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DNA unwinding and inhibition of mouse leukemia L1210 DNA topoisomerase I by intercalators

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ABSTRACT

The DNA unwinding effects of some 9-aminoacridine derivatives were compared under reaction conditions that could be used to study drug-induced topoisomerase II inhibition. An assay was designed to determine drug-induced DNA unwinding by using L1210 topoisomerase I. 9-aminoacridines could be ranked by decreasing unwinding potency: compound C > 9-aminoacridine > o-AMSA > compound A > compound B > m-AMSA. Ethidium bromide was more potent than any of the 9-aminoacridines. This assay is a fast and simple method to compare DNA unwinding effects of intercalators. It led to the definition of a drug intrinsic unwinding constant (k). An additional finding was that all 9-aminoacridines and ethidium bromide inhibited L1210 topoisomerase I. Enzyme inhibiton was detectable at low enzyme concentrations (< 1 unit) and when the kinetics of topoisomerase I-mediated DNA relaxation was studied. Topoisomerase I inhibition was not associated with DNA swivelling or cleavage.

INTRODUCTION

Many DNA intercalators have been synthesized in the acridine series. Acridine orange is used for physicochemical studies of DNA; quinacrine is a potent antimalarial and antihelmintic drug and amsacrine (m-AMSA) is used as an anticancer agent in human acute leukemia (1-7). The antitumor effect of m-AMSA is associated with the production protein-linked DNA strand breaks in mammalian cells in culture (8). It is now established that these peculiar DNA breaks result from topoisomerase II inhibition (9-11). m-AMSA is thought to trap topoisomerase II by freezing abortive intermediates of the DNA strand passing reaction, in which the enzyme is bound to the 5'-termini of the breaks it catalyzes (9-12). Although this effect can be observed easily in purified systems, it is not known whether the inhibitory effect of m-AMSA upon topoisomerase II-DNA complexes is due solely to DNA intercalation.

Before undertaking our studies with topoisomerase II (13), we sought to

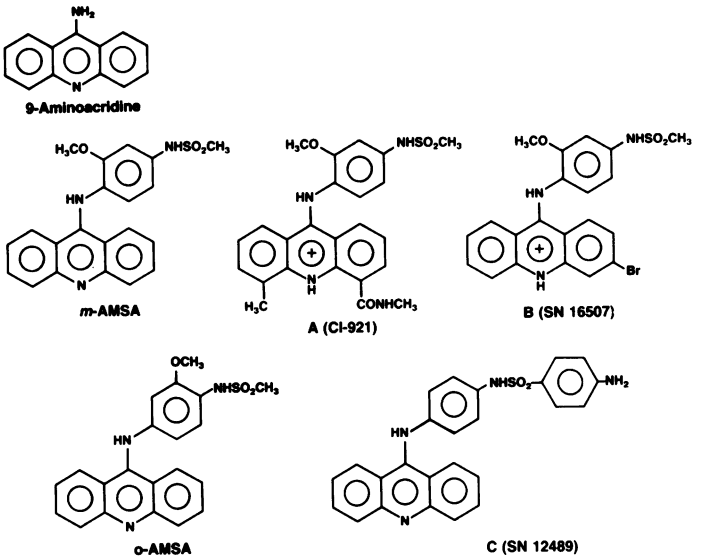

Figure 1: Structure of the 9-aminoacridine derivatives used in the present study. m-AMSA is for 4'-(9-acridinylamino) methanesulfon-m-anisidide (Amsacrine), and o-AMSA for 4'-(9-acridinylamino)methanesulfon-o-anisidide. The 3 other 9-aminoacridine derivatives have been abbreviated A, B and C for convenience.

compare the DNA intercalative properties of various acridine derivatives under reaction conditions that could be used to study topoisomerase II. The 9-aminoacridines selected (Figure 1) were chosen with the help of Dr. Bruce Baguley (Cancer Research Laboratory, University of Auckland, New Zealand), on the basis of their differential DNA affinities and sequence selectivity of binding. For instance, compounds A and B have similar DNA binding affinities but differential sequence selectivity, with compound A binding preferentially poly(dG·dC)-poly(dG·dC) , whereas compound B binds preferentially poly(dA·dT)-poly(dA·dT) (Dr. B. Baguley, personal communication).

The DNA intercalative potencies of the 9-aminoacridines were determined by using a DNA unwinding assay using L1210 topoisomerase I. In the course of these experiments, we found that 9-aminoacridine intercalators and ethidium bromide could inhibit L1210 topoisomerase I-mediated DNA relaxation.

MATERIALS AND METHODS

Materials: DNA, Drugs and Enzymes

SV40 DNA (> 90% form I) and agarose were purchased from Bethesda Research Laboratories (Gaithersburg, MD).

4'-(9-acridinylamino)methanesulfon-m-anisidide (m-AMSA, Amsacrine) (NSC 249992), 4'-(9-acridinylamino)methanesulfon-o-anisidide (o-AMSA) (NSC 123127) and ethidium bromide were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI. 9-aminoacridine was purchased from Aldrich Chemical Company (Milwaukee, WI). Compounds A (CI 921, NSC 343499), B (SN 16507) and C (SN 12489, NSC 140701) were given by Dr. Bruce Baguley, Cancer Research Laboratory, University of Auckland School of Medicine, New Zealand. Drugs were dissolved at 10 mM in dimethylsulfoxide and stored at -20°C.

Topoisomerase I was purified from mouse leukemia (L1210) cell nuclei, as described previously (9). Enzyme purity was checked by sodium dodecyl-sulfate polyacrylamide gels (one 100 kD band). One unit topoisomerase I was defined as the lowest enzyme activity required to relax 0.4 µg native SV40 DNA completely, in the conditions described below.

Topoisomerase Reactions

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 Na_2EDTA, 15 µg/ml bovine serum albumin), in the absence of ATP. 0.4 µg SV40 DNA was reacted with the indicated amounts of L1210 topoisomerase I, in the absence or presence of drugs, for 30 min at 37°C. Reactions volumes were typically 30 µl. Reactions were stopped by adding sodium dodecylsulfate ($NaDoSO_4$) and proteinase K (Merck, Darmstadt, West Germany) (1% and 0.5 mg/ml, final concentrations respectively). After an additional 30 min at 37°C, an appropriate volume of (10 x) loading buffer (0.3% bromophenol blue, 16% Ficoll, 0.01 M Na_2HPO_4) was added. None of the drugs modified DNA migration.

Agarose Gel Electrophoresis

One percent agarose gels were made in Tris-Acetate-EDTA (TAE) buffer (0.04 M Tris·Acetate, pH 7.6, 0.01 M Na_2EDTA). Gels were run at 1 V/cm overnight and then stained with 1 µM ethidium bromide for 45 min. After an additional 30 min destaining in 1 mM Mg_2SO_4, the gels were photographed under U.V. light with Polaroid 55 or 57 films. To obtain the curves shown on Figure 4, the distance between the migration positions of negatively supercoiled and nicked relaxed SV40 DNA was measured from the densitometer

scanning (Beckman DU 8B) of the negative of a given gel and set as control migration. Migration distances of each individual DNA topoisomer resulting from topoisomerase I action in the absence or presence of drug, was also measured relative to the position of nicked relaxed DNA. The ratio between this distance and that of control migration was computed and plotted versus number of superhelical turns per SV40 molecule, as determined by band counting (14-16), knowing that neighbor topoisomers differ by one superlical turn.

Determination of Intercalator Intrinsic Unwinding Constants (k)

The total drug-induced reduction of DNA twist for a N base-pair duplex circular DNA molecule is:

$$2 N r \Phi$$

where r is the number of intercalated drug molecules per nucleotide and Φ the DNA unwinding angle for each intercalated molecule. Topoisomerase I action adjusts DNA linking number (Lk : number of crossover points of one DNA single-strand over the other) to the modified DNA twist (14-18), in such a way that:

$$2 N r \Phi = 360 \times \Delta Lk$$

Lk change (ΔLk) was determined by band counting assuming $\Delta Lk = 1$ between adjacent DNA topoisomers, and 360° is the unwinding angle between adjacent DNA topoisomers. The above equation can be rewritten:

$$r \Phi = 360 \Delta Lk / 2N \quad (1)$$

$r\Phi$ corresponds to the intercalator-induced unwinding per nucleotide. Knowing Φ (Table II), the bound drug concentration (b) can be calculated:

$$b = r \times [Nt]$$

where [Nt] is the nucleotide concentration. [Nt] was 43 µM in all experiments. The free drug concentration can be expressed:

$$C = Ct - b = Ct - r [Nt] \quad (2)$$

The relative DNA unwinding potencies of drugs were compared by defining a drug intrinsic unwinding constant (k), which corresponds to the intercalator-induced DNA unwinding per nucleotide and per free drug concentration:

$$k = r \Phi / C \quad (3)$$

k can be defined also from the Scatchard equation:

$$r / C = Kap (n - r) \quad (4)$$

where Kap is the drug apparent intrinsic binding constant and n the number of drug binding sites per nucleotide (n = 0.25 in the neighbor exclusion model for intercalation). Equations (3) and (4) can be combined and then:

$$k = r \Phi / C = Kap \Phi (n - r) \quad (5)$$

Thus, Kap, and Φ can be calculated from the same equation.

Quantification of topoisomerase I inhibition by DNA intercalators

Topoisomerase I-mediated DNA relaxation was quantified by densitomer scanning of the negatives of agarose gels. The fraction of DNA in the supercoiled band (S) was calculated in untreated native DNA (S_O), in enzyme-treated DNA (S_E), and in DNA treated with enzyme and various drug concentrations (S_{ED}). The percent inhibition of relaxation at a given drug concentration was calculated by using the formula:

$$[(S_{ED} - S_E) / (S_O - S_E)] \times 100\%$$

Drug concentrations yielding 30% inhibition [Ci(30%)] were then derived.

RESULTS

Determination of Intercalator-induced DNA Unwinding by a Topoisomerase I Unwinding Assay

DNA intercalation unwinds closed circular duplex DNA molecules and modifies their torsional tension. DNA relaxation by mammalian DNA topoisomerase I relieves DNA torsional tension within negatively or positively

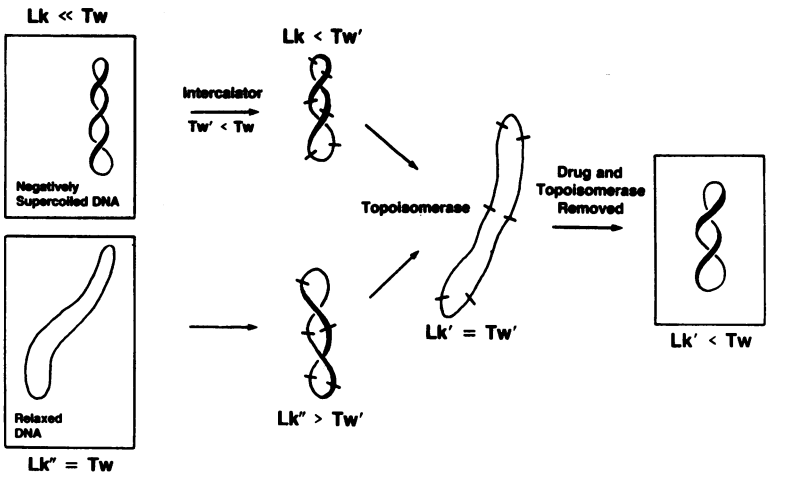

Figure 2: Principle of the DNA unwinding assay using DNA topoisomerase I. The upper pathway describes the assay with supercoiled native SV40 DNA. Intercalation reduces the DNA twist to Tw' (Tw' < Tw). Upon topoisomerase I addition the linking number (Lk) is adjusted to the twist of the intercalated DNA (Lk' = Tw'). Finally, drug removal, while topoisomerase I is inactivated (by 1% $NaDoSO_4$ and proteinase K) leaves the DNA in a new supercoiling state (Lk' < Tw). The lower pathway describes the assay with relaxed SV40 DNA.

supercoiled DNA. Since DNA intercalators were not known to inhibit topoisomerase I-mediated DNA relaxation (19), we designed a DNA unwinding assay that could be performed in the same buffer and DNA conditions as those that would be used to study the effects of 9-aminoacridines upon mammalian topoisomerase II. The assay is described on Figure 2. The starting DNA substrate is either negatively supercoiled or relaxed SV40 (boxed areas on Figure 2). Upon DNA intercalation, the DNA twist (Tw) is reduced to a new value (Tw'). In the absence of topoisomerase inhibition, the DNA linking number is ajusted to the twist of the intercalated DNA (Lk' = Tw') (17-21). Finally, removal of the drug while topoisomerase I is inactivated (by $NaDoSO_4$ addition, dilution and proteinase K digestion) leaves the DNA in a supercoiled state defined by a linking number (Lk') less than the twist (Tw). The assay was performed both with supercoiled (upper left panels, Figure 2) and relaxed closed circular duplex DNA (lower left panels, Figure

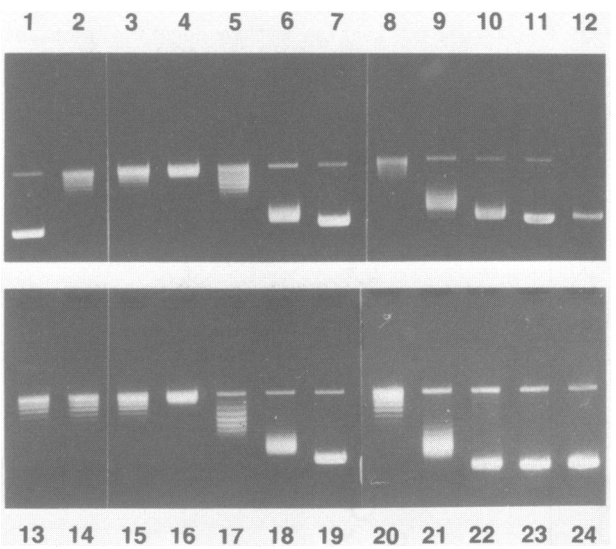

Figure 3: Two examples of the DNA unwinding assay using either m-AMSA or compound C and L1210 topoisomerase I. The upper and lower panels show the assay performed with native supercoiled (lane 1) or topoisomerase I-relaxed (lane 13) SV40 DNA, respectively. 0.4 µg DNA was reacted with excess topoisomerase I in the absence (lanes 2 and 14) or presence of m-AMSA (lanes 3-7 and 15-19) or compound C (lanes 8-12 and 20-24) for 30 min at 37°C. Drug concentrations were 1 µM (lanes 3, 8, 15, 20), 10 µM (lanes 4, 9, 16, 21), 20 µM (lanes 5, 10, 17, 22), 50 µM (lanes 6, 11, 18, 23) and 100 µM (lanes 7, 12, 19, 24). Reactions were stopped by adding $NaDoSO_4$ and proteinase K and run in 1% agarose TAE buffer gels.

2) for the following reasons. First, if only the assay with relaxed DNA were used and no final modification of the DNA occurred, it would be impossible to know whether the drug inhibited topoisomerase I or did not intercalate. The parallel use of supercoiled DNA eliminates lack of intercalation as an explanation for an absence of supercoiled DNA relaxation. A second reason for performing the unwinding assay both with supercoiled and relaxed DNA is that the observation of similar DNA end-products with both DNA substrates ensures that no drug remained bound during electrophoresis and that the measured DNA linking number change ($\Delta Lk = |Lk' - Lk| = |Lk' - Lk''|$) is an accurate measurement of drug unwinding ($\Delta Tw = |Tw - Tw'|$) (Figure 2). Finally, DNA intercalation is characterized by a DNA unwinding that occurs independently of the supercoiling state of the DNA substrate (22). Thus, this characteristic effect can be easily monitored by using both negatively supercoiled and closed circular DNA.

Figure 3 shows the example of m-AMSA and compound C intercalation in the DNA unwinding assay using L1210 topoisomerase I. Supercoiled native SV40 DNA was used as a starting substrate in the upper panel (lane 1). m-AMSA produced a concentration-dependent alteration of topoisomerase I-

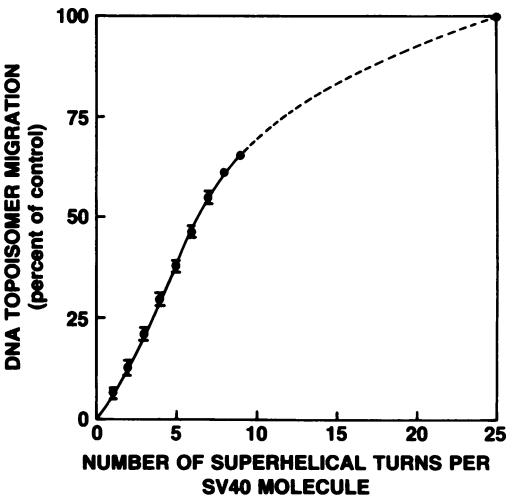

Figure 4: Relationship between DNA migraton and number of superhelical turns per SV40 molecule in 1% agarose gels in TAE buffer. The distance between nicked relaxed and native SV40 DNA was measured from the densitometer scanning of agarose gels and set as 100% migration (control) with 25 superturns. The distance between individual DNA topoisomers and nicked relaxed DNA was also measured and normalized to control.

Table I: Comparison of the DNA Unwinding Characteristics of Intercalators in the Topoisomerase I Unwinding Assay

Drug	Ct (μM)	ΔLK	rΦ	C(μM)	k	k (mean ± SD)
	1	2	0.0687	0.826	0.083	
	3	5.5	0.1888	2.522	0.075	0.070
9-AA *	5	7.5	0.2575	4.349	0.059	± 0.011
	10	16	0.5493	8.570	0.064	
	10	3	0.1030	9.784	0.011	0.010
m-AMSA	20	6	0.2060	19.568	0.011	± 0.002
	30	7	0.2403	29.496	0.008	
	10	7	0.2403	9.50	0.025	0.027
o-AMSA	20	16	0.5493	18.848	0.029	
	5	3.5	0.1202	4.748	0.025	
A	10	6.5	0.2232	9.532	0.023	0.026
	20	16	0.5493	18.848	0.029	± 0.003
	10	5	0.1717	9.640	0.018	
B	20	10	0.3433	19.280	0.018	0.018
	1	2	0.0687	0.856	0.080	0.073
C	5	9	0.3090	4.352	0.071	± 0.007
	10	17	0.5836	8.776	0.067	
	0.3	2	0.0687	0.186	0.369	0.331
Ethidium	1	6	0.2060	0.659	0.313	± 0.033
Bromide	3	18	0.6180	1.978	0.312	

Drug unwinding studies were performed in buffer B (0.01 M Tris.HCl, pH 7.5, 0.05 M KCl, 5 mM $MgCl_2$, 0.01 mM Na_2EDTA, 15 μg/ml bovine serum albumin).
* 9-aminoacridine is abbreviated 9-AA
Ct is the total drug concentration.
ΔLk is the DNA linking number change (absolute value) measured in the topoisomerase I unwinding assay.
r is the number of drug molecules bound per nucleotide.
Φ is the unwinding angle produced by intercalated drug molecule.
rΦ is the drug-induced DNA unwinding per nucleotide [equation (1)].
C is the free drug concentration [equation (2)].
k is the intrinsic unwinding constant for drug [equation (3)].

mediated DNA relaxation. This can be seen by comparing DNA relaxation in the absence (lane 2) or presence of drug (lanes 3-7). More than 50 μM m-AMSA (lane 6) was required to relax DNA and prevent topoisomerase I action. Compound C (lanes 8-12) produced more DNA unwinding than m-AMSA at equal concentrations. The observed alterations of topoisomerase I-mediated DNA relaxation measured accurately DNA intercalation because similar DNA end products were observed when the assay was performed with relaxed closed

Table II: Relationship between Intercalator-induced DNA Unwinding and Topoisomerase I Inhibition

Drug	Ci(30%) (μM)	k	k x Ci(30%)	r
9-AA *	5	0.070	0.35	0.021
m-AMSA	6.5	0.010	0.07	0.003
o-AMSA	12.5	0.027	0.33	0.016
A	7.5	0.026	0.20	0.009
B	20	0.018	0.36	0.018
C	5	0.073	0.37	0.018
Ethidium	1	0.331	0.33	0.013

* 9-aminoacridine is abbreviated 9-AA.
Ci(30%) is the drug concentration required to produce 30% topoisomerase I inhibition.
k is the intrinsic unwinding constant for drug [equation (3) and Table I].
k Ci(30%) = r Φ (see equation 3, assuming equality between total and DNA bound drug) is equal to drug-induced DNA unwinding (equation 1).
r is the number of drug molecules bound per nucleotide and was calculated assuming: r Φ = k x Ci(30%), and Φ = 17° for 9-AA, 20.5° for the other 9-aminoacridines and 26° for ethidium bromide.

circular SV40 DNA as a substrate (lower panel, Figure 3).

Using the topoisomerase I unwinding assay, we then compared the DNA unwinding potencies of the six 9-aminoacridines shown on Figure 1 to that of ethidium bromide. This was done by determining experimentally topoisomerase I-mediated DNA linking number change (ΔLk) (see Materials and Methods). This enabled us to establish first a relationship between the number of superhelical turns per SV40 DNA molecule and DNA topoisomer migration distance in agarose gel electrophoresis (Figure 4). DNA topoisomer bands could be identified up to the 9th topoisomer (solid line in Figure 4) and DNA topoisomer migration distance was proportional to the number of superhelical turns up to 7 superhelical turns. Native SV40 DNA has 25 negative superturns (15, 16), but between 9 and 25 superturns the individual bands were too close to each other to be separated (dashed line in Figure 4). Because of the differences (essentially $MgCl_2$) between the

reaction and electrophoresis buffers, topoisomerase I-relaxed DNA had usually between 2 and 3 positive superhelical turns in electrophoresis buffer (see Figures 3, 7, 8 and 10). Since intercalation induces topoisomerase I-mediated Lk changes towards negative values, ΔLk could be easily determined by band counting up to ΔLk values of -12 [$|\Delta Lk| = |(-9)-(+3)|$]. For simplification, the absolute value of ΔLk was used in the text, figures and Table I. The curve shown on Figure 4 was used for determining ΔLk values greater than 12. By this method, ΔLk values were determined over a range of drug concentrations for each compound (Table I). The conversion from total to free drug concentration was performed as described under Materials and Methods and Table I. $r\phi$, which corresponds to the intercalator-induced DNA unwinding per nucleotide, was calculated for each drug at various concentrations (Equation (1) and Table I). Assuming that ϕ was 17° for 9-aminoacridine, 20.5° for m-AMSA, o-AMSA, and compounds A, B and C (22, 23), and 26° for ethidium bromide (24), r (number of intercalated drug molecules per nucleotide) was determined (Table II). Apparent drug binding affinity constants (Kap) could be derived from these values by using the McGhee and von Hippel treatment (Equation (5)) (25). For instance, Kap = 5.74×10^4 M^{-1} for ethidium bromide in the topoisomerase I unwinding assay.

Similar results were obtained by independent fluorimetric measurements with calf thymus DNA in topoisomerase I buffer at 37°C and Scatchard analysis (Kap = 6.5×10^4 M^{-1} for ethidium bromide). Fluorimetric measurements with calf thymus DNA, showed also that the apparent binding affinity constant for ethidium bromide was 2- to 3-fold greater at 25°C than at 37°C (18.5 versus 6.5×10^4 M^{-1}). Therefore, equilibrium drug binding is lower at higher temperatures.

Figure 5 shows the relationship between ΔLk (drug-induced DNA unwinding) and free drug concentrations. 9-aminoacridine compounds varied widely in their DNA unwinding potencies and where all less potent than ethidium bromide. For all compounds, ΔLk was proportional to the free drug concentration. Therefore, even at the highest drug concentrations, the DNA was not saturated, and drug-induced DNA unwinding was independent of DNA superhelicity. Drug intrinsic unwinding constants (k) are proportional to the slopes of the lines shown on Figure 5 (see equations (1) and (3); Materials and Methods). k was independent of the drug concentration for a given drug but was specific of each intercalator (Table I). Ethidium bromide appeared 4-5-fold more potent than 9-aminoacridine or compound C, which were 3-fold more potent than compound A and o-AMSA, which were 1.5-fold more potent

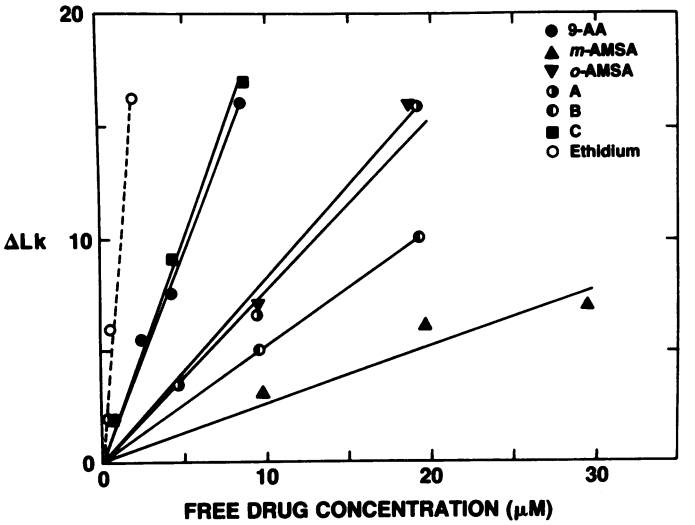

Figure 5. DNA unwinding produced by 9-aminoacridines and ethidium bromide as determined by the DNA unwinding assay with topoisomerase I. Topoisomerase I-relaxed SV40 DNA was reacted with drugs in the presence of excess enzyme. Reactions were stopped and agarose gels were run, as described in the legend of Figure 3. Drug-induced topoisomerase I-mediated DNA topoisomer shift (ΔLk) was determined by band counting (see Materials and Methods). Free drug concentration was calculated as described under Materials and Methods.

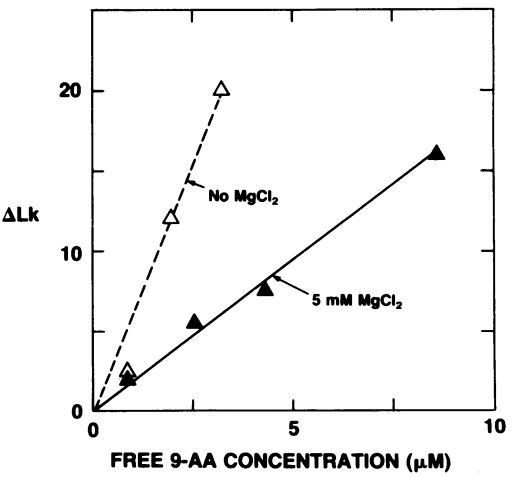

Figure 6: Effect of $MgCl_2$ upon 9-aminoacridine-induced DNA unwinding as measured by the topoisomerase I unwinding assay. Native SV40 DNA and topoisomerase I were reacted with 9-aminoacridine in the presence of excess enzyme. Reactions were stopped and gels run as described in the legend of Figure 3. Drug-induced DNA unwinding and free drug concentration were determined as described in the legend of Figure 5.

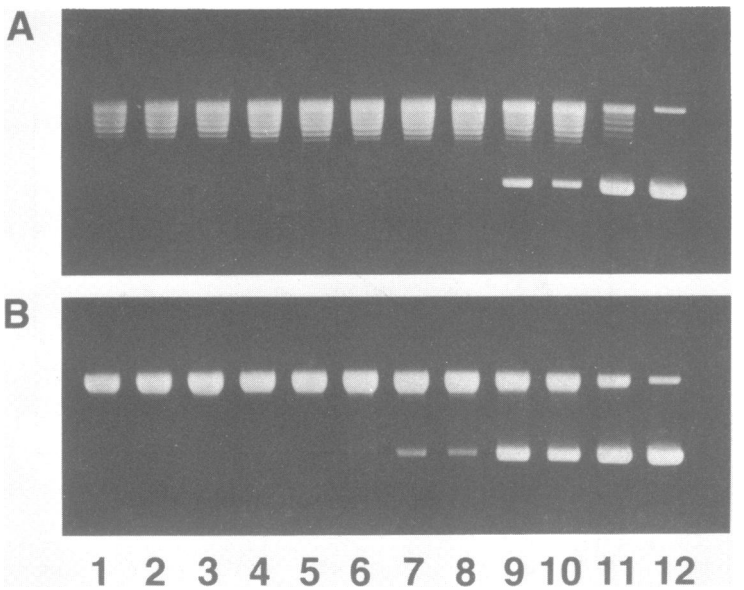

Figure 7: Effect of m-AMSA upon topoisomerase I-mediated DNA relaxation. Decreasing concentrations of L1210 topoisomerase I were reacted with native SV40 DNA for 30 min at 37°C in buffer B in the absence (panel A) or presence of 20 μM m-AMSA (panel B). Topoisomerase I per reaction was 0.15, 0.10, 0.09, 0.08, 0.07, 0.06, 0.05, 0.04, 0.03, 0.02, 0.01, and 0 μl in lanes 1-12, respectively. Reactions were stopped and gel run as described in the legend of Figure 3. One unit topoisomerase I activity is defined as the lowest enzyme amount required to relax DNA completely (0.04 μl = 1 unit, lane 7, panel A).

than compound B, which was 1.8-fold more potent than m-AMSA. It is particularly striking that o-AMSA was 2.7-fold more potent than its stereoisomer m-AMSA in unwinding DNA. Since both drugs have a similar unwinding angle (Φ = 20.5°), this difference has to be due to differences in DNA binding constants (22, 23).

The topoisomerase I unwinding assay can be used to study various buffer conditions. Figure 6 shows that the 9-aminoacridine-induced DNA unwinding was more pronounced in the absence of $MgCl_2$. The intrinsic unwinding constant was approximately 3-fold greater than in the presence of 5 mM $MgCl_2$ (k = 0.21 versus 0.07). k did not change upon lowering the KCl concentration from 50 to 25 mM; data not shown). However, topoisomerase I catalytic activity was reduced at these lower ionic concentrations and was abolished when KCl was omitted from buffer B.

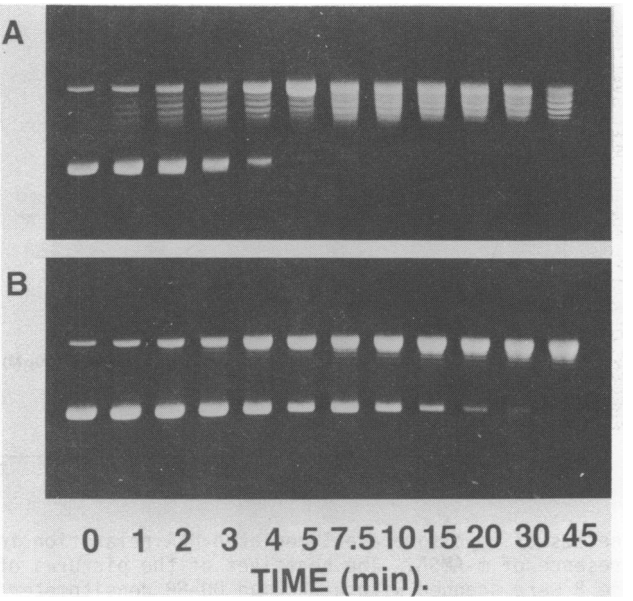

Figure 8: Kinetics of topoisomerase I-mediated DNA relaxation in the absence (panel A) and presence of 20 μM m-AMSA (panel B). One unit enzyme was used in both reactions. At the indicated times, aliquots of the reactions were stopped as described in the legend of Figure 3. Agarose gels were also run as described in the legend of Figure 3.

Inhibition of L1210 Topoisomerase I by DNA Intercalators

Additional experiments were performed to determine whether intercalator-induced DNA unwinding was independent of the amount enzyme used in the assay (Figure 7). Decreasing enzyme concentrations were employed together with a constant DNA concentration in the absence (panel A) or presence of 20 μM m-AMSA (panel B). m-AMSA-induced DNA unwinding was similar over a wide range of enzyme concentrations (lanes 1-5, panel B). Identical unwinding was observed with 4 μl (100 units) enzyme (not shown). Thus, drug-induced DNA unwinding is not influenced by the amount of enzyme used. Panel A (Figure 7) shows also a sharp transition zone for the enzyme alone when the amount of enzyme was under 1 unit (lanes 8-9). No intermediate DNA topoisomer distribution can be seen. The DNA was either fully supercoiled or fully relaxed. This observation confirms the processive activity of mammalian topoisomerase I (26, 27).

20 μM m-AMSA (panel B), actually inhibited topoisomerase I at concentrations below 1.5 units (lane 7). This inhibition was not accompanied by the

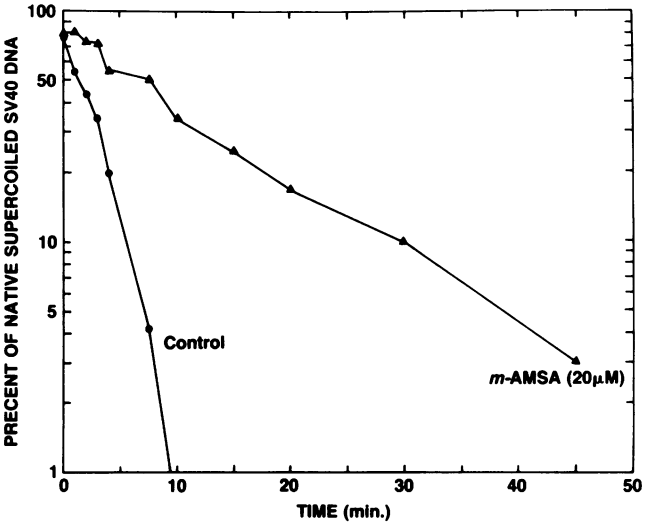

Figure 9: Kinetics of topoisomerase I-mediated DNA relaxation in the absence and presence of m-AMSA. The negatives of the pictures of the gels shown on Figure 8 were scanned with a Beckman DU-8B densitometer and the percentage of native supercoiled SV40 DNA calculated.

appearance of topoisomers of intermediate superhelicity. Moreover, no DNA breaks could be detected in agarose gels (not shown) run in the presence of chloroquine in order to separate nicked from relaxed circular DNA (28, 29).

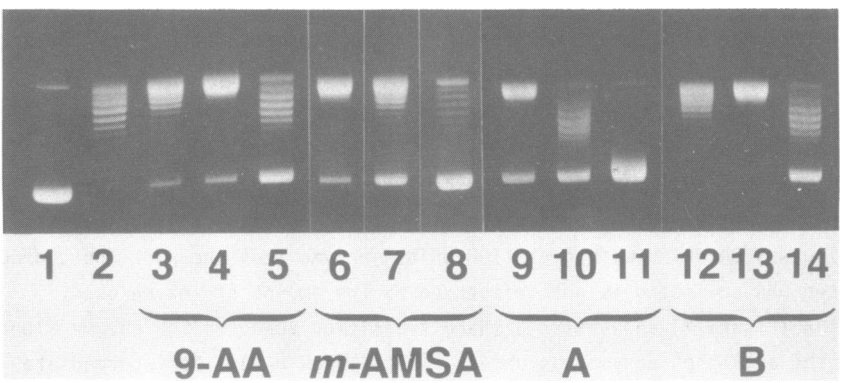

Figure 10: Drug and concentration-dependence of the inhibitory effect of some 9-aminoacridines upon L1210 topoisomerase I. Native SV40 DNA (lane 1) was reacted with 1 unit enzyme for 30 min at 37°C in the absence of drug (lane 2) or in the presence of 9-aminoacridine (9-AA) (1, 2, and 5 μM in lanes 3, 4, and 5), m-AMSA, compound A or compound B (5 μM in lanes 6, 9, 12; 10 μM in lanes 7, 10, 13; and 20 μM in lanes 8, 11, 14). Reactions were stopped and gels run as described in the legend of Figure 3.

Topoisomerase I inhibition by m-AMSA was analyzed further by looking at enzyme kinetics in the absence or presence of 20 μM m-AMSA (Figures 8 and 9). In the absence of m-AMSA, 1 unit topoisomerase I relaxed SV40 DNA with nearly first order kinetics and a half-time of 1.5 min (Figure 9). Again, at the earliest time points the DNA was either fully relaxed or fully supercoiled (panel A, Figure 8) confirming the processive nature of the reaction. In the presence of m-AMSA, the DNA relaxation kinetics was much slower, although still first order ($t\ ^{1}/_{2}$ = 10 min, Figure 9) and without DNA topoisomers of intermediate superhelicity (panel B, Figure 8). Increasing the incubation time to 45 min (Figure 8, lower right lane) allowed the reaction to go to completion in the presence of m-AMSA. Therefore the inhibitory effect of m-AMSA seems to result from a reversible inhibition of topoisomerase I either before or after one full catalytic cycle or from a slowing of the enzyme reaction in the presence of bound drug.

The other 9-aminoacridines and ethidium bromide also inhibited topoisomerase I-mediated DNA relaxation. The inhibition was detectable at low enzyme concentrations and was drug concentration-dependent (Figure 10). The inhibitory effects of DNA intercalators upon 1 unit of topoisomerase I were then compared (Table II). 30% inhibition was chosen for comparison because of the weak potency of some derivatives at concentrations that relaxed significantly the DNA and produced topoisomer shifts that made difficult the identification of a true topoisomerase I inhibition. The drug concentrations producing 30% topoisomerase I inhibition [Ci(30%)] varied between 1 μM (ethidium bromide) and 20 μM (compound B) (Table II). Intercalators could be ranked by decreasing topoisomerase I inhibitory effect: ethidium bromide > 9-aminoacridine = compound C > m-AMSA > compound A > o-AMSA > compound B. The relationship between topoisomerase I inhibition and intercalator-induced DNA unwinding was analyzed by using the drug intrinsic unwinding constants [(k), equation 3, Table I]. Drug-induced DNA unwinding being proportional to $r\Phi$ (equation 1), and $r\Phi$ equal to kC (equation 3), kC was determined for each drug at their Ci(30%) (Table II). This calculation gives only an approximative value for r since it was performed by assuming equivalence between total (C_t) and free drug concentration (C). However, Table I shows that such an assumption can be made for all 9-aminoacridines. Only in the case of ethidium, using C instead of C_t would give a smaller unwinding angle (0.22 instead of 0.33). Nearly similar values were obtained for 9-aminoacridine, o-AMSA, compounds B and C. Therefore,

topoisomerase I inhibition seems to related to DNA unwinding for these drugs. However, in the case of m-AMSA and compound A, 30% topoisomerase I inhibition was produced by drug concentrations that produced less DNA unwinding.

DISCUSSION

The most generally accepted criteria for drug intercalation are that: 1) the DNA helix is extended and 2) locally unwound by drug binding, and that 3) the plane of the aromatic chromophore of the bound drug is parallel to that of the base pairs (2, 22, 30). Drug-induced DNA unwinding is the most selective criterion of drug intercalation although the fulfillment of all three criteria should be met in order to classify a compound as an intercalator. Intercalation leads to a typical biphasic response in assays measuring drug-induced DNA unwinding of native DNA (22, 31-33). Negative superhelical turns are only partially removed at low levels of drug intercalation. Increasing the bound intercalator increases the unwinding up to an "equivalence-point", where all superturns are removed. Relaxed DNA migrates more slowly than supercoiled DNA in sedimentation and electrophoresis techniques. At drug concentrations exceeding the equivalence-point, the unwinding effect of intercalation introduces positive superturns and the DNA migrates faster. The DNA unwinding assay using mammalian topoisomerase I is based upon the above concept. However, instead of putting the drug into the running buffers, it is added to the reaction mixtures prior to electrophoresis (33-35). Each electrophoresis lane is used to assay a single drug concentration. Buffer, temperature, DNA or topoisomerase I conditions can be studied because mammalian topoisomerase I processes DNA under variable conditions (36-38). This is not the case of prokaryotic topoisomerase I, which cannot relax positively supercoiled DNA (36-38). The determination of drug intrinsic unwinding constants ($k = r \Phi / C$) can be done easily from the agarose gels results (which give $r \phi$), but requires the concurrent determination of C (free drug concentration). C can be calculated only if Φ is known. However, at low drug binding densities, C can be equated to C_t (total drug concentration). Table I shows that it was the case for 9-aminoacridine compounds.

Small changes in the substituents on the anilino moiety resulted in marked changes in the DNA unwinding characteristics of 9-aminoacridines. This was particularly clear for the two isomers, m-AMSA and o-AMSA since the intrinsic unwinding constant of o-AMSA was 2- to 3-fold greater than that of

m-AMSA. Waring et al. (22) had obtained similar results using viscosity measurements with PM2 DNA. This difference is not due to different DNA unwinding angles, but to differences in DNA binding affinity constants (22, 23). Similarly, we found that 9-aminoacridine was more potent in unwinding SV40 DNA than m-AMSA. This difference has already been observed and can be attributed to the higher DNA binding constant of 9-aminoacridine, because its DNA unwinding angle is even narrower (17°) than that of m-AMSA (20.5°) (23). Our finding that the intrinsic unwinding constants of compounds A and B were greater than that of m-AMSA is also in agreement with their greater DNA binding constants (B. Baguley, personal communication). Moreover, the apparent DNA binding constants for ethidium bromide were nearly identical in the topoisomerase I unwinding assay and in fluorimetric measurements and Scatchard analysis (5.7 and 6.5 x 10^4 M^{-1}, respectively) (see Results). Therefore, the DNA unwinding assay using mammalian topoisomerase I could be used to detect and quantify DNA intercalation.

All 9-aminoacridines and ethidium bromide appeared to inhibit topoisomerase I-mediated DNA relaxation. Ellipticines have also been reported to inhibit trypanosoma cruzi topoisomerase I (39). Enzyme inhibition by 9-aminoacridines and ethidium bromide was best detectable at low enzyme concentrations and in enzyme kinetics experiments. Intercalator-induced topoisomerase I inhibition differs from topoisomerase II inhibition because it is not associated with the production of enzyme-linked DNA strand breaks. Topoisomerase I inhibition appeared reversible since it was overcome by high enzyme concentrations and increasing incubation time. The fact that, under inhibitory conditions, the DNA topoisomers were either fully relaxed or fully supercoiled, suggests that intercalators inhibited topoisomerase I by preventing the enzyme from entering its processive catalytic cycle.

The results of Table II suggest that drug-induced DNA unwinding could be responsible for the inhibition. We estimated that approximately one drug molecule was intercalated per 2 to 5 helical turns when topoisomerase I inhibition was 30% (r values in Table II). Less DNA unwinding was required to inhibit topoisomerase I in the case of m-AMSA (one drug molecule per 16 helical turns). Therefore, m-AMSA is not only a potent topoisomerase II inhibitor, but also a potent topoisomerase I inhibitor. m-AMSA could produce some specific alterations of DNA structure or interact directly with topoisomerase II and topoisomerase I enzymes. The observation that compound A was twice more potent than compound B in inhibiting topoisomerase I raises the possibility of a DNA sequence selectivity of the inhibitory

effect since compound A binds preferentially G·C rich DNA, while compound B binds A·T rich DNA. The relevance of topoisomerase I inhibition by intercalators and particularly m-AMSA, with respect to their antitumor effects is still unclear, however this effect should be considered when using these drugs in cellular systems.

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