produce this cleavage using HL-60 extract. Of additional importance, high concentrations of both mitoxantrone and BIDA blocked the generation of catenanes (i.e., topoisomerase II mediated DNA strand passage) (Figure 4, middle panel). Lower concentrations of those drugs actually produced *more* cleavage than did the higher concentrations, parallelling the finding in intact cells (Figure 2). Finally, note that at higher concentrations of mitoxantrone, BIDA, and actinomycin D, the characteristic alteration in mobility of the supercoiled SV40 produced by intercalation was detectable [e.g., see Andersson et al. (1987)].

In contrast, camptothecin and actinomycin D produced single-strand cleavage, but not double-strand cleavage. This cleavage was not stimulated by ATP (Castora & Kelly, 1986) and required SDS for detection and was thus topoisomerase I mediated. This cleavage was comparable whether HL-60 or HL-60/AMSA nuclear extract was the source of topoisomerase I (data not shown). In our cell lines, actinomycin D seems to target topoisomerase I, but the concentration range needed to detect topoisomerase I mediated effects in cells or biochemical systems is much greater than that needed to produce cytotoxicity. It is questionable whether either topoisomerase I or topoisomerase II inhibition plays a major role in the cytotoxic activity of actinomycin D (Johnson et al., 1990).

When the SDS–KCl [^{32}P]DNA–protein complex precipitation assay was used to quantify the actions of mitoxantrone and BIDA in a biochemical system (Liu et al., 1983; Bakic et al., 1987), biphasic drug dose–response curves were seen as had been seen in mitoxantrone- and BIDA-treated cells (Figure 2). The resistance of the topoisomerase II from HL-60/AMSA to these drugs was also seen in this assay (data not shown). This strongly indicates that the basis for this biphasic curve was not artifactual and was probably a result of an alteration of the topoisomerase II–DNA interaction

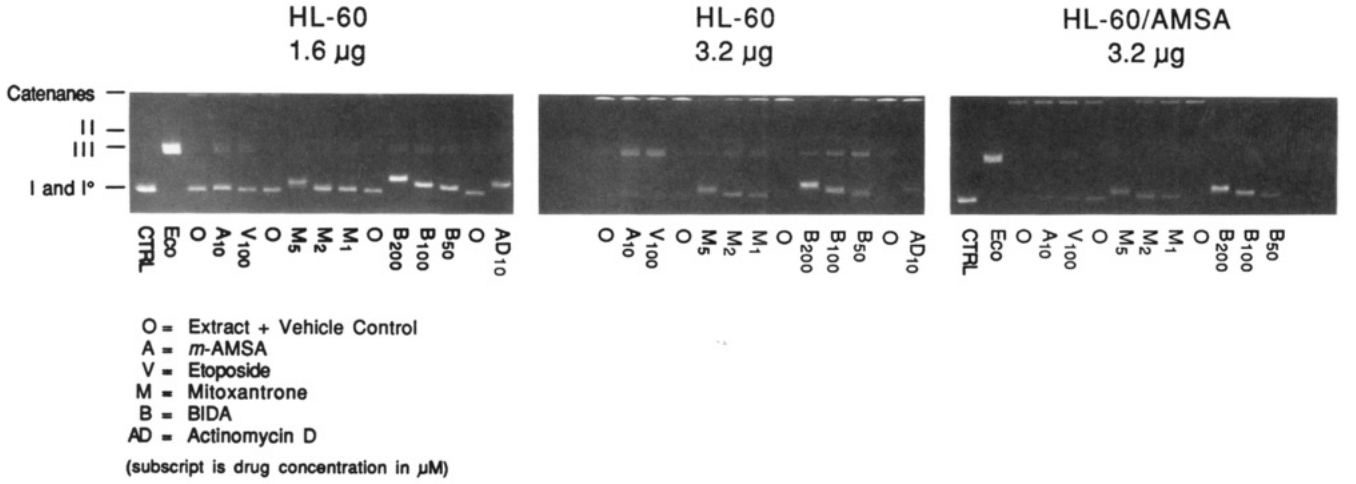

FIGURE 4: Drug-induced topoisomerase II mediated DNA cleavage of closed, circular supercoiled SV40 DNA using nuclear extracts from HL-60 and HL-60/AMSA cells. Incubations were for 30 min at 37 °C in the presence of 1 mM ATP, after which reactions were stopped with SDS and proteinase (see Experimental Procedures) and reaction products separated by electrophoresis on agarose gels in the presence of 0.5 μg/mL ethidium (Zwelling et al., 1989; Experimental Procedures). DNA forms are as indicated on the left of the figure. Form I is covalently closed, supercoiled; form II is nicked; form III is linearized. Drug concentrations are given in micromolar in the subscripts as indicated. Extract plus vehicle controls were run for each agent as solvents for the various compounds were not identical.

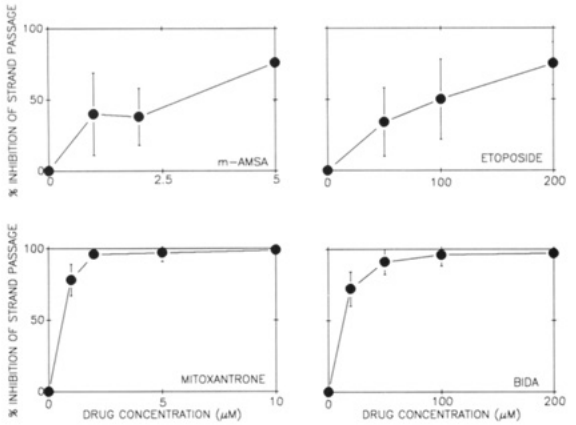

FIGURE 5: Ability of various concentrations of *m*-AMSA, etoposide, mitoxantrone, and BIDA to inhibit the strand-passing activity of HL-60 topoisomerase II. Strand passage was measured by using the decatenation of [^{3}H]kDNA (see Experimental Procedures). These experiments were performed with 189 ng of HL-60 nuclear extract, which produced a fraction of strand passage of 0.66 ± 0.2 (n = 4) (see Experimental Procedures for the method of calculation). Values are means ± 1 SD of at least three independent determinations.

produced by high concentrations of these drugs. Again, as in intact cells (Table I, Figure 3), mitoxantrone and BIDA inhibited DNA–protein cross-linking and DNA cleavage produced by *m*-AMSA and etoposide in biochemical systems (data not shown).

The data in Figure 4 showing the inhibition of topoisomerase II mediated strand passage (catenation) suggested the mechanism by which high concentrations of mitoxantrone and BIDA were producing their inhibitory effects on DNA cleavage and DNA–protein cross-linking. If those drugs act at the step in the cycle of topoisomerase II strand passage *prior to* DNA covalent binding and cleavage (see Figure 6 under Discussion), they could inhibit strand passage, the vital function of the enzyme, without producing DNA cleavage or protein cross-linking. Direct examination of this revealed that both mitoxantrone and BIDA were more potent inhibitors of strand passage than was either *m*-AMSA or etoposide (Figure 5). In other experiments, we demonstrated that preincubation of topoisomerase II with *m*-AMSA, etoposide, mitoxantrone, or BIDA did not inhibit the enzyme's subsequent ability to cleave DNA (data not shown). Incubating 1 μM *m*-AMSA

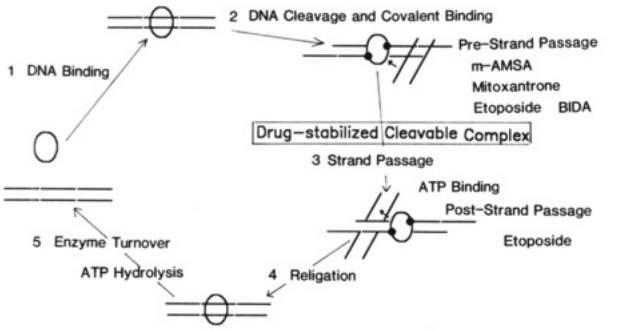

FIGURE 6: Model of the strand-passing cycle of topoisomerase II [after Osheroff (1986)].

with 20 μM mitoxantrone over 2 h produced less than a 5% variation in the concentration of either drug individually or in admixture as analyzed by HPLC (data not shown).

Discussion

In the present work, we demonstrate that drugs from different intercalator classes have different relative propensities to interfere with the various steps in the topoisomerase II cycle (Figure 6). *m*-AMSA and the nonintercalator etoposide mainly produce protein-associated DNA cleavage at all concentrations. Their actions, however, must differ in some way because HL-60/AMSA and its topoisomerase II are inhibited by etoposide but *not* by *m*-AMSA (Zwelling et al., 1989). At low concentrations, mitoxantrone and BIDA appear to act like *m*-AMSA. HL-60/AMSA and its topoisomerase II are resistant to those actions. At higher concentrations, however, mitoxantrone and BIDA inhibit their own abilities to stabilize topoisomerase II–DNA complexes (Capolongo et al., 1990; Figure 2) as well as those of *m*-AMSA and etoposide (Figure 3 and Table I). Thus, mitoxantrone (Capolongo et al., 1990) and BIDA (Andersson et al., 1987; Hsiang et al., 1989), which can produce DNA cleavage but which are much more potent inhibitors of strand passage than are *m*-AMSA and etoposide (Figure 5), inhibit topoisomerase II at a position in its cycle prior to the step at which the "cleavable complex" even forms. In this way, mitoxantrone and BIDA most resemble merbarone (Drake et al., 1989b), another topoisomerase II reactive agent that has its major actions on DNA strand passage and can inhibit the DNA cleaving actions of *m*-AMSA.

Other topoisomerase II reactive drugs have been reported to produce biphasic dose–response curves of their own DNA cleaving and cross-linking activities in cells (Potmesil et al., 1983; Capranico et al. 1986; Pierson et al., 1988, 1989), in isolated nuclei (Pommier et al., 1985a), and in isolated biochemical systems (Tewey et al., 1984b; Pommier et al., 1985b; Vilarem et al., 1986; Multon et al., 1989; Fosse et al., 1990). Even intercalators that do not stabilize topoisomerase II–DNA complexes can affect the actions of topoisomerase II–reactive drugs (Rowe et al., 1985). The most likely explanation is that the high concentration of mitoxantrone and BIDA (and other self-inhibiting intercalators) establish a new relationship between topoisomerase II and its DNA binding sites. The target for the drug-induced production of protein-associated strand cleavage, the topoisomerase II–DNA complex, is disrupted so that drugs that stabilize this configuration can no longer do so and their cytotoxic actions are inhibited. However, the establishment of this new relationship can in itself be cytotoxic, particularly when mitoxantrone is the drug employed (Figure 1).

It is also possible that topoisomerase II mediated DNA cross-linking and cleavage could *increase* following mitoxantrone or BIDA removal as the intracellular drug concentration falls. This effect has been produced by high concentrations of an ellipticine derivative (Pierson et al., 1988). However, this would not explain the effects of BIDA and mitoxantrone on the actions of *m*-AMSA and etoposide.

The relative weakness of BIDA's cytotoxic actions, even at concentrations that inhibit its own ability to form cross-links (100 μM), allowed us to perform the experiments shown in Figure 3. Although the inhibition of etoposide's cross-linking actions can occur even 55 min after the addition of etoposide to cells (bottom), the ability of BIDA to protect cells from the cytotoxic actions of etoposide is lost with time (top). This confirms previous work demonstrating (1) that the magnitude of the protein-associated DNA cleavage produced by *m*-AMSA (Zwelling et al., 1981) or etoposide (Long et al., 1984) is reached rapidly, reverses rapidly, and thus probably represents a kinetic equilibrium (on–off phenomenon) rather than a static, limited accumulation of DNA effects and (2) that the *duration* of *m*-AMSA (Zwelling et al., 1982) or etoposide (Chatterjee et al., 1990) treatment has a greater influence on cytotoxicity than does drug *concentration*. Together, these findings imply that the cytotoxic effects of topoisomerase II reactive agents derive from the sustained interference of enzyme-mediated DNA strand passage. Recent evidence suggests that this interference leads to aberrant recombination (as manifested by sister chromatid exchange induction) (Chatterjee et al., 1990) as well as to a cascade of other biochemical events ending in cell death (Jaxel et al., 1988; Kaufmann, 1989; Tanizawa et al., 1989).

Referring to Figure 6 again allows us to speculate about the sites within the topoisomerization cycle at which the various drugs act. Clearly, *m*-AMSA, etoposide, and low concentrations of mitoxantrone and BIDA can stabilize the cleavable complex. This complex may be stabilized before or after strand passage, an event requiring ATP binding. Previous work has demonstrated that the topoisomerase II from HL-60/AMSA not only is resistant to the actions of the various intercalators but also is resistant to etoposide in the *absence* of ATP (Zwelling et al., 1989). The addition of ATP, however, renders HL-60/AMSA topoisomerase II sensitive to stabilization in a complex with DNA by etoposide but not by the intercalators. Perhaps etoposide can stabilize cleavable complexes in the pre- *and* post-strand-passage configurations whereas the intercalators only act in the pre-strand-passage configuration. Thus, if HL-60/AMSA topoisomerase II cannot attain a stabilizable pre-strand-passage configuration, then it and the cell in which it acts will be resistant to intercalators.

Although HL-60/AMSA cells are resistant to the cytotoxic actions of *m*-AMSA, mitoxantrone, and BIDA, the cells can be killed by very high doses of these drugs [Figure 1 and Zwelling et al., (1989)]. Additionally, though high concentrations of BIDA completely block the cytotoxicity of *m*-AMSA in HL-60, those concentrations only partially block the effects of *m*-AMSA in HL-60/AMSA (Table II). This suggest that while high concentrations of mitoxantrone and BIDA might be killing HL-60 cells through inhibition of topoisomerase II mediated DNA strand passage (Figure 5), high concentrations of drugs in HL-60/AMSA may be killing the cells by another mechanism not related to topoisomerase II at all (Wong et al., 1984a,b).

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Specificities and Kinetics of Uracil Excision from Uracil-Containing DNA Oligomers by *Escherichia coli* Uracil DNA Glycosylase†

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ABSTRACT: Uracil DNA glycosylase excises uracil residues from DNA that can arise as a result of deamination of cytosine or incorporation of dUMP residues by DNA polymerase. We have carried out a detailed study to define the specificities and the kinetic parameters for its substrates by using a number of synthetic oligodeoxyribonucleotides of varying lengths and containing uracil residue(s) in various locations. The results show that the *Escherichia coli* enzyme can remove a 5′-terminal U from an oligomer only if the 5′-end is phosphorylated. The enzyme does not remove U residues from a 3′-terminal position, but U residues can be excised from oligonucleotides with either pd(UN)p or pd(UNN) 3′-termini. The oligomer d(UUUUT) can have the second or third U residues from the 5′-end excised even when the neighboring site is an abasic site (3′ or 5′, respectively). On the basis of these findings, pd(UN)p was anticipated to be the smallest size substrate. Results show detectable amounts of U release from the substrate pd(UT)p; however, significantly higher amounts of U release were observed from pd(UT-sugar) or pd(UTT). Determinations of the K_m and V_{max} values show that the different rates of U excision from oligomers of different sizes (trimeric to pentameric) but containing U in the same position are largely due to the differences in the K_m values, whereas the different rates of U excision from the substrates of the same size but containing U in different positions are largely due to different V_{max} values.

The DNA glycosylases excise damaged or unconventional bases from DNA and initiate the DNA base excision repair pathway (Friedberg et al., 1978). These enzymes have been identified and purified from both prokaryotic and eukaryotic sources (Friedberg et al., 1978; Duncan, 1981; Lindahl, 1982; Morgan & Chlebek, 1989). Uracil DNA glycosylase excises uracil residues from DNA that can arise as a result of either deamination of deoxycytosine or incorporation of dUMP

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