Effects of DNA Intercalating Agents on Topoisomerase II Induced DNA Strand Cleavage in Isolated Mammalian Cell Nuclei

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Received March 12, 1985

ABSTRACT: Intercalator-induced DNA double-strand breaks (DSB) presumably represent topoisomerase II DNA cleavage sites in mammalian cells. Isolated L1210 cell nuclei were used to determine the saturability of this reaction at high drug concentrations. 4′-(9-Acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) and 5-iminodaunorubicin (5-ID) both produced DSB in a concentration-dependent manner, and the production of these breaks leveled off above 10 μM. Addition of *m*-AMSA to 5-ID-treated nuclei did not raise the plateau level. Thus, both drugs seemed to interact similarly on identical targets. The ellipticine derivative 2-methyl-9-hydroxyellipticinium (2-Me-9-OH-E$^+$) had two effects on the production of DSB. Below 10 μM, 2-Me-9-OH-E$^+$ produced DSB as did ellipticine, *m*-AMSA, or 5-ID. Above 10 μM, 2-Me-9-OH-E$^+$ did not induce DSB and inhibited the DSB induced by *m*-AMSA, 5-ID, or ellipticine. 2-Me-9-OH-E$^+$ and *m*-AMSA competed with each other to produce either double-strand break formation (*m*-AMSA-induced reaction) or double-strand break inhibition (2-Me-9-OH-E$^+$-induced reaction at concentrations greater than 10 μM). Because these results were reproduced in experiments using DNA topoisomerase II isolated from L1210 nuclei, it is likely that the intercalator-induced protein-associated DNA breaks detected by alkaline elution in nuclei represent DNA topoisomerase II–DNA complexes. The intercalators appear to interact reversibly and competitively, but in different ways, with these complexes. *m*-AMSA, 5-ID, ellipticine, and 2-Me-9-OH-E$^+$, the last two at concentrations below 10 μM, probably stabilize the enzyme–DNA complexes in an open (cleaved) conformation of DNA, whereas high concentrations of 2-Me-9-OH-E$^+$ destabilize the DNA topoisomerase II DNA cleavage intermediates.

DNA intercalating drugs induce both DNA single-strand breaks (SSB) and DNA double-strand breaks (DSB) in mammalian cells. There is now good evidence that both types of DNA breaks result from the action of topoisomerase II: (1) the breaks produced by intercalators in cells are associated with DNA–protein links (Ross et al., 1979) which prevent DNA swiveling in 2 M salt (Pommier et al., 1984a,b); (2) the formation of protein-associated DNA breaks by intercalators in intact cells has the kinetic characteristics of a reversible enzymatic reaction (Zwelling et al., 1981); (3) the formation and resealing of intercalator-induced DNA breaks can be prevented by DNA topoisomerase II inhibitors (Marshall et al., 1983; Pommier et al., 1984b,c); (4) the enzymatic activity producing the intercalator-induced DNA breaks and DNA–protein cross-links is extractable from isolated L1210 cell nuclei at 0.35 M NaCl (Filipski et al., 1983), and purification of this activity shows that it consists of topoisomerase II (Minford et al., 1984); (5) purified calf thymus DNA topoisomerase II induces both DNA breaks and DNA–protein cross-links in the presence of intercalators (Nelson et al., 1984; Tewey et al., 1984a,b). It has been proposed that intercalators uncouple the DNA breaking–rejoining cycle of DNA topoisomerase II and stabilize those intermediates that consist of one topoisomerase II molecule bound at each break site induced by the enzyme (Pommier et al., 1984b; Nelson et al, 1984).

We wished to study the saturability of intercalator-induced protein-associated DNA double-strand breaks (DSB) in isolated mammalian cell nuclei by using wide concentration ranges of various drugs or combination of drugs (Figure 1). DSB were the most satisfactory measure of altered topoisomerase II action, because they were less susceptible than SSB to spurious nicking of DNA during incubation and could be measured over a wider range of drug concentrations by a single filter elution protocol (Pommier et al., 1984d). Isolated L1210 cell nuclei were used because the DSB frequencies produced by intercalators are usually similar to those found in whole cells (Pommier et al., 1984c) and because the nucleus preparation avoids the problems of drug cellular uptake or metabolism (Pommier et al., 1984c; Bachur et al., 1982). In the course of this study, we observed that 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) and 2-methyl-9-hydroxyellipticinium (2-Me-9-OH-E$^+$) differed in their ability to produce DSB at concentrations above 10 μM. Indeed 2-Me-9-OH-E$^+$, but not *m*-AMSA, inhibited DSB production at high concentration. The fact that comparable results were obtained with DNA topoisomerase II purified from similarly isolated L1210 cell nuclei [see the following paper (Pommier et al., 1985)] is in agreement with the hypothesis that intercalators produce DSB in mammalian cell nuclei through the mediation of DNA topoisomerase II action. Our results suggest a model consisting of reversible equilibria leading to several types of drug–DNA–topoisomerase II ternary complexes.

Materials and Methods

Cells and Radioactive Labeling. L1210 mouse leukemia cells were grown in suspension culture in RPMI 1630 medium supplemented with 15% fetal calf serum as described previously (Zwelling et al., 1981). Cellular DNA was radioactively

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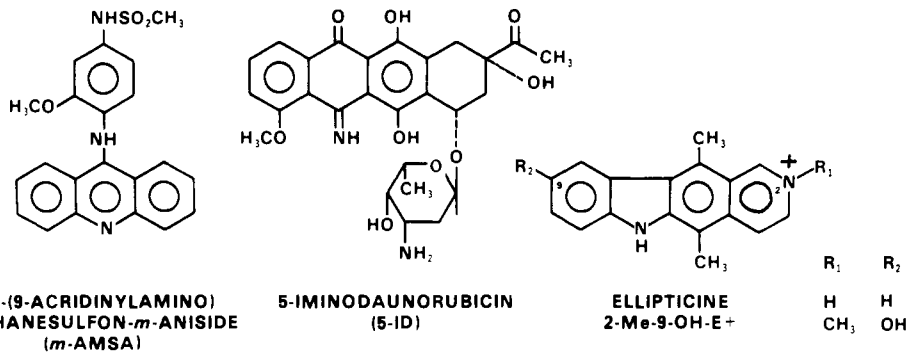

FIGURE 1: Chemical structures of 4'-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA), 5-iminodaunorubicin (5-ID), ellipticine, and 2-methyl-9-hydroxyellipticinium (2-Me-9-OH-E$^+$).

labeled in exponentially growing cells by incubation with [2-^{14}C]thymidine (0.02 μCi mL^{-1}) for 20 h at 37 °C (New England Nuclear, Boston, MA). One hour before preparation of nuclei, radioactive label was removed by centrifugation and resuspension of the cells in RPMI 1630 medium containing no [^{14}C]thymidine.

Isolation of L1210 Cell Nuclei. L1210 mouse leukemia cells were centrifuged and resuspended in nucleus buffer [150 mM NaCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM ethylene glycol bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA), 0.1 mM dithiothreitol, pH 6.4] at ice temperature. These cells were centrifuged again and resuspended in $^1/_{10}$ volume of ice-cold nucleus buffer. Nine-tenths volume of ice-cold nucleus buffer containing 0.3% Triton X-100 was then added and the mixture incubated for 10 min at 4 °C. The nuclei were pelleted by centrifugation at 1200 rpm for 5 min and resuspended in nucleus buffer at 37 °C (Filipski & Kohn, 1982; Pommier et al., 1982).

Drug Treatments and Cell Irradiation. 4'-(9-Acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) (NSC 249992) and ellipticine (NSC 71795) were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI. *m*-AMSA was dissolved in dimethyl sulfoxide at 10 mM and ellipticine in 10 mM HCl at 2.5 mM. 2-Methyl-9-hydroxyellipticinium (2-Me-9-OH-E$^+$) was a gift from Dr. J. B. Le Pecq, Institut Gustave Roussy, Villejuif, France. 2-Me-9-OH-E$^+$ was dissolved at 10 mM in glass-distilled water. 5-Iminodaunorubicin (5-ID) was obtained from Dr. E. Acton, Stanford Research Institute, and was dissolved in water at 1 mM. *m*-AMSA, 2-Me-9-OH-E$^+$, and 5-ID were stored frozen at −20 °C in stock solution. Ellipticine stock solution was kept at 4 °C in a plastic tube.

Drug treatments were for 30 min in nucleus buffer at 37 °C. When the interactive effects between two intercalators were studied, the first intercalator was added to isolated nuclei in suspension in nucleus buffer at 37 °C. After 5 min the second intercalator was added to the reaction mixture for the next 25 min, while the concentration of the first intercalator remained the same. All drug treatments were stopped by a 50-fold dilution of treated nuclei in ice-cold nucleus buffer.

Irradiation of nuclei was performed by using a ^{137}Cs source as described previously (Pommier et al., 1982). Nuclei were kept in ice until they were assayed by alkaline elution.

Measurement of DNA Double-Strand Breaks (DSB) by Filter Elution. DNA double-strand breaks were measured as described previously (Pommier et al., 1984d). The method used derives from the procedure of Bradley & Kohn (1979) but extends the range of measurement of DSB frequencies up to 20000 DSB rad equiv. It uses no more than 2×10^5 nuclei loaded per filter, a faster elution pump speed (0.12–0.16 mL min^{-1}), and 10-min interval fractions over a total elution time

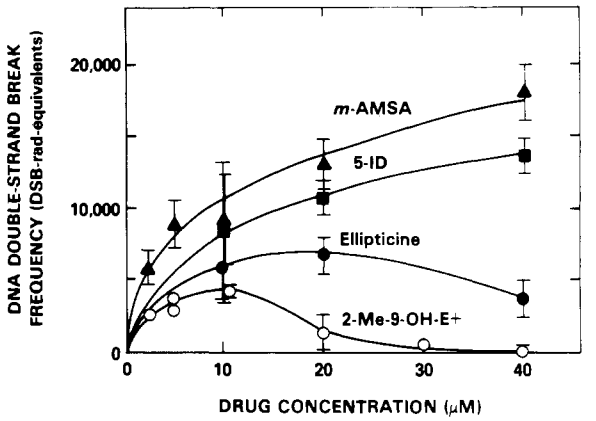

FIGURE 2: DNA double-strand breaks produced by *m*-AMSA (▲), 5-ID (■), ellipticine (●), and 2-Me-9-OH-E$^+$ (○) in isolated L1210 cell nuclei. Nuclei were treated with intercalators for 30 min at 37 °C. Reactions were stopped by a 50-fold dilution of treated nuclei in ice-cold nucleus buffer. DSB were assayed by filter elution (pH 10). Error bars denote standard deviations of at least three independent determinations.

of 60 min. Under these conditions the fraction of ^{14}C-labeled DNA retained per filter after 30 min of elution (Y) was inversely proportional to the administered dose of γ irradiation (X). The DNA double-strand break calibration curve of γ-irradiated L1210 cell nuclei was identical with that determined previously (Pommier et al., 1984d):

$$Y = 0.911 - (3.23 \times 10^{-5})X \quad (1)$$

Intercalator-induced DSB gave nearly linear elution curves. The similarity between these curves and those of irradiated nuclei (Figure 1) allowed the expression of the intercalator-induced DSB in DSB rad equivalents (Kohn et al., 1981). The DSB rad equivalent values for intercalator-treated nuclei were obtained from the fraction of ^{14}C-labeled DNA retained on the filter after 30 min of elution. This value was set equal to Y in eq 1, which was then solved for X.

Results

Drug Concentration Dependence of Trapping of DNA Double-Strand Breaks (DSB). The production of DSB due to the action of DNA intercalating agents on isolated cell nuclei was measured by pH 10 elution from polycarbonate filters (see Materials and Methods). These DSB have been shown to be protein-associated (Pommier et al., 1982), presumably due to covalent linkage of DNA to topoisomerase II. In the assays, the bound protein was removed by treatment of the nuclei with proteinase K prior to elution.

At concentrations up to 10 μM, the four intercalators studied (*m*-AMSA, 5-ID, ellipticine, and 2-Me-9-OH-E$^+$) produced DSB that increased with increasing drug concen-

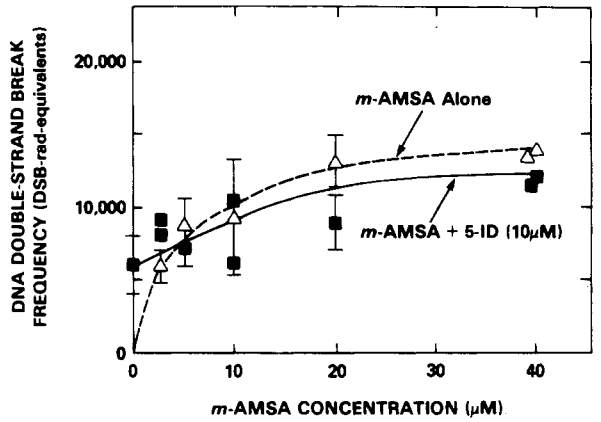

FIGURE 3: Comparison between the production of DSB by *m*-AMSA alone (△) and by *m*-AMSA in the presence of 5-ID (■) in isolated L1210 cell nuclei. Drug treatments were for 30 min at 37 °C (see Materials and Methods). Reactions were stopped by a 50-fold dilution of drug-treated nuclei in ice-cold nucleus buffer. Errors bars denote standard deviations for at least three independent determinations.

tration (Figure 2). At higher concentrations, *m*-AMSA and 5-ID produced greater numbers of DSB, although in both cases the curves tended to level off, as if approaching saturation (Figure 2). The saturation level appeared to be slightly higher for *m*-AMSA than for 5-ID. The number of ellipticine-induced DSB rose to a maximum at 20 μM (Figure 2) and then decreased as the drug concentration was further increased. In the case of 2-Me-9-OH-E$^+$, it rose to a maximum at 10 μM and then decreased to undetectable levels at 30 μM. Thus 2-Me-9-OH-E$^+$ and, to a much lesser extent, ellipticine, are self-inhibitory at high concentrations with regard to DSB production. This result is consistent with our previous finding that 2-Me-9-OH-E$^+$ did not produce protein-associated DNA strand breaks at concentrations above 30 μM in isolated nuclei (Pommier et al., 1982).

Interactions between Different Intercalators. The next question addressed was whether different intercalators interact with the same class of DSB-generating processes. Treatment of L1210 cell nuclei with 10 μM 5-ID produced approximately 6000 rad equiv of DSB (Figures 2 and 3). When nuclei were exposed simultaneously (see Materials and Methods) to 5-ID and *m*-AMSA, the resulting DSB frequency was lower than would have been expected if the DSB induced by each drug were additive (Figure 3). Below 5 μM *m*-AMSA, the 5-ID treatment increased the *m*-AMSA-induced DSB frequency, whereas above 5 μM *m*-AMSA, 5-ID had no additional effect (Figure 3). Thus, the saturation level of the DSB induced by *m*-AMSA alone or in the presence of 5-ID was similar, which suggests that both drugs may interact with the same class of targets whose number is somehow limited in mammalian cell nuclei.

We next asked whether self-inhibitory concentrations of 2-Me-9-OH-E$^+$ would also inhibit DSB production by other intercalators. Treatment of isolated nuclei with various concentrations of 2-Me-9-OH-E$^+$ in the presence of 20 μM *m*-AMSA, 5-ID, or ellipticine for 25 min resulted in an inhibition of the *m*-AMSA-, 5-ID-, or ellipticine-induced DSB (Figure 4). This inhibitory effect of 2-Me-9-OH-E$^+$ occurred in the concentration range that was self-inhibitory.

When nuclei were treated with 20 μM 2-Me-9-OH-E$^+$ plus various concentrations of *m*-AMSA, it was seen that high concentrations of *m*-AMSA overcame the 2-Me-9-OH-E$^+$ inhibition (Figure 5). An 80 μM concentration of *m*-AMSA produced 15 000 rad equiv of DSB in the presence of 20 μM 2-Me-9-OH-E$^+$, as did 20 μM *m*-AMSA in the absence of

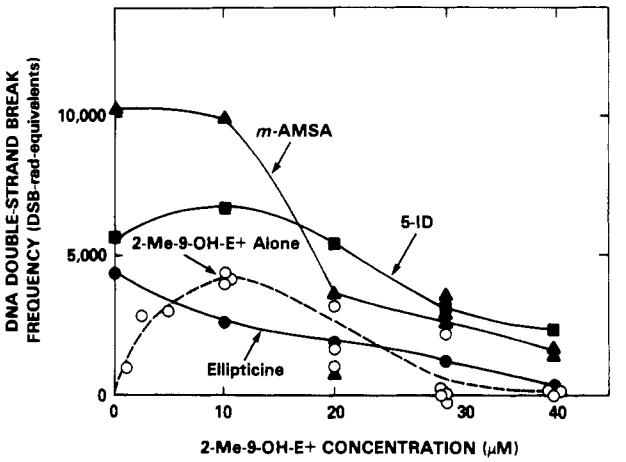

FIGURE 4: Inhibition of intercalator-induced DSB production by high concentrations of 2-Me-9-OH-E$^+$. Isolated nuclei were treated for 30 min at 37 °C with various concentrations of 2-Me-9-OH-E$^+$ alone (○) or 2-Me-9-OH-E$^+$ together with 20 μM of either *m*-AMSA (▲), 5-ID (■), or ellipticine (●).

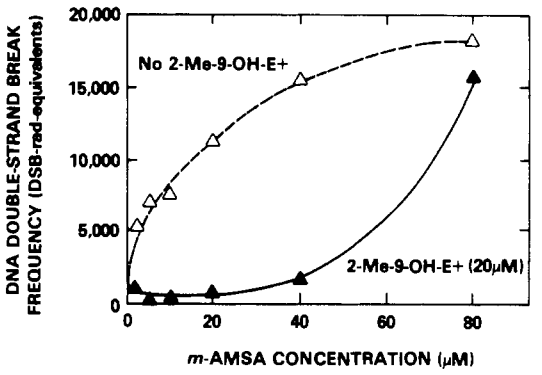

FIGURE 5: Dependence of the inhibitory effect of 2-Me-9-OH-E$^+$ on *m*-AMSA concentration. Isolated nuclei were treated for 30 min at 37 °C with various concentrations of *m*-AMSA alone (△) or *m*-AMSA together with 20 μM 2-Me-9-OH-E$^+$ (▲).

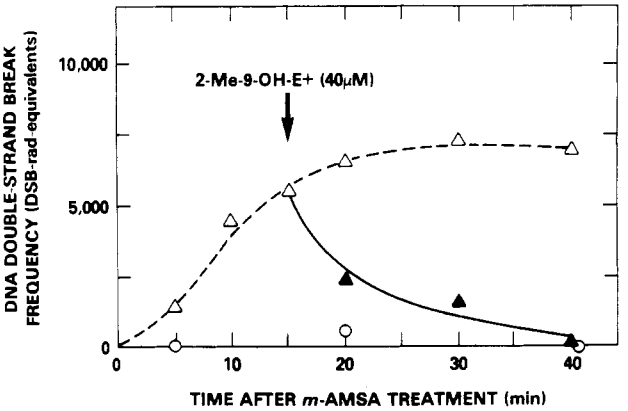

FIGURE 6: Kinetics of the inhibitory effect of 2-Me-9-OH-E$^+$ upon *m*-AMSA-induced DSB. Isolated L1210 cell nuclei were first treated with 20 μM *m*-AMSA alone; after 15 min, 40 μM 2-Me-9-OH-E$^+$ was added to a portion of the nuclear suspension (▲) while another portion was maintained with *m*-AMSA alone (△).

2-Me-9-OH-E$^+$ (Figures 5 and 2). This result suggested that the 2-Me-9-OH-E$^+$ inhibition of *m*-AMSA-induced DSB was reversible and perhaps competitive.

Reversibility Kinetics in Isolated Nuclei. Treatment of isolated nuclei with 20 μM *m*-AMSA rapidly produced DSB (Figure 6); a plateau was reached after 15–20 min. When nuclei were treated with *m*-AMSA for 15 min and 2-Me-9-OH-E$^+$ was then added, the *m*-AMSA-induced DSB were

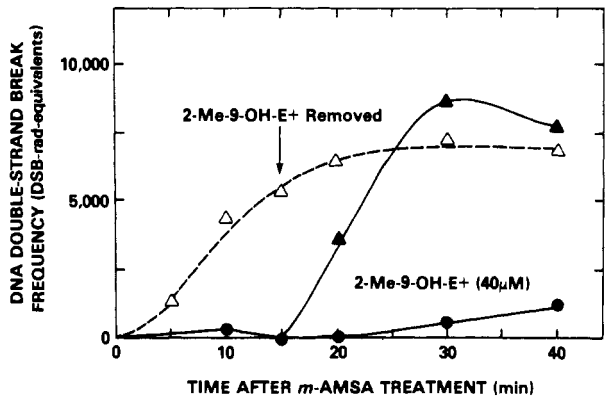

FIGURE 7: Kinetics of reversal of the 2-Me-9-OH-E$^+$ inhibition of *m*-AMSA-induced DSB. Isolated nuclei were treated either with *m*-AMSA alone (20 μM) (△) or with 2-Me-9-OH-E$^+$ (40 μM) plus *m*-AMSA (20 μM) (●). Fifteen minutes after the addition of *m*-AMSA (arrow), a portion of the 2-Me-9-OH-E$^+$-containing nuclear suspension was diluted 50-fold in nucleus buffer at 37 °C containing only *m*-AMSA (▲).

completely reversed (Figure 6). More than half of the *m*-AMSA-induced DSB disappeared within 5 min of the addition of 2-Me-9-OH-E$^+$. This kinetics of reversal of *m*-AMSA-induced DNA breaks by 2-Me-9-OH-E$^+$ is similar to that observed after *m*-AMSA removal from isolated nuclei (Pommier et al., 1984c).

We then tested directly whether the inhibitory effect of 2-Me-9-OH-E$^+$ was reversible (Figure 7). Nuclei that had been treated with 2-Me-9-OH-E$^+$ plus *m*-AMSA were used. Removal of the 2-Me-9-OH-E$^+$ by a 50-fold dilution of the nuclei with nucleus buffer at 37 °C containing only *m*-AMSA was followed by the reappearance of *m*-AMSA-induced DSB (Figure 7). After 15 min the DSB frequency reached the plateau level that was seen in nuclei that had been treated with *m*-AMSA alone. This result shows that the inhibitory effect of 2-Me-9-OH-E upon the nuclear target of *m*-AMSA was reversible upon 2-Me-9-OH-E$^+$ removal.

Discussion

The results obtained with *m*-AMSA and 5-ID in isolated nuclei suggest that these two intercalating drugs may interact with the same class of nuclear targets to produce DSB. The concentration–response curves were similar (Figure 2) as were the saturation amounts of DSB produced by *m*-AMSA alone or in the presence of 5-ID (Figure 3). These nuclear targets are likely to be DNA topoisomerase II–DNA complexes since both drugs can trap such complexes in the presence of DNA and purified enzyme (Minford et al., 1984; Nelson et al., 1984; Tewey et al., 1984a,b; Pommier et al., 1985). *m*-AMSA and, to a lesser extent, 5-ID induce not only DSB but also true SSB in whole cells (Zwelling et al., 1981; Zwelling et al., 1982a) and isolated nuclei (Pommier et al., 1984c); therefore, both SSB and DSB intermediates could be trapped or induced by *m*-AMSA and 5-ID. The DSB formed at low concentrations of 2-Me-9-OH-E$^+$ and ellipticine could also correspond to DNA topoisomerase II–DNA complexes (Tewey et al., 1984a,b; Pommier et al., 1985). Since no true SSB could be detected after treatment of whole cells or isolated nuclei with either 2-Me-9-OH-E$^+$ or ellipticine (Zwelling et al., 1982c; Pommier et al., 1984c), these intercalators would trap very few (or no) SSB intermediates and would thereby differ from *m*-AMSA and 5-ID. The trapping of topoisomerase II–DNA complexes by intercalators does not seem to require long-range torsional strain (Pommier et al., 1984d) and can be demonstrated with supercoiled, relaxed, or linear DNA (Tewey et al., 1984a,b; Pommier et al., 1985). These data support the view that the site of intercalation that traps the complex is near the DNA–topoisomerase II bond. It could, in principle, be either on the cleaved strand or on the passing strand. These possibilities are discussed in detail in the following paper (Pommier et al., 1985). In any case, the present finding that intercalator-induced DSB tended toward saturation in nuclei suggests that the number of DNA topoisomerase II molecules that can be trapped at their DNA cleavage site by intercalators is limited in mammalian cell nuclei. This result is in agreement with previous results obtained with *m*-AMSA in whole cells (Zwelling et al., 1981; Pommier et al., 1983). Assuming that 1000 rad yields one DSB per 5×10^6 nucleotides (Kohn et al., 1981; Blöcher, 1982), the plateau level of 20 000 DSB rad equiv observed in the case of *m*-AMSA or 5-ID would correspond to one DNA topoisomerase II cleavage site per 2×10^5 nucleotides.

The absence of DSB at concentrations of 2-Me-9-OH-E greater than 20 μM corresponds to an active inhibition of intercalator-induced DSB (Figure 3). The fact that *m*-AMSA and 2-Me-9-OH-E$^+$ competed to induce either DSB production (Figure 5) (*m*-AMSA effect) or DSB inhibition (Figure 6) (2-Me-9-OH-E$^+$ effect at concentrations above 20 μM) suggests that both drugs acted on a single class of nuclear targets (topoisomerase II–DNA complexes) but in different ways. High concentrations of 2-Me-9-OH-E$^+$ could stabilize intermediates of the DNA breaking–rejoining reaction mediated by DNA topoisomerase II at stages in which the enzyme is either dissociated from DNA or bound to DNA without inducing cleavage. In the latter case, the binding of topoisomerase II to DNA would not be covalent, since no DNA–protein cross-links have been detected by alkaline elution (data not shown) at the concentration at which 2-Me-9-OH-E$^+$ inhibited *m*-AMSA-induced DSB.

The results of this study suggest that intercalating drugs differ in their ability to stabilize or induce topoisomerase II–DNA intermediate complexes in mammalian cell nuclei.

Acknowledgments

We thank Madie Tyler for typing the manuscript and Drs. Michael R. Mattern and Jon K. Minford for valuable discussion and critical review of the manuscript.

Registry No. *m*-AMSA, 51264-14-3; 5-ID, 72983-78-9; 2-Me-9-OH-E$^+$, 58337-34-1; topoisomerase, 80449-01-0; ellipticine, 519-23-3.

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Effects of the DNA Intercalators 4′-(9-Acridinylamino)methanesulfon-*m*-anisidide and 2-Methyl-9-hydroxyellipticinium on Topoisomerase II Mediated DNA Strand Cleavage and Strand Passage

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Received March 12, 1985

ABSTRACT: DNA topoisomerase II is believed to be the enzyme that produces the protein-associated DNA strand breaks observed in mammalian cell nuclei treated with various intercalating agents. Two intercalators—4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA, amsacrine) and 2-methyl-9-hydroxyellipticinium (2-Me-9-OH-E$^+$)—differ in their effects on protein-associated double-strand breaks in isolated nuclei. *m*-AMSA stimulates their production at all concentrations, whereas 2-Me-9-OH-E$^+$ stimulates at low concentrations and inhibits at high concentrations. We have reproduced these differential effects in experiments carried out in vitro with purified L1210 DNA topoisomerase II, and we have found that concentrations of 2-Me-9-OH-E$^+$ above 5 μM prevent the trapping of DNA–topoisomerase II cleavable complexes irrespective of the presence of *m*-AMSA. It also stimulated topoisomerase II mediated DNA strand passage, again with or without inhibitory amounts of *m*-AMSA (this result suggests that extensive intercalation by 2-Me-9-OH-E$^+$ destabilized the cleavable complexes). From these data, it is concluded that intercalator-induced protein-associated DNA strand breaks observed in intact eukaryotic cells and isolated nuclei are generated by DNA topoisomerase II and that intercalators can affect mammalian DNA topoisomerase II in more than one way. They can trap cleavable complexes and inhibit DNA topoisomerase II mediated DNA relaxation (*m*-AMSA and low concentrations of 2-Me-9-OH-E$^+$) or destabilize cleavable complexes and stimulate DNA relaxation (high concentrations of 2-Me-9-OH-E$^+$).

Intercalator-induced DNA breaks in mammalian cells (Ross et al., 1979; Zwelling et al., 1981; Pommier et al., 1984a,b) and in isolated nuclei (Pommier et al., 1982, 1984c,d) have been postulated to result from altered DNA topoisomerase II action. Results obtained with purified DNA topoisomerase II are in agreement with such a hypothesis. Intercalators stimulate topoisomerase II induced DNA strand breaks and DNA–protein links within single topoisomerase II–DNA complexes (Nelson et al., 1984; Tewey et al., 1984a,b; Minford et al., 1984). These "cleavable complexes" (Liu et al., 1983) are analogous to the protein-associated DNA breaks induced by intercalators in cells, in that (1) enzyme molecules are bound to the 5′ termini of both DNA strands at the break site (Liu et al., 1983; Sander & Hsieh, 1983; Marshall et al., 1983) and (2) DNA strands are prevented from swiveling at the break site, presumably by bound DNA topoisomerase II (Liu et al., 1983; Pommier et al., 1984a,b). Further evidence that intercalator-induced protein-associated DNA breaks observed in mammalian cells correspond to DNA topoisomerase II–DNA cleavable complexes is that the intercalator-dependent DNA–protein-linking activity from mouse leukemia (L1210) nuclei has been purified and identified as DNA topoisomerase II (Minford et al., 1984).

In the preceding paper (Pommier et al., 1985), we report that 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA, amsacrine) at all concentrations stimulated the formation of DNA double-strand breaks, whereas 2-methyl-9-

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