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ROLE OF DNA INTERCALATION IN THE INHIBITION OF PURIFIED MOUSE LEUKEMIA (L1210) DNA TOPOISOMERASE II BY 9-AMINOACRIDINES

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Abstract—An attempt was made to analyze the mechanism by which 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) inhibits mammalian DNA topoisomerase II. The effects of various 9-aminoacridine derivatives differing by their DNA affinities and DNA sequence selectivity of binding were compared in the presence of purified mouse leukemia L1210 DNA topoisomerase II. No correlation was found between DNA unwinding and topoisomerase II inhibition. 9-Aminoacridine was inactive as a topoisomerase II inhibitor and *o*-AMSA was only weakly active. The location of L1210 topoisomerase II mediated DNA breaks produced in the absence or presence of 9-aminoacridines were studied in [^{32}P]-end-labeled pBR 322 DNA. All 9-aminoacridines, even those differing by their DNA sequence selectivity of binding, produced similar DNA cleavage patterns. Most drug-induced topoisomerase II mediated DNA breaks appeared at sites that were already cleaved by the enzyme in the absence of drug. The present results suggest that 9-aminoacridines inhibit L1210 DNA topoisomerase II by interacting at or near enzyme–DNA complexes by some unknown DNA effect or by direct protein interaction.

DNA topoisomerases are ubiquitous enzymes found both in prokaryotes and eukaryotes [1–3]. DNA topoisomerases have been classified into type I and type II enzymes. Type I topoisomerases change the DNA linking number by steps of one, and the type II by steps of two. Both types of enzyme have been isolated from mammalian cell nuclei [4–7]. Antitumor DNA intercalators and epipodophyllotoxin derivatives inhibit eukaryotic type II topoisomerases by stabilizing enzyme–DNA complexes, in which the enzyme is covalently bound to the 5′-terminus of an enzyme-induced DNA break [6, 8–13]. These complexes are thought to be abortive intermediates of the DNA strand passing reactions of topoisomerase II.

The nature of the interactions between DNA intercalators and DNA topoisomerase II is unknown. Intercalators bind DNA and could affect the enzyme by modifying its DNA substrate. One of the most potent DNA intercalators to inhibit mammalian DNA topoisomerase II is 4′-(9-acridinylamino)methanesulfon-*m*-aniside (*m*-AMSA) [6, 13]. *m*-AMSA is an acridine derivative substituted on the 9-position with an anilino substituted group. The isomer of *m*-AMSA, *o*-AMSA, which bears the methoxy group in the ortho position instead of the meta (Fig. 1), is markedly less effective than *m*-AMSA in inhibiting mammalian topoisomerase II [13]. This observation raises the possibility that drug intercalation might not be sufficient to trap topoisomerase II–DNA complexes.

In the present study, we have investigated the interaction of *m*-AMSA and several other 9-aminoacridine derivatives with topoisomerase II–DNA complexes in order to determine the role of DNA intercalation in the trapping of enzyme–DNA complexes. The compounds that were used are shown on Fig. 1. *m*-AMSA and *o*-AMSA were the derivatives of reference because of their differential inhibitory effects upon mammalian topoisomerase II. 9-Aminoacridine was chosen because it is a better intercalator than *m*-AMSA [14, 15] and does not have the bulky anilino group on the 9-position of the acridine ring. Compounds A, B and C were chosen with Dr. Bruce Baguley (Cancer Research Laboratory, Auckland University School of Medicine, New Zealand), who sent us these compounds. Compound C has a higher DNA affinity than *m*-AMSA but has a longer side chain substitution. Compounds A and B have different DNA binding sequence selectivity. Compound A binds preferentially poly[d(G-C)]·poly[d(G-C)] over poly[d(A-T)]·poly[d(A-T)], whereas compound B does the opposite. After having determined the DNA unwinding properties of the six compounds using a topoisomerase I assay, we studied the potency of the compounds to inhibit mouse leukemia L1210 topoisomerase II and the location of the trapped topoisomerase II–DNA complexes using [^{32}P]-end-labeled pBR 322 DNA and DNA agarose or sequencing gel electrophoresis.

MATERIALS AND METHODS

Materials. pBR 322, SV40 and λ Hind III DNAs, EcoR I and Hind III restriction enzymes, T_4

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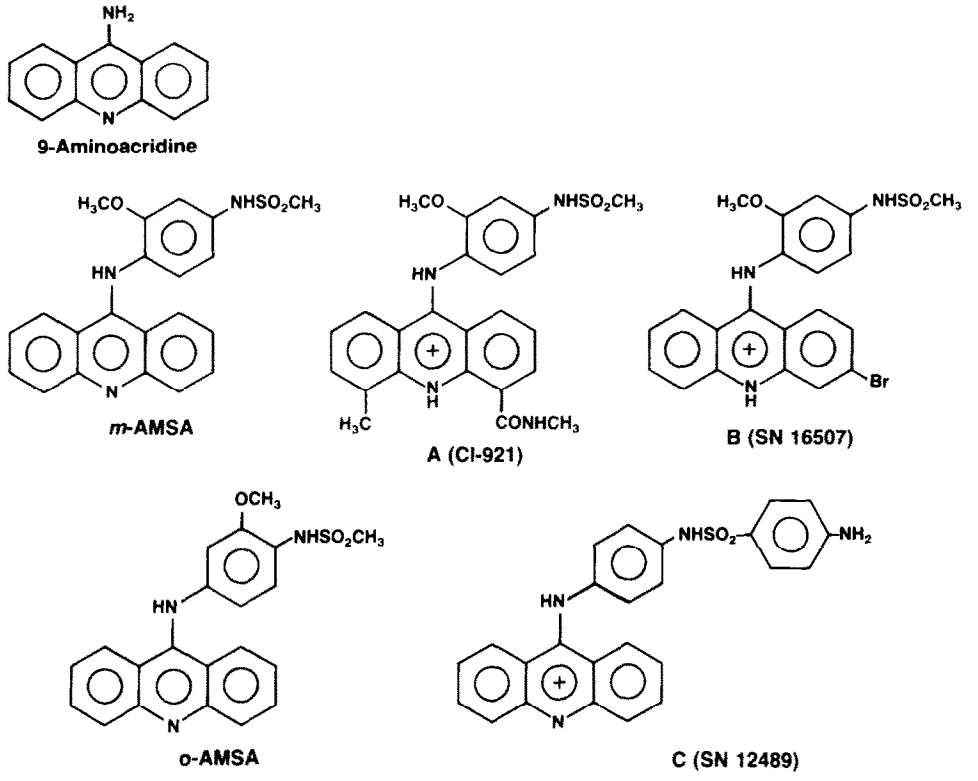

Fig. 1. Structures of the 9-aminoacridine derivatives used in the present study.

polynucleotide kinase, and agarose were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Phosphatase was purchased from New England Biolabs (Beverly, MA). Polyacrylamide and [^{32}P-γ]ATP were purchased from Bio-Rad, Inc. (Richmond, CA) and New England Nuclear Research Products (Boston, MA) respectively. XAR-5 films (Eastman Kodak Co., Rochester, NY) were used for autoradiography.

pBR 322 DNA was end-labeled as described previously [13]. Briefly, the DNA was first cut with Hind III at position 29 of the pBR 322 genome [16], and its termini were dephosphorylated with calf alkaline phosphatase. The 5′-termini were then labeled with [^{32}P-γ]ATP and T_4 polynucleotide kinase. [^{32}P]-End-labeled pBR 322 Hind III DNA was further cut with EcoR I at position 0 of the genome [16]. Such a procedure generates two [^{32}P]-5′-end-labeled fragments, one of 4333 base pairs and the other of 29 base pairs. Because of the different sizes of these two fragments, reactions were performed with the two DNA fragments present in the reaction mixtures. The topoisomerase II mediated DNA breaks generating DNA fragments longer than 30 base pairs could then be localized into the pBR 322 genome. The DNA markers used in the agarose gels were similarly labeled at their 5′-termini with [^{32}P-γ]ATP and polynucleotide kinase. They were either λ Hind III or λ Hind III EcoR I fragments.

Type II DNA topoisomerase was purified from mouse leukemia (L1210) cell nuclei, as described previously [6].

Adriamycin (NSC 123127), 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA, Amsacrine) (NSC 249992), 4′-(9-acridinylamino)-methanesulfon-*o*-anisidide (*o*-AMSA) (NSC 56306) and 5-iminodaunorubicin (NSC 254681) were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI. 9-Aminoacridine was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Compounds A (CI-921, NSC 343499), B (SN 16507) and C (SN 12489) were a gift from Dr. Bruce Baguley, Cancer Research Laboratory, University of Auckland, School of Medicine, New Zealand. 2-Methyl-9-hydroxyellipticinium (NSC 264137) was a gift from Dr. J. B. Le Pecq, Institut Gustave Roussy, Villejuif, France. Teniposide (VM-26) was a gift from the Bristol Myers Co. (Syracuse, NY). All the 9-aminoacridine derivatives were dissolved in dimethyl sulfoxide at 10 mM. Adriamycin and 5-iminodaunorubicin were dissolved in distilled water at 1 mM. Drug stock solutions were kept frozen at −20°. Teniposide was dissolved in dimethyl sulfoxide at 10 mM immediately before use.

*Comparison of intercalator-induced DNA unwinding**. Native SV40 DNA was first reacted with purified L1210 topoisomerase I for 5 min. Various drug concentrations were then added to the reaction mixture for an additional 30 min. Drug-induced DNA unwinding was then determined as a shift of DNA

* Pommier *et al.*, manuscript submitted for publication.

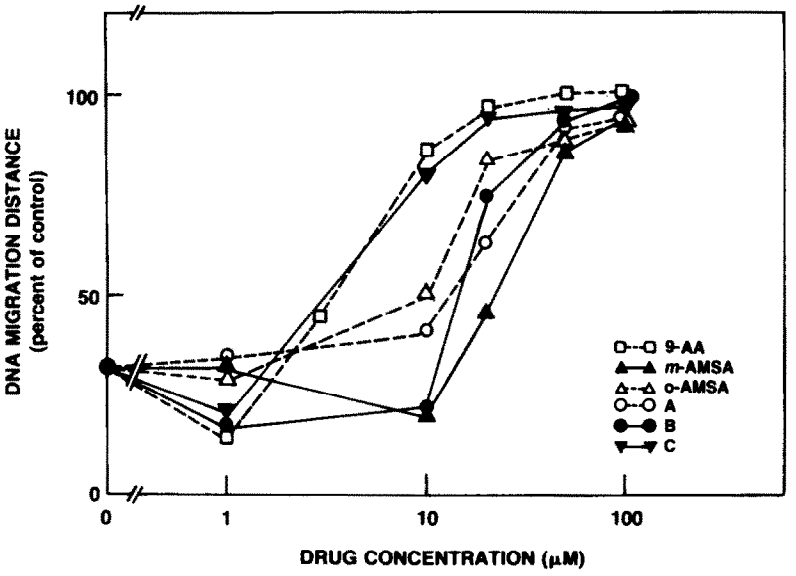

Fig. 2. DNA unwinding produced by 9-aminoacridines as determined by using a topoisomerase I unwinding assay. L1210 topoisomerase I relaxed SV40 DNA was reacted with drugs in the presence of excess enzyme. Reactions were stopped, and agarose gels were run as described under Materials and Methods. The migration distances between the leading DNA topoisomer and that of nicked relaxed DNA were measured from the electrophoresis pictures and normalized by expressing them as a percentage migration distance of native supercoiled SV40 DNA.

topoisomer distribution after agarose gel electrophoresis (see Fig. 2).

Reaction conditions. Reactions were performed in buffer B (0.01 M Tris·HCl, pH 7.5, 0.05 M KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/ml bovine serum albumin) for 30 min at 37°. Reactions were stopped by adding sodium dodecyl sulfate ($NaDoSO_4$) and proteinase K (Boehringer Mannheim, Indianapolis, IA) (1% and 0.5 mg/ml final concentrations respectively). After an additional 30 min of incubation at 37°, samples were processed as follows: (1) in the case of topoisomerase reactions such as those shown in Figs. 2–4, samples were directly loaded into agarose gels after addition of loading buffer [10x solution consisting of 0.3% bromophenol blue, 16% Ficoll and 0.01 M Na_2HPO_4]; (2) in the case of DNA cleavage reactions in [^{32}P]-end-labeled pBR 322 DNA, each sample was extracted after proteinase K digestion with an equal volume of a 1:1 mixture of phenol–chloroform, and then supplemented with an appropriate volume of loading buffer and heated at 65° for 2–5 min immediately before being loaded into the agarose gel; (3) in the case of samples to be run into sequencing gels, the DNA was precipitated in the presence of 2.5 M sodium acetate, 1 μg tRNA and 3 vol. of ethanol overnight at −20°, washed once with cold ethanol, and dryed. The DNA pellets were resuspended into 3 μl loading buffer for Maxam and Gilbert sequencing gels (80% formamide, 0.01 M NaOH, 1 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue). Samples were counted for [^{32}P] cpm and heated at 65° for 2–5 min. Equal counts were loaded into each lane of sequencing gels. DNA sequencing reactions of [^{32}P]-end-labeled pBR 322 DNA were performed as described previously [17].

DNA gels and autoradiography. (1) Agarose gels for DNA topoisomer detection were made in 1% agarose in TEA buffer (0.04 M Tris·acetate, pH 7.6, 0.01 M EDTA). Gels were run at 2–3 V/cm overnight, stained with ethidium bromide (1 μM), and destained with 1 mM $MgSO_4$ for 30–45 min and then photographed under u.v. with Polaroid 57 or 55 films. (2) Agarose gels for topoisomerase II mediated DNA cleavage reactions within [^{32}P]-end-labeled pBR 322 DNA were made in 1 or 1.5% agarose in TBE buffer (Tris·borate·EDTA). Gels were run at 2–3 V/cm overnight and then dried on a 3 MM paper with a lyophilizer. Dried gels were autoradiographed with Kodak XAR-5 films. (3) DNA sequencing gels were made in 6% polyacrylamide (29:1, acrylamide:bis), 7 M urea in TBE buffer. Gels were preelectrophorized until their temperature reached 50–55°. Samples were loaded, and gels were run at 1600–1700 V (70 W) for approximately 2 hr and then transferred to a 3 MM paper sheet, dried and autoradiographed as above.

Densitometer scanning and computer analysis of topoisomerase II mediated DNA cleavage sites. Autoradiography films and negatives of agarose gel pictures were scanned with a DU-8B Beckman spectrophotometer. The densitometer was connected to a computer in order to store, graph and analyze the data. λ Hind III or λ Hind III EcoR I markers were run in the two outer lanes of all gels in order to check the uniformity of the DNA migration throughout the gel. A line was drawn between two bands of similar migration of the markers in order to give a position reference for each lane. The 6557 and 4973 base pairs fragments were the reference lines usually chosen for the λ Hind III and λ Hind III EcoR I sets of markers respectively. The

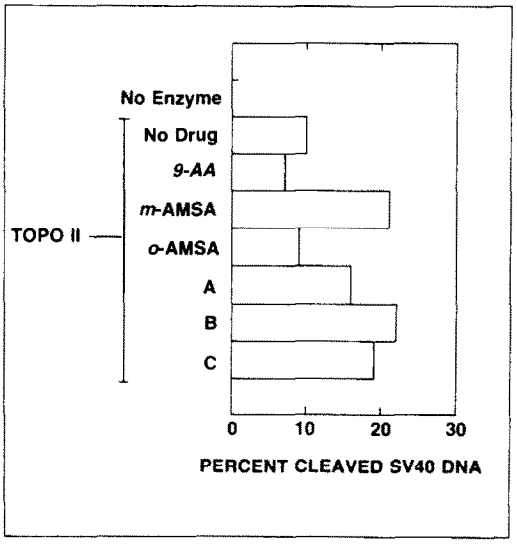

Fig. 3. DNA topoisomerase II mediated DNA cleavage induced by 9-aminoacridines at equal DNA unwinding concentrations. Native SV40 DNA (control) was reacted with purified L1210 topoisomerase II in the absence of drug or in the presence of 3.5 μM 9-aminoacridine (9-AA), 22 μM *m*-AMSA, 10 μM *o*-AMSA, 12.5 μM compound A, 12.5 μM compound B, or 2 μM compound C for 30 min at 37° in the absence of ATP. Reactions were stopped, and agarose gels were run as described under Materials and Methods. Negatives of the pictures of a typical gel were scanned and the fraction of linear DNA was calculated.

regression lines of the markers were then determined for each gel by calculating the regression line of the logarithm of the fragment size (in base pairs) versus the migration distance of each fragment from the reference line. Reaction lanes were scanned and the size of each fragment generated by topoisomerase II in the absence or presence of drug was then determined. The absorbance versus migration distance graph was transformed to generate an intensity versus strand length graph by computer on the basis of the principle of equivalent area elements. A final correction was made to determine the genomic position of topoisomerase II mediated DNA cuts by taking into account the position of the label relative to the conventional 0 position of the pBR 322 genome [16].

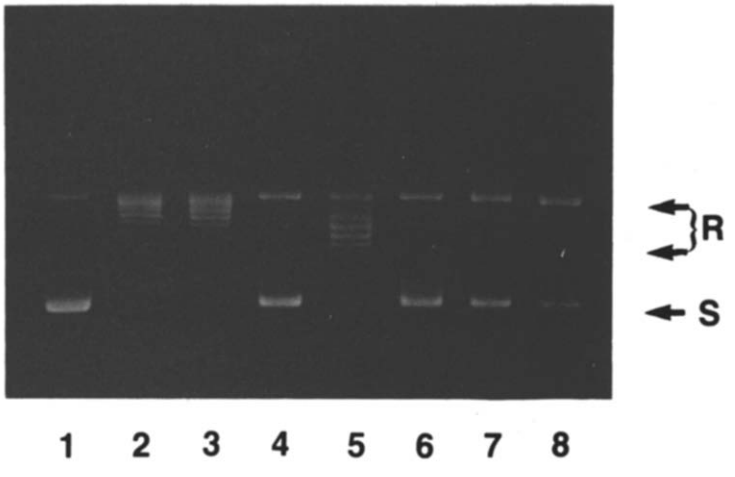

Fig. 4. Inhibitory effects of 9-aminoacridine derivatives upon L1210 DNA topoisomerase II mediated DNA relaxation in the presence of 1 mM ATP. Native SV40 DNA (0.4 μg, lane 1) was reached with purified L1210 topoisomerase II in the absence of drug (lanes 2) or in the presence of drug concentrations giving similar DNA unwinding [3.5 μM 9-aminoacridine (lane 3), 22 μM *m*-AMSA (lane 4), 10 μM *o*-AMSA (lane 5), 12.5 μM compound A (lane 6), 12.5 μM compound B (lane 7), 2 μM compound C (lane 8)]. Reaction mixtures were stopped by adding $NaDoSO_4$ (1% final concentration) and run into a 1% agarose gel in Tris·acetate·EDTA buffer. R: relaxed DNA topoisomers; S: native supercoiled SV40 DNA.

RESULTS

Relative potency of 9-aminoacridines in inhibiting L1210 DNA topoisomerase II. The relative DNA unwinding potencies of the 9-aminoacridine derivatives shown in Fig. 1 were determined by a DNA unwinding assay using L1210 topoisomerase I in the buffer and DNA conditions in which topoisomerase II reactions were carried out. The potencies of the compounds varied widely (Fig. 2). Each compound was then tested against topoisomerase II at drug concentrations giving a similar unwinding of native SV40 DNA. A 50% migration distance shift was chosen as a common unwinding concentration and was produced by 3.5 μM 9-aminoacridine, 20 μM *m*-AMSA, 10 μM *o*-AMSA, 12.5 μM compounds A and B, and 2 μM compound C. Topoisomerase II mediated DNA breaks were not produced by all drugs under these conditions (Fig. 3). Neither 9-aminoacridine nor *o*-AMSA induced topoisomerase II-mediated DNA breaks, whereas *m*-AMSA and compounds A, B and C did. The inhibition of the enzyme catalytic activity was also tested at equal drug-induced DNA unwinding concentrations (Fig. 4). 9-Aminoacridine (lane 3) had no effect, *o*-AMSA (lane 5) was weakly inhibitory, and *m*-AMSA and compounds A, B and C inhibited completely topoisomerase II mediated DNA relaxation (lanes 4, 6, 7 and 8 respectively). Taken together, these results show that, at drug concentrations giving similar DNA unwinding, compounds A, B, C and *m*-AMSA inhibited L1210 topoisomerase II, whereas 9-aminoacridine had no effect and *o*-AMSA only a weak effect. These observations indicate that the DNA topoisomerase II inhibitory effect of 9-aminoacridine derivatives is not directly related to DNA unwinding.

DNA topoisomerase II induced DNA cleavage patterns of 9-aminoacridines in pBR 322 DNA. The next question was whether the 9-aminoacridines that inhibited L1210 topoisomerase II were doing so by stabilizing enzyme–DNA complexes at similar or different sites along DNA. The DNA cleavage patterns of topoisomerase II mediated DNA breaks induced by *m*-AMSA and compounds A, B, and C were studied over a wide range of drug concentrations in [^{32}P]-end-labeled pBR 322 DNA (Fig. 5). No difference was found between the DNA cleavage patterns induced by the four compounds. A comparison of the intensity of DNA cleavage at 0.1 μM drug concentration (lanes 3, 6, 9, and 12) indicated that the compounds could be ranked by decreasing activity: C > *m*-AMSA > A > B. In

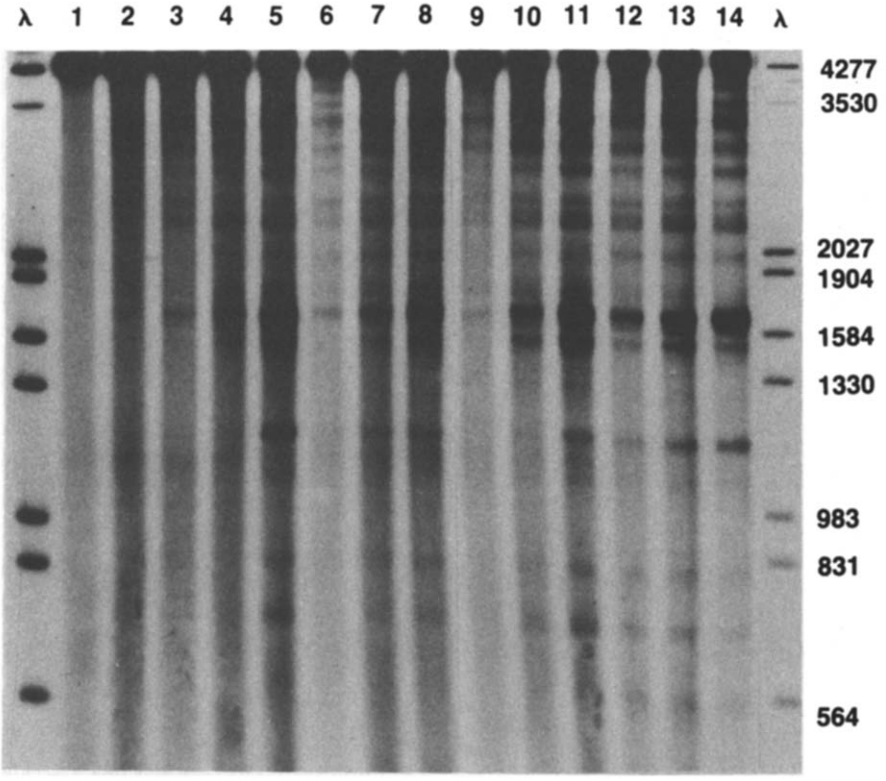

Fig. 5. DNA double strand break patterns of L1210 DNA topoisomerase II in the absence or presence of 9-aminoacridines in [^{32}P]-end-labeled pBR 322 DNA. pBR 322 DNA (lane 1) was reacted with purified L1210 topoisomerase II in the absence (lane 2) or presence of various concentrations of either *m*-AMSA (0.1, 1, 10 μM in lanes 3, 4, 5), compound A (0.1, 1 and 10 μM in lanes 6, 7 and 8), compound B (0.1, 1 and 10 μM in lanes 9, 10 and 11) or compound C (0.1, 1 and 10 μM in lanes 12, 13 and 14). Reactions were performed in the absence of ATP for 30 min at 37° and then stopped by adding $NaDoSO_4$ and proteinase K (1% and 0.5 mg/ml final concentrations respectively). Reaction mixtures were further incubated for 30 min at 37° and run into a 1% agarose gel in Tris·borate·EDTA buffer. The gel was then dried and autoradiographed. λ: Hind III/EcoR I digest of lambda DNA. The size (in base pairs) of the λ fragments is indicated at the right of the picture.

addition, at 10 μM none of the drugs inhibited topoisomerase II mediated DNA cleavage. Therefore, 9-aminoacridine intercalators seem to differ from the other intercalators, such as the ellipticines [8, 12], anthracyclines [9], or ethidium bromide [18].

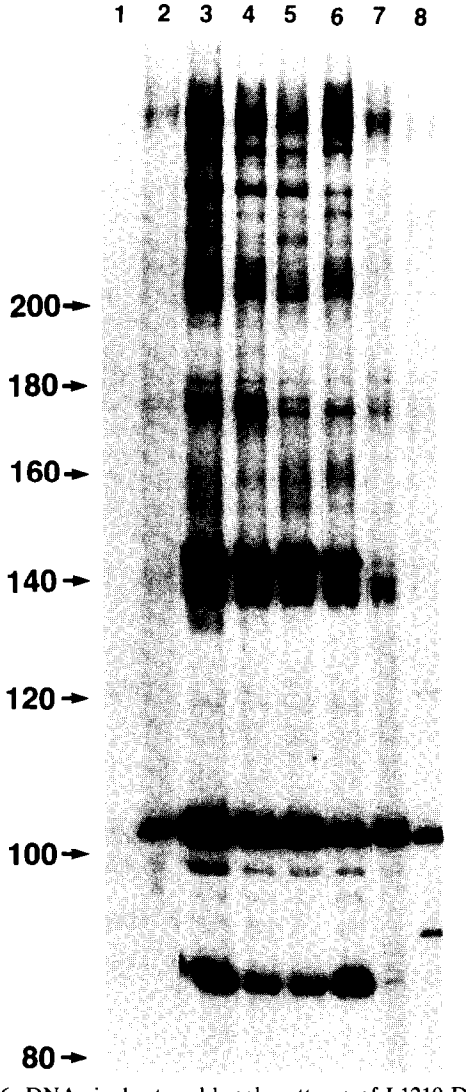

Fig. 6. DNA single-strand break patterns of L1210 DNA topoisomerase II in the absence or presence of 9-aminoacridines in [^{32}P]-end-labeled pBR 322 DNA. pBR 322 DNA that had been labeled at the 5′-termini of the Hind III cutting site and further cut with EcoR I (lane 1) was reacted with purified L1210 topoisomerase II in the absence (lane 2) or presence of 10 μM of either *m*-AMSA (lane 3), compound A (lane 4), compound B (lane 5), compound C (lane 6), 2-methyl-9-hydroxyellipticinium (1 μM, lane 7), or 5-iminodaunorubicin (2 μM, lane 8). Reactions were performed in the absence of ATP for 30 min at 37° and then were stopped by adding $NaDoSO_4$ and proteinase K (1% and 0.5 mg/ml respectively). After an additional 30-min incubation at 37°, DNA was precipitated in ethanol overnight at −20° and resuspended into 4 μl Maxam and Gilbert loading buffer. Reaction products were run into a 6% urea-polyacrylamide gel in Tris·borate·EDTA buffer. The gel was then dried and autoradiographed. Genomic positions of the cutting sites were determined by running concurrently sequencing reactions of similarly end-labeled pBR 322 DNA (not shown); they are indicated at the left of the picture.

A more detailed comparison was performed by studying the topoisomerase II mediated DNA fragments in a sequencing gel system (Fig. 6). In this kind of analysis, only the first two or three hundred nucleotides from the 5′-[^{32}P]-end-labeled terminus can be analyzed. Maxam and Gilbert DNA sequencing reactions were run simultaneously in order to localize the positions of the DNA breaks (number and arrows on the left, Fig. 6). Under the experimental condition of Fig. 6, L1210 topoisomerase II alone produced only one major cut at position 102 or 103 (lane 2). *m*-AMSA and compounds A, B and C induced topoisomerase II mediated DNA breaks not only at position 103, but at multiple other sites, the strongest being around positions 87 and 142–143 (lanes 3–6). A more detailed analysis of the positions of the DNA cutting sites will be discussed later. At the moment, the important point is the analogy between the effects of the four compounds, although their DNA sequence selectivity of binding was known to be different. Reactions were also performed with 2-methyl-9-hydroxyellipticinium (lane 7) and 5-iminodaunorubicin (lane 8). The distribution of the cleavages sites differed from that of the 9-aminoacridines, which shows that the gel system used could detect true differences in DNA cleavage patterns. The concentration of the 9-aminoacridines used in Fig. 6 was 10 μM. Similar reactions performed with drug concentrations as low as 0.1 μM did not show any difference between the topoisomerase II mediated DNA cleavage patterns induced by 9-aminoacridines in pBR 322 DNA.

Location of the DNA topoisomerase II mediated DNA breaks induced by m-*AMSA in pBR 322 DNA.* *m*-AMSA was then chosen to determine more precisely the location of 9-aminoacridine-induced topoisomerase II mediated DNA breaks in pBR 322 DNA. The reasons of this choice were: (1) *m*-AMSA is a well established topoisomerase II inhibitor, (2) *m*-AMSA is the only 9-aminoacridine currently used in cancer chemotherapy, and (3) the *m*-AMSA-induced topoisomerase II mediated DNA cleavage pattern in pBR 322 DNA was similar to those of the other 9-aminoacridines (Figs. 5 and 6).

m-AMSA-induced topoisomerase II mediated DNA breaks were first studied by the procedure summarized on Fig. 7. pBR 322 DNA that had been [^{32}P]-end-labeled at the 5′-termini of the Hind III cutting site and further cut at the EcoR 1 site (see Materials and Methods) was reacted with various concentrations of *m*-AMSA in the absence or presence of L1210 DNA topoisomerase II. Reaction products were run into a 1.5% agarose gel along with [^{32}P]-end-labeled λ Hind III fragments. The gel was then dried and autoradiographed (panel A, Fig. 7). The film was scanned with a Beckman DU-8B densitometer (panel B, Fig. 7). Lane 2 shows the densitometer scanning of the DNA fragments produced by L1210 topoisomerase II alone and lanes 3, 4 and 5 those of the DNA fragments produced by 0.1, 1 and 10 μM *m*-AMSA added to L1210 topoisomerase II. The *m*-AMSA-induced topoisomerase II mediated DNA breaks were selectively distributed, and the intensity of cleavage was con-

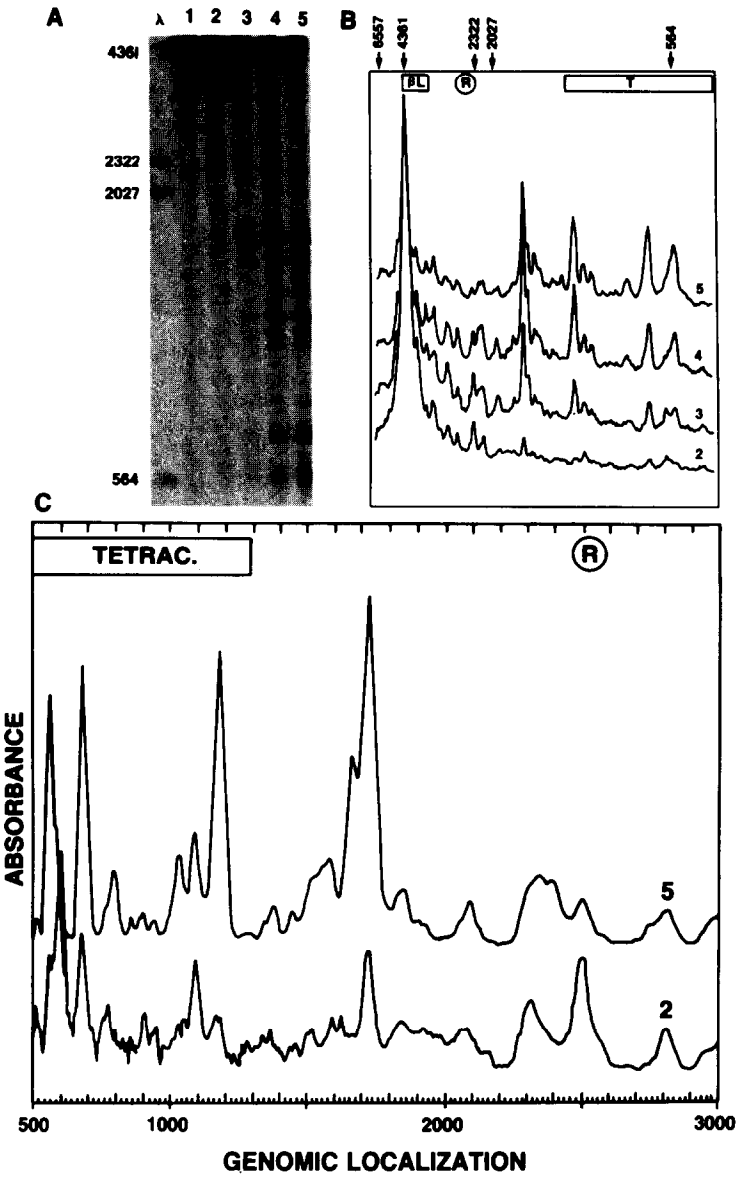

Fig. 7. Location of L1210 topoisomerase II-mediated DNA double-strand breaks in pBR 322 DNA. pBR 322 DNA that had been [^{32}P]-end-labeled at the 5′-termini of the Hind III cutting site (lane 1) was reacted with purified L1210 topoisomerase II in the absence (lane 2) or presence of *m*-AMSA (0.1 μM in lane 3, 1 μM in lane 4, and 10 μM in lane 5). Reactions were performed without ATP for 30 min at 37° and were stopped with $NaDoSO_4$ and proteinase K (1% and 0.5 mg/ml respectively). After an additional 30-min incubation at 37°, reaction mixtures were run into a 1.5% agarose gel in Tris·borate·EDTA buffer and autoradiographed (panel A). The film was scanned (panel B). Using the migration position of the λ Hind III fragments and computer analysis (see Materials and Methods), the densitometer scanning was converted into a densitometer pattern (panel C), which allows the determination of the location (within approximately 50 bp) of the topoisomerase II mediated DNA breaks. Boxes correspond to the tetracycline and β-lactamine resistance genes and the circle to the origin of replication of pBR 322.

centration dependent (compare lanes 3 and 4 of panel B, Fig. 7). A further step in the analysis was to determine the genomic localization of the topoisomerase II mediated DNA breaks produced in the absence or presence of *m*-AMSA. This was done by a computer analysis of the densitometer scanning shown in panel B. A regression line of the λ Hind III fragments was first established by expressing the migration distance versus the logarithm of the size (in nucleotide) of the fragments (see Materials and Methods). The correlation coefficient of this line was 0.99. The size (in nucleotides) of the topoisomerase II induced pBR 322 DNA fragments was then determined from the λ Hind III fragments regression line

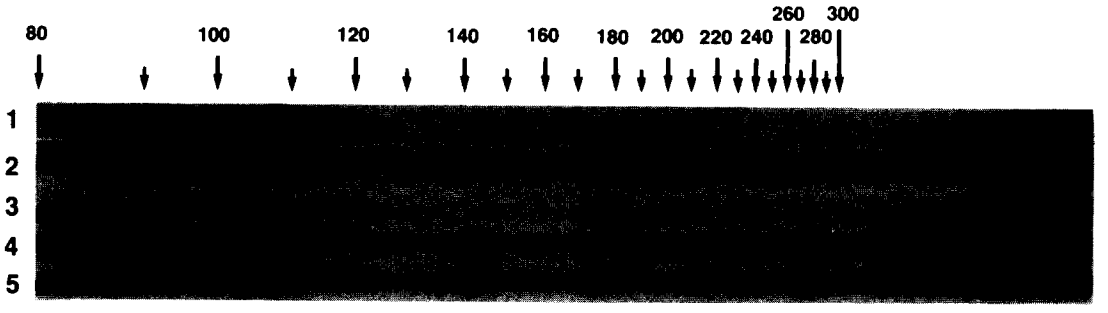

Fig. 8. Location of L1210 DNA topoisomerase II mediated DNA single-strand breaks in pBR 322 DNA. pBR 322 DNA that had been [^{32}P]-end-labeled at the 5'-terminus of the Hind III cutting site (lane 1) was reacted with purified L1210 topoisomerase II in the absence (lane 2) or presence of adriamycin (10 μM, lane 3), *m*-AMSA (10 μM, lane 4), or teniposide (10 μM, lane 5). Reactions were performed and analyzed as described in the legend of Fig. 6.

and the DNA breaks location expressed as genomic positions by correcting their fragment size according to the [^{32}P]-label position (see Materials and Methods). Panel C (Fig. 7) shows such an analysis for lanes 2 and 5 of panel B. The tracing of lane 2 was amplified 2-fold relative to that of lane 5 in order to show more clearly the DNA cleavage pattern induced by L1210 topoisomerase II alone. Most *m*-AMSA-induced topoisomerase II mediated DNA breaks were located at DNA sites that were already observed in the absence of drug. However, the *m*-AMSA enhancement was not uniform, and the drug seemed to enhanced selectively certain sites that were not always the most pronounced in the absence of *m*-AMSA. For example, site 1170 was a strong cutting site in the presence of *m*-AMSA (lane 5), whereas it was a weak site in the absence of drug (lane 2). This result suggests, therefore, that sensitivity of topoisomerase II induced DNA cutting sites to the effect of *m*-AMSA was dependent upon DNA sequence. Similar analysis of two other gels of L1210 topoisomerase II mediated DNA breaks in the absence or presence of *m*-AMSA in pBR 322 DNA (data not shown) led to similar conclusions. In these three independent gels, the determination of the genomic position of the cutting sites shown on Fig. 7 was accurate $\pm$50 nucleotides. Figure 7 also shows that the DNA breaks induced by L1210 topoisomerase II in the absence or presence of *m*-AMSA did not occur at any preferential location with respect to the tetracycline or the β-lactamine resistance genes or the origin of replication.

The location of the topoisomerase II mediated DNA breaks produced in the absence or presence of *m*-AMSA was also determined in similarly [^{32}P]-end-labeled pBR 322 DNA by sequencing gel electrophoresis (Fig. 8). The numbers and arrows in Fig. 8 represent genomic positions as determined by DNA sequencing reactions. Control DNA had an unexplained cut near position 108–109 (lane 1). L1210 topoisomerase II produced major cuts in positions 102–103, 182–183 and 272–273, and some other minor cuts (lane 2). Adriamycin (lane 3) was more selective than *m*-AMSA (lane 4). All the teniposide-induced DNA cutting sites (lane 5) were similar to those induced by *m*-AMSA, although the relative intensity at each site was different. Similarly, the adriamycin-induced topoisomerase II-mediated DNA cuts (lane 3) also belonged to the *m*-AMSA cleavage pattern. This result is in agreement with the possibility that each type of drug could selectively enhance topoisomerase II mediated DNA cleavage by interfering preferentially with certain enzyme binding sites. Comparison of Figs. 6 and 8 shows, however, some quantitative differences between the *m*-AMSA-induced topoisomerase II mediated DNA cleavage patterns within the first 200 nucleotides of pBR 322. In Fig. 6 (lane 3), the three major cutting sites are around positions 87, 103 and 140, whereas the major cutting sites in Fig. 8 (lane 4) are around positions 100, 103 and 180. Nonetheless, in both cases the same sites could be seen, but their relative intensities varied. The most likely reason for this difference is that two different enzyme preparations were used.

Thus, other DNA sequencing experiments similar to those shown on Figs. 6 and 8 were performed. Taken together, these sequencing experiments showed that the relative intensity of L1210 topoisomerase II mediated DNA breaks produced in the absence or presence of *m*-AMSA or other 9-aminoacridines varied from one enzyme preparation to another but were reproducible. A summary of the location of the DNA cuts produced by L1210 topoisomerase II in the absence or presence of 9-aminoacridines is shown on Table 1. The exact

Table 1. Location of the DNA cleavage sites induced by L1210 DNA topoisomerase II alone or in the presence of 9-aminoacridines in pBR 322 DNA

	Topoisomerase II alone	Topoisomerase II + *m*-AMSA
Strong	103, 183, 273	87, 103, 143, 273
Weak	143, 174, 240	174, 183, 228, 240

pBR 322 DNA that had been [^{32}P]-end-labeled at the 5'-termini of the Hind III cutting site was reacted with purified L1210 DNA topoisomerase II in the absence or presence of *m*-AMSA. Reactions were performed and results analyzed as described in Figs. 6 and 8. The EcoR 1 restriction site was taken as position 0 of the pBR 322 genome [16].

location of the cuts is probably ±1 nucleotide because in most cases the migration distance of the topoisomerase II generated DNA fragments was in between two consecutive nucleotides of the Maxam and Gilbert sequencing lanes. This shift is probably due to the fact that the 3′-DNA terminal are different in the case of topoisomerase II- and piperidine-mediated DNA breaks. Topoisomerase II mediated DNA breaks result in a 3′-hydroxyl-end, whereas piperidine cleavage generates a 3′-phosphate-end [17, 19].

DISCUSSION

The present study shows that some but not all 9-aminoacridine intercalators inhibit mammalian DNA topoisomerase II mediated DNA relaxation. This inhibition is associated with increased formation of topoisomerase II–DNA complexes that can be detected as DNA strand breaks upon sodium dodecyl sulfate denaturation and proteinase K digestion. Similar effects have already been observed with many DNA intercalators [8–11], which suggested that DNA intercalation could be responsible for the trapping of topoisomerase II–DNA complexes. One of the most specific consequences of DNA intercalation is the reduction of DNA twist at the drug intercalation site. This DNA twist reduction produces, in turn, a propagated DNA unwinding. In the case of 9-aminoacridines, drug-induced DNA unwinding does not seem sufficient to inhibit L1210 DNA topoisomerase II. This is particularly clear by comparing the effects of *m*-AMSA and *o*-AMSA. *o*-AMSA has the same DNA unwinding angle (20.5°) as *m*-AMSA [14, 15]. However, at similar DNA unwinding concentrations, *o*-AMSA was markedly less potent than *m*-AMSA in inhibiting and trapping topoisomerase II. Striking also is the case of 9-aminoacridine which is a better DNA intercalator than either *m*- or *o*-AMSA [15], but was devoid of inhibitory effect upon L1210 topoisomerase II. A recent study comparing the inhibitory effects of several 9-aminoacridines including *m*-AMSA, *o*-AMSA and 9-aminoacridine upon calf thymus DNA topoisomerase II gave similar results [20]. Therefore, it seems unlikely that drug-induced DNA unwinding could produce mammalian topoisomerase II inhibition by itself.

Such a view is consistent with our previous findings that *m*-AMSA could induce similar formation of topoisomerase II–DNA complexes in untreated and in X-irradiated L1210 cell nuclei in which intercalator-induced DNA unwinding could not be propagated over a long distance [21]. Thus, the inhibitory effect of 9-aminoacridines upon mammalian DNA topoisomerase II seems to involve some special interaction at or nearby the topoisomerase II binding site to DNA.

L1210 topoisomerase II is likely to have specific recognition sites within DNA since the enzyme-mediated DNA breaks produced in the absence of drug do not appear randomly with respect to DNA sequence (Figs. 5–8) [8–10, 13, 18–20]. In particular, the L1210 topoisomerase II cleavage site at position 103 of pBR 322 is similar to that of the Drosophila enzyme [19]. The other cleavage sites do not share any obvious consensus DNA sequence with this site, which suggests that mammalian DNA topoisomerases II recognize some features of DNA structure, which are not strictly dependent upon DNA sequence at the DNA cutting site. Since 9-aminoacridines markedly distort the shape of the DNA helix at their intercalation site (removal of 20.5° from the average 36° helical twist of adjacent base pairs), it seems unlikely that enzyme receptor sites would recognize the intercalated sites. The observation that 9-aminoacridines enhanced previously existing enzyme sites rather than induced new sites (Fig. 7) is consistent with such an hypothesis. Also consistent with this hypothesis is the finding that compounds A and B, which were selected for their different DNA sequence selectivity of binding, induced similar topoisomerase II mediated DNA cleavage patterns. However, it is known from crystallographic studies [22] that intercalators can alter DNA structure nearby their site of intercalation. These smaller distortions could be abnormally handled by the enzyme receptor site and lead to the trapping of topoisomerase II–DNA complexes. Such an hypothesis implies that only some DNA intercalators would produce these distortions since 9-aminoacridine was ineffective and *o*-AMSA only weakly effective. Thus, crystallographic studies would be needed to elucidate the comparative DNA effects of *m*-AMSA, *o*-AMSA and 9-aminoacridine.

Another alternative is that some 9-aminoacridine derivatives could interact directly with mammalian DNA topoisomerase II. The side chains of 9-aminoacridine derivatives occupy the DNA grooves [14] and could interact with the enzyme. This hypothesis is supported by the fact that 9-aminoacridine which has no bulky side chain had no topoisomerase II inhibitory effect, while compound C, which has a bulky side chain, was the most potent topoisomerase II inhibitor.

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