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INHIBITION OF EPIPODOPHYLLOTOXIN CYTOTOXICITY BY INTERFERENCE WITH TOPOISOMERASE-MEDIATED DNA CLEAVAGE*

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Abstract—This laboratory and others previously proposed that the antitumor effects of the epipodophyllotoxin compounds are based on their abilities to stimulate DNA cleavage by a DNA topoisomerase. To explore this relationship further, we studied the intercalating agent ethidium bromide and found that it blocked epipodophyllotoxin-induced DNA cleavage by DNA topoisomerase II *in vitro* as well as *in vivo*. Using an *in vitro* assay consisting of purified calf thymus DNA topoisomerase II, end-labeled DNA, and the epipodophyllotoxin teniposide, we found that ethidium bromide markedly interfered with the enzyme-mediated DNA cleavage. Furthermore, ethidium bromide also blocked the formation of DNA single- and double-strand breaks in mouse L1210 cells when exposed to the epipodophyllotoxin etoposide. This effect cannot be explained by alterations in drug accumulation since steady-state drug concentrations were unchanged, and the effect was also observed in isolated nuclei. In addition to its effects on epipodophyllotoxin-mediated DNA breakage, ethidium bromide also potently inhibited the cytotoxic effects of etoposide but only when present during drug treatment. Thus, we believe that ethidium bromide may be a useful tool to investigate drug-induced perturbations of topoisomerase activity and their relationship to antitumor effect. Our data strongly support the hypothesis that the antitumor activity of epipodophyllotoxins is based on the ability to stimulate the formation of a cleavable complex between DNA topoisomerase and DNA.

Accumulating evidence suggests that DNA topoisomerase II, an enzyme which breaks and rejoins DNA strands in concert, mediates the formation of DNA single- and double-strand breaks following exposure of mammalian cells to a variety of anticancer agents. These agents include a number of drugs which bind to DNA by intercalation such as adriamycin, *m*-AMSA (amsacrine) and ellipticine [1], as well as members of the epipodophyllotoxin group which include etoposide and teniposide [2]. While the details of the drug–topoisomerase interaction have yet to be fully elucidated, it appears that, in the presence of the drugs, a complex is formed between enzyme and DNA which, upon exposure to a denaturing agent, results in cleavage of one or both DNA strands. Thus, it is believed that the drug interferes with the rejoining action of the enzyme. In the course of investigating this interaction, Tewey *et al.* [3] observed that several intercalating agents share the peculiar property of actually inhibiting topoisomerase-mediated DNA cleavage at high concentrations, suggesting that some property of these particular agents confers the ability to interfere with the nicking action as well. Based upon this observation, we have further determined that for at least one intercalating agent, ethidium bromide, this property appears to dominate, as little DNA cleavage was observed in the presence of DNA topoisomerase II, DNA, and the drug. Indeed, we found that ethidium bromide actually inhibited the topoisomerase-mediated DNA cleavage induced by other drugs. This characteristic of ethidium bromide is a useful tool for studying the implications of drug–topoisomerase interaction *in vivo*. Specifically, since the relationship between epipodophyllotoxin-mediated DNA cleavage and cytotoxicity has not been established conclusively, we have examined the effects of ethidium bromide on these two variables in mouse L1210 cells treated with etoposide. We found that ethidium bromide inhibited both DNA double- and single-strand break formation as well as cytotoxicity and that this effect was observed only when both drugs were present simultaneously. Our data support a role for DNA strand breakage in the antitumor effect of the epipodophyllotoxins.

METHODS

Procedures for L1210 cell culture, isolation of nuclei, colony-forming assays and DNA alkaline elution have been published previously [4, 5]. Etoposide and teniposide were obtained from the NCI and dissolved in dimethylsulfoxide (DMSO). Drug treatments were for 1 hr at 37° unless otherwise noted. ^{3}H-Labeled etoposide (200 mCi/mmole) was obtained from Moravek Biochemicals (Brea, CA) and was 92% pure by high pressure liquid chromatographic assay.

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Purification of calf thymus DNA-topoisomerase II is described in detail elsewhere.* Briefly, freshly-obtained calf thymus glands were trimmed of adventiae and homogenized in a Waring blender. The resulting nuclei were collected by centrifugation, washed, and lysed by high-speed blending. Polymin-P (10%, pH 7.8) was added to a final concentration of 0.35% and the precipitate was collected. The pellet was resuspended and proteins were extracted by the addition of 5 M NaCl until a final concentration of 0.55 M was achieved. Nucleic acids were then reprecipitated with additional Polymin-P, and the precipitate was removed by centrifugation. Supernatant protein was precipitated with ammonium sulfate, resuspended, dialyzed, and then applied to a BioRex 70 column and eluted with potassium phosphate. Pooled fractions containing topoisomerase II activity were then applied to a hydroxylapatite column, again eluted with potassium phosphate and then loaded onto a blue agarose column and eluted with 2 M NaCl, 1% Triton X-100. The resulting enzyme fractions were then concentrated on a small hydroxylapatite column and then dialyzed against storage buffer. Topoisomerase II activity was assayed throughout the purification procedure using the P4 phage unknotting assay described by Liu *et al.* [6]. This assay is highly specific for topoisomerase II activity. Homogeneity of the enzyme preparation was documented by sodium diodecyl sulfate (SDS)-polyacrylamide electrophoresis using a Coomassie blue stain. In addition to possessing P4 unknotting activity, the enzyme also demonstrated ATP-dependent DNA relaxing activity and ATPase activity.

The cleavage assay has been characterized previously in detail elsewhere [1]. *Eco* R1 digested PBR 322 DNA was labeled at its 3′ ends with [^{32}P]dATP using the large fragment of *Escherichia coli* DNA polymerase I. The end-labeled PBR 322 DNA was then digested again with Hind III restriction enzyme to remove a 31 BP fragment. Topoisomerase II cleavage reactions were done in reaction mixtures (20 μl each) containing 40 mM Tris, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 0.5 mM dithiothreitol, 0.5 mM EDTA, 30 μg/ml bovine serum albumin, 20 μg of calf thymus DNA topoisomerase II and drugs as indicated. Reactions were incubated at 37° for 30 min and then terminated by the addition of 1 μl of 10% sodium dodecyl sulfate and 1.5 mg/ml proteinase K (50°, 30 min). Five microliters of 50% sucrose, 50 mM EDTA, pH 8.0, and 0.05 mg/ml bromophenol blue were added to each sample before loading onto a 1% agarose gel. Autoradiography was performed as previously described [6].

Uptake of [^{3}H]etoposide was determined by incubating L1210 cells (5×10^6/ml) with radiolabeled drug for 60 min in the presence or absence of ethidium bromide (added 30 min before etoposide). At the end of the incubation, cells were diluted with 10 vol. of ice-cold phosphate-buffered saline (PBS), centrifuged at 500 *g* for 2 min, and then washed twice with ice-cold PBS. The washed pellet was drawn up into a plastic pipette tip, extruded onto a polyethylene tare, and dried overnight at 70°. The dried pellets were weighed, placed in a scintillation vial,and dissolved in 0.25 ml of 1 N NaOH for 2 hr at 70°. The digest was neutralized with 0.25 ml of 1 N HCl, 4.5 ml of Safety-Solv (Research Products International Corp., Mt. Prospect, IL) was added, and radioactivity was determined on a liquid scintillation spectrometer. Counting efficiencies were determined using [^{3}H]toluene standards.

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RESULTS

Effects of ethidium bromide on topoisomerase-mediated DNA cleavage were studied using a highly purified calf thymus topoisomerase II and linearized plasmid DNA bearing a ^{32}P-label on the 3′ end. Site-specific double-strand cleavage results in fragmentation of the DNA into discrete sizes represented by bands when subjected to agarose gel electrophoresis and autoradiography. As shown in Fig. 1, there was a low level of topoisomerase-mediated DNA cleavage in the absence of any drug (Lane B),

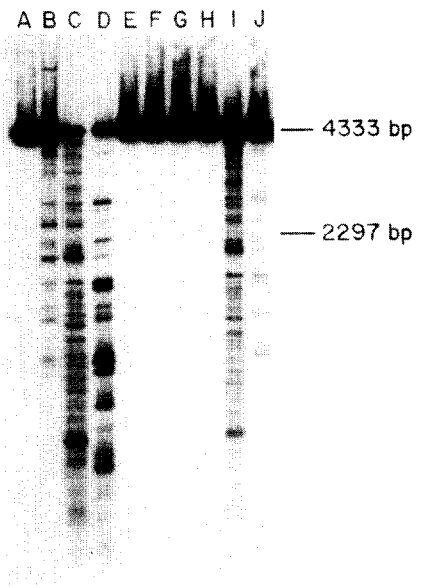

Fig. 1. Inhibition of topoisomerase-mediated DNA cleavage by ethidium bromide. Reactions (20 μl) containing 1.25 μg/ml 3′-end-labeled pBR322 DNA and 20 μg/ml calf thymus DNA topoisomerase II were incubated at 37° with various drugs. Lanes A–G were incubated for 30 min before terminating with 2 μl containing 10% SDS, 1 mg/ml proteinase K. Samples were then incubated for an additional 30 min at 50° before analyzing by gel electrophoresis and autoradiography. Lane A, the control, contained no enzyme. Lanes B–G contained topoisomerase and no addition (Lane B), 15 μM VM-26 (Lane C), 25 μM *m*-AMSA (Lane D), 25 μM ethidium bromide (Lane E), 15 μM VM-26 and ethidium bromide (Lane F), and 25 μM *m*-AMSA and ethidium bromide (Lane G). Lane H–J were identical to Lanes B–D, respectively, except that, following 10 min incubation, ethidium bromide was added to a final concentration of 10 μg/ml. The reactions were then incubated for an additional 30 min at 37° before terminating with 2 μl of 10% SDS, 1 mg/ml proteinase K.

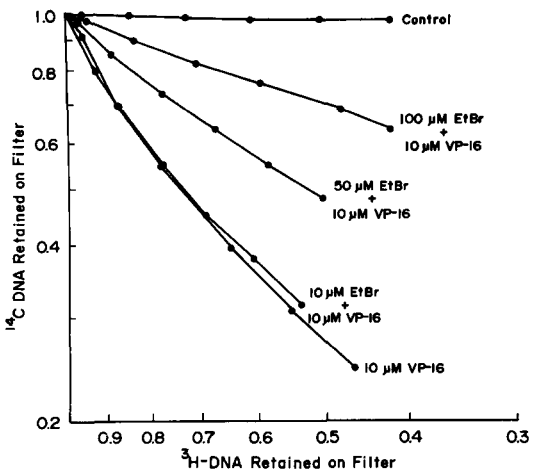

Fig. 2. Effect of ethidium bromide on etoposide-induced DNA single-strand breaks in L1210 cells. Cells containing ^{14}C-labeled DNA were incubated in the presence of various concentrations of ethidium bromide for 30 min prior to the addition of 10 μM etoposide. After another hour of incubation, the cells were washed with ice-cold medium, and strand break frequency was determined at pH 12.1 by the alkaline elution method. Cells containing [^{3}H]DNA received 2000 rads and were used as internal standards.

and this was markedly augmented in the presence of the epipodophyllotoxin VM-26 (Lane C) and the intercalating agent *m*-AMSA (Lane D). Similar results for these drugs and for the epipodophyllotoxin etoposide have been published previously [1, 2]. Topoisomerase-mediated DNA cleavage stimulation by teniposide can be observed at concentrations as low as 1 μM (unpublished observations). When ethidium bromide (25 μM) was included in the reaction mixture, however, the baseline DNA cleavage by topoisomerase was completely

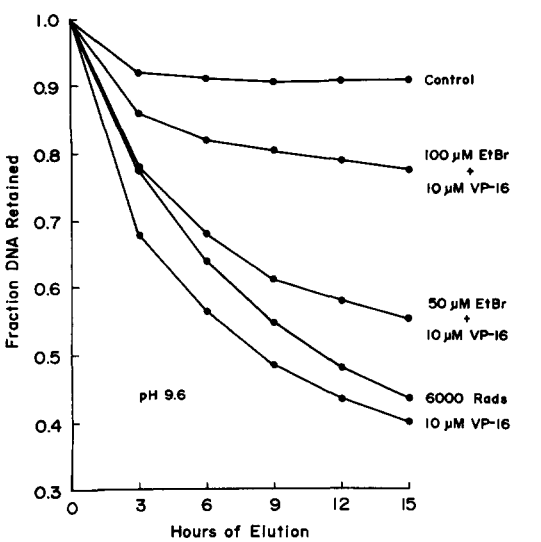

Fig. 3. Effect of ethidium bromide on etoposide-induced DNA double-strand breaks. Cells were treated as in Fig. 2 but elution of DNA was performed at a non-denaturing pH (9.6) according to Ross and Bradley [7].

inhibited (Lanes E–G), suggesting that some feature of ethidium bromide interferes with the topoisomerase cleavage activity. A similar phenomenon was observed when etoposide was substituted for teniposide. Interestingly, when ethidium bromide was added 10 min after incubation of teniposide with enzyme and DNA was begun, DNA cleavage was reversed but only partially (Lane I).

To study this phenomenon further *in vivo*, L1210 cells were exposed to various concentrations of ethidium bromide for 30 min prior to a 60-min treatment with 10 μM etoposide at 37°. At the end of the drug treatment period, both drugs were removed by washing cells in ice-cold fresh drug-free media, and DNA single-strand break frequency was assessed using the alkaline elution method. As expected, etoposide alone caused a high frequency of single-strand breaks (Fig. 2). These breaks were progressively reduced in the presence of increasing concentrations of ethidium bromide. The intercalating agent alone had no effect on the DNA elution rate (not shown). We also examined the effects of ethidium bromide on repair of single-strand breaks following removal of etoposide and found no change in the rate of repair. We have shown previously that etoposide causes DNA double-strand breaks in excess of those that can be accounted for on the basis of coincident single-strand breaks. Since these lesions may have particular significance with respect to lethality, we also studied the effect of ethidium bromide on etoposide-induced double-strand breaks using the same treatment protocol as before except for eluting the DNA at a non-denaturing pH (9.6) as previously described [7]. Again, the presence of ethidium bromide caused a dose-dependent inhibition of double-strand break formation by etoposide (Fig. 3).

This laboratory and others have suggested that the cytotoxicity of etoposide is based on the ability of the drug to cause DNA strand breakage [5, 8, 9]. It was, thus, of interest to determine if the effects of ethidium bromide, as seen in Figs. 1–3, may alter cytotoxicity. L1210 cells were incubated with ethidium bromide and etoposide as described in Fig. 2, and the cells were then seeded in soft agar to assess colony forming ability. Ethidium bromide alone resulted in cloning efficiencies of 86, 56 and 23% at concentrations of 25, 50, and 100 μM respectively. When combined with etoposide, however, ethidium bromide caused a dose-dependent reduction in the lethality of etoposide. At 100 μM ethidium bromide, there was virtually complete inhibition of etoposide cytotoxicity (Fig. 4). Since ethidium bromide does intercalate into DNA and may well cause transient perturbations in macromolecular synthesis and cell cycle traverse, we sought to determine if its protective effects on etoposide cytotoxicity would be manifest if cells were not exposed to the two drugs concurrently but rather if the ethidium bromide was added immediately after etoposide washout when repair processes are operative but presumably new strand break formation would not occur. We thus incubated cells with 10 μM etoposide for 60 min and then immediately washed and resuspended them into medium containing 100 μM ethidium bromide. As represented by the open symbol in Fig. 4, the ethidium bromide had no effect on etoposide cytotoxicity

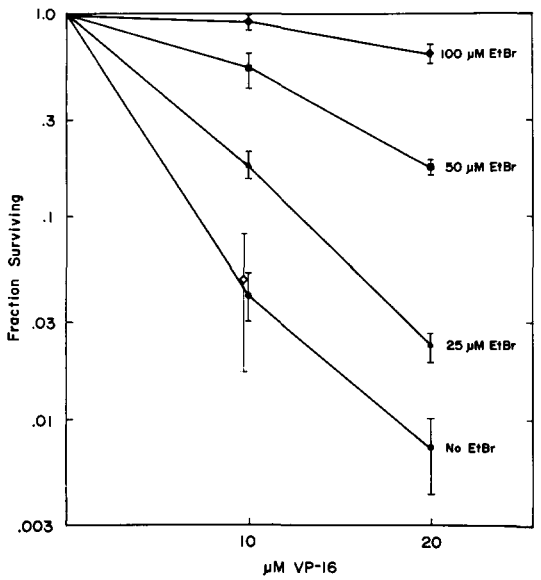

Fig. 4. Ethidium bromide reduction of etoposide cytotoxicity. L1210 cells were treated as in Figs. 2 and 3 but were washed with warm drug-free medium and then seeded into soft agar. Each point represents the mean ± S.D. of at least three experiments and is corrected for survival of control cells. Colony forming efficiencies of cells treated with 0, 25, 50 and 100 μM ethidium bromide were 90, 86, 56 and 23% respectively.

under these circumstances. We have independently determined the influence of macromolecular synthesis inhibition by exposing cells to a concentration of cytosine arabinoside (10^{-4} M) and cordycepin (4×10^{-4} M) which inhibits DNA and RNA synthesis, respectively, more than 90% for various periods of time after etoposide treatment. Neither agent significantly altered etoposide cytotoxicity.

Both DNA strand breakage and log cell kill by etoposide are directly proportional to the intracellular steady-state drug concentration [10]. It was, therefore, important to rule out the possibility that ethidium bromide interfered with the accumulation of intracellular etoposide. L1210 cells were incubated with 50 or 100 μM ethidium bromide for 30 min prior to the addition of [^{3}H]etoposide (10 μM; specific activity 200 mCi/mmole). After 60 min of further incubation, the cells were washed and collected as a pellet, and the intracellular drug concentration was determined. In the absence of ethidium bromide, the pellets contained 32.5 ± 6.6 nmoles etoposide/mg dry weight, while in the presence of 50 or 100 μM ethidium bromide the pellets contained 34.3 ± 7.3 nmoles/mg and 26.1 ± 3.0 nmoles/mg etoposide respectively. These figures represent the mean ± standard deviation of four experiments. Thus, ethidium bromide had no significant effect on accumulation of etoposide. This result was further corroborated by the observation that ethidium bromide also blocked etoposide-induced strand breaks in isolated nuclei, an experimental model in which problems of membrane permeability are obviated (data not shown).

DISCUSSION

In previous studies, our laboratory, as well as others, has shown that exposure of mammalian cells to epipodophyllotoxins causes DNA strand breakage [5, 8, 11]. Although we initially hypothesized, and provided circumstantial evidence for, a mechanism of DNA strand breakage involving an oxidation-reduction process, we believe that subsequent studies render this mechanism less likely. Minoccha and Long [12] first suggested a topoisomerase-mediated mechanism for epipodophyllotoxins based on the observation that DNA catenation by a partially purified topoisomerase II was inhibited by these compounds. DNA cleavage was not observed in these studies. In examining the drug-induced DNA strand breakage phenomenon in isolated nuclei, we found that drug-induced damage was temperature dependent, stimulated by ATP, and required a heat labile nuclear component extractable in 0.35 M NaCl [13]. Subsequently, we have shown that, using a highly purified topoisomerase II, DNA cleavage is markedly enhanced in the presence of etoposide and teniposide ([2]; Fig. 1). Thus, we believe that it is highly likely though not proven that topoisomerase mediates the DNA strand breakage observed in whole cells after exposure to epipodophyllotoxins.

While the mechanism of strand breakage is now becoming defined, the relationship of the DNA damage to cytotoxicity is less well established. Long *et al.* [8] have studied a series of epipodophyllotoxin congeners and found an excellent correlation in their ability to induce DNA single-strand breaks and cause cytotoxicity in human lung adenocarcinoma cells. Our laboratory has demonstrated that micromolecular concentrations of disulfiram, a sulfhydryl blocking agent, inhibit both epipodophyllotoxin-induced cytotoxicity and strand breakage in mouse L1210 cells [9]. To these pieces of evidence, we now add the results of our current work. Taking advantage of the observation that ethidium bromide does not induce DNA cleavage by topoisomerase II *in vitro* as would be expected for an intercalating agent, we have asked whether it might actually prevent cleavage induced by other agents and whether this effect would have biological consequences. As shown here (Fig. 1), ethidium bromide did inhibit the topoisomerase-mediated DNA cleavage caused by *m*-AMSA, and the epipodophyllotoxin teniposide (VM-26) in a reconstituted system of pure enzyme and pure DNA. It was, thus, of great interest to examine this interaction *in vivo* as well. The concomitant reduction in etoposide-induced DNA single- and double-strand breaks and cytotoxicity observed in L1210 cells in the presence of ethidium bromide (Figs. 2–4) provides important, though still circumstantial, support for the mechanism of action we have proposed.

Unresolved is the issue of how the epipodophyllotoxin–topoisomerase interaction actually results in cell death. The DNA strand breaks we observe in alkaline elution assays are largely a function of detergent denaturation of the topoisomerase–DNA "cleavable complex." Exactly what form this complex takes in the cell and what its consequences are for replication and transcription have yet to be

defined. It is known that these drugs cause chromosomal aberrations of the chromatid type and that these aberrations appear to occur principally in the G_2 and S phase of the cell cycle [14]. This would suggest that strand breakage does occur *in vivo* and that chromosomal damage may underlie the cytotoxicity. In addition to causing the formation of the cleavable complex, however, epipodophyllotoxins also inhibit the catalytic or "strand passing" effect of topoisomerase II [12, 15]. It is this activity which allows topoisomerase II to decatenate DNA circles or remove knots in topologically constrained DNA. In considering which of these two effects of epipodophyllotoxins on topoisomerase activity results in their antitumor effect, we favor the formation of the cleavable complex as being primary. This is because in screening a number of intercalating agents and epipodophyllotoxins we have found that the ability to cause formation of the complex correlates better with cytotoxic potency than does inhibition of catalytic activity. For instance, the aminoacridine isomers *o*-AMSA and *m*-AMSA are nearly equipotent with respect to inhibition of catalytic activity, yet *m*-AMSA is far more active with respect to stimulating DNA cleavage and producing cytotoxicity than is the ortho isomer [1]. A similar phenomenon has been observed using ellipticine derivatives [15]. Ethidium bromide represents another example of this principle. This intercalating agent does not induce cleavable complex formation, but it does block topoisomerase-mediated unknotting of phage DNA [3]. Yet ethidium bromide is much less potent with respect to cytotoxicity than other intercalating agents. Further, it is useful to point out an analogous circumstance in a bacterial system. The antibiotics nalidixic acid and oxolinic acid induce a cleavable complex between DNA and DNA gyrase from *E. coli* [16]. They also inhibit the strand passing activity of this form of topoisomerase II. Yet, when drug-resistant gyrase mutants are hybridized with sensitive wild type cells to form partial bacterial diploid cells, the sensitive allele is dominant [17]. While not conclusive, this result is most easily accounted for by a mechanism which requires cleavable complex formation rather than one which requires substantial inhibition of available strand passing activity.

The mechanism by which ethidium bromide interferes with topoisomerase function is not obvious. Of the three drugs shown in Fig. 1, *m*-AMSA binds to DNA by intercalation while teniposide belongs to a class of drug which does not bind to DNA [2]. Thus, while one could speculate that ethidium bromide simply competitively displaces the intercalating drugs from DNA binding sites and then fails to elicit DNA cleavage, the interaction with teniposide implies that ethidium bromide actually directly interferes with the nicking function of the enzyme. This latter action may result from a direct interaction between ethidium bromide and the enzyme or perhaps from masking of critical binding sites on DNA by the bulky side group of the intercalating agent. Since the DNA cleavage assay shown in Fig. 1 is performed using linearized plasmid DNA, it seems unlikely that ethidium bromide exerts its effect by alterations in DNA topology. Whatever the mechanism, this property of ethidium bromide, plus its lack of cytotoxicity, makes it a useful probe to determine the role of topoisomerase-mediated DNA cleavage in cytotoxicity.

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