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DNA Binding by Epipodophyllotoxins and *N*-Acyl Anthracyclines: Implications for Mechanism of Topoisomerase II Inhibition

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SUMMARY

Previous evidence suggests that epipodophyllotoxins, such as etoposide and teniposide, and the *N*-acyl anthracycline AD41 inhibit topoisomerase II resealing even though they apparently do not bind to DNA. Using experimental conditions designed to detect limited numbers of DNA binding sites, we now report that both epipodophyllotoxins and the *N*-acyl anthracyclines AD41 and AD32 bind to DNA. Binding was greater to kinetoplast DNA than to pUC18 plasmid DNA. There was also greater etoposide binding to single-stranded DNA than to double-stranded linear or supercoiled DNA. Based on binding competition experiments, etoposide and teniposide appear to have equal affinity for DNA, in spite of the fact that the latter is more potent as a topoisomerase inhibitor. This suggests that the difference in the drugs relates to protein interaction. There are 3- to 7-fold more binding sites for AD41 than for AD32, depending on the DNA substrate employed, and both drugs, unlike adriamycin, exhibit saturation of binding sites over a concentration range of 0–50 μM when kinetoplast DNA is the substrate. Evidence for DNA intercalation by AD41 is provided by the observation that the drug introduces positive supercoils into covalently closed plasmid DNA. Based on these data, a hypothesis is proposed that would provide a general mechanism whereby intercalating agents and epipodophyllotoxins alter topoisomerase function and presumably exert their antitumor effects.

The nuclear enzyme DNA topoisomerase II is the principal intracellular target for a number of important anticancer agents (1, 2). These include classical intercalating agents (e.g., anthracyclines, aminoacridines, anthracenediones, and ellipticines) as well as epipodophyllotoxins (e.g., etoposide and teniposide) and *N*-acyl anthracyclines. Each of these agents shares the property of trapping a DNA-topoisomerase II complex which, upon exposure to denaturing agents, results in the formation of protein-associated DNA breaks (3–5). Both single- and double-strand breaks have been observed. It is likely that this drug effect results from interference with the normal DNA breaking-resealing action of topoisomerase II. Interestingly, each of the different groups of drugs produces cleavage that is site specific and unique for that drug group (6–8). In virtually all respects, the action of these drugs is similar to that of the bacterial topoisomerase II (DNA gyrase) inhibitors nalidixic acid and oxolinic acid, and the latter bears some structural similarity to the epipodophyllotoxins (Fig. 1).

Little is known about how the previously cited anticancer agents disrupt the normal breaking-resealing action of topoisomerase II. Indeed, one of the most elementary issues, i.e., whether the drug target is enzyme, DNA, or both, has not been resolved. Some of the difficulty in articulating a reasonable hypothesis derives from the fact that, although the DNA binding characteristics of intercalating agents have been well documented, published evidence would suggest that the epipodophyllotoxins (7) and *N*-acyl anthracyclines (9) do not bind to DNA. The problem has been further compounded by technical difficulties in obtaining quantities of pure mammalian topoisomerase II sufficient for establishing whether these drugs interact directly with enzyme. Similar issues regarding the action of nalidixic acid congeners on DNA gyrase have only recently become clarified. Initial evidence indicated that nalidixic acid did not bind to DNA (10), and thus it was presumed that the enzyme was the principal drug target. A more recent examination of the problem by Shen and Pernet (11), however, has established that these drugs, indeed, bind to DNA and that their affinity for DNA closely correlates with their potency as enzyme inhibitors. These investigators could not demonstrate drug binding to the enzyme. Clearly, if the model of Shen and Pernet can be extrapolated to the inhibitors of mammalian topoisomerase II, there are important implications with respect to designing new drugs and to understanding the mechanism itself. We have, therefore, experimentally re-examined the issue of DNA binding by epipodophyllotoxins and *N*-acyl anthracy-

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ABBREVIATIONS: KDNA, kinetoplast DNA; AD41, *n*-trifluoroacetyl adriamycin; AD32, *N*-trifluoroacetyl adriamycin valerate; EtBr, ethidium bromide; *m*-AMSA, 4′-(-acridinylamino)methanesulfon-*m*-anisidine.

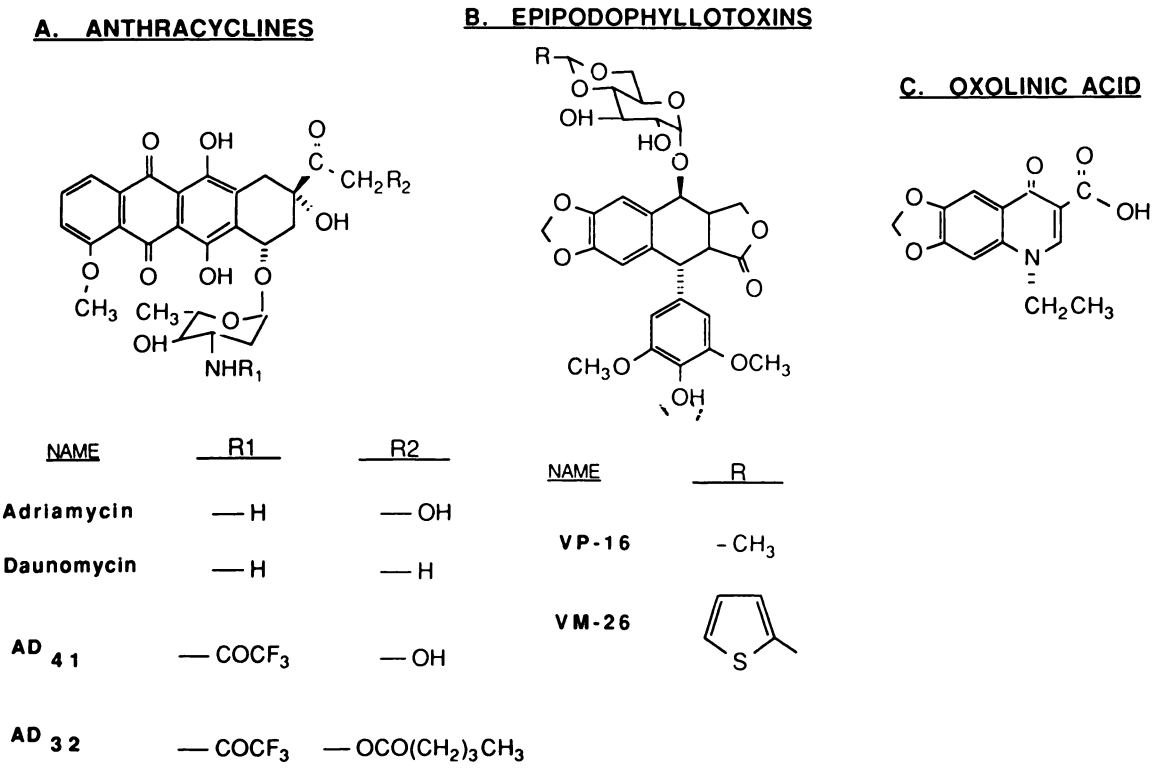

Fig. 1. Structures of anthracyclines, epipodophyllotoxins, and the bacterial DNA gyrase inhibitor oxolinic acid.

clines under experimental conditions that would allow detection of infrequent binding sites on DNA such as was described for the gyrase inhibitors. We have found that both epipodophyllotoxins and *N*-acyl anthracyclines bind to DNA and that many of the characteristics of this DNA binding are similar to those of the binding of the gyrase inhibitors. Based on this evidence, we discuss a mechanistic model that could account for how intercalating agents, epipodophyllotoxins, and *N*-acyl anthracyclines share a common mechanism for interfering with DNA topoisomerase II.

Experimental Procedures

Materials

pUC18 DNA was isolated from HB101 *Escherichia coli* and purified by CsCl gradient centrifugation followed by column (Biogel A-1.5m; Bio-Rad; Rockville Centre, NY) chromatography. kDNA was isolated from *Crithidia fasciculata* (12), RNase A treated, and spun-column (Sephadex G-50; Pharmacia Fine Chemicals, Piscataway, NJ) purified. pUC18 DNA was linearized using *Sma*I or *Hind*III (Bethesda Research Laboratories, Gaithersburg, MD) restriction enzymes, and kDNA was linearized with *Xba*I. Aquassure was from New England Nuclear (Boston, MA). General lab supplies were from Fisher Scientific (Orlando, FL) or Bio-Rad. All other materials were reagent grade from Sigma Chemical Co. (St. Louis, MO), Bio-Rad, or Pharmacia.

[^{3}H]Etoposide (VP-16, 400 mCi/mmol) was obtained from Moravek Biochemicals (Brea, CA). The purity was greater than 97% by reverse phase thin layer chromatography assay. [^{14}C]AD41 and [^{14}C] AD32 were kindly provided by Merv Israel (University of Tennessee, Memphis). The label is located on the carbonyl carbon of the trifluoroacetamide, and the purity was established by high performance liquid chromatography analysis to be greater than 99%. The specific activity of both [^{14}C]AD41 and [^{14}C]AD32 is 2.66 mCi/mmol as determined by quantitative UV/visible spectrophotometry and liquid scintillography. [^{14}C]Adriamycin was synthesized by SRI International (Menlow Park, CA) and kindly provided by Dr. Milan Potmesil. The label is on the C-14, and the specific activity is 23.5 mCi/mmol.

Drug Binding Assays

kDNA binding assay. A 100-μl reaction mixture containing 2–5 pmol of kDNA and various amounts of drug in PMD (10 mM KH_2PO_4, pH 7.4, 5 mM $MgCl_2$, 4 mM dithiothreitol buffer was incubated at 37° for various periods of time. A pmol of kDNA is herein defined as the amount of network DNA containing 6×10^{11} minicircles based on an average size of 2500 base pairs per minicircle. The kDNA was then spun down for 5 min in a tabletop Fisher microcentrifuge (13). No detectable kDNA remained in the supernatant under these conditions. Exactly 75 μl of supernatant was carefully removed and measured for unbound drug. The remaining volume was aspirated, measured, and then used to calculate the amount of drug bound to kDNA by subtracting the presumed amount of unbound drug (calculated from the supernatant) from the total amount of labeled drug observed in the presence of kDNA. There was no difference in drug distribution in the reaction mixture after centrifugation in the absence of kDNA. Drug concentrations were always well within the limits of their solubility.

Reversibility of drug binding to kDNA was studied as follows. Radiolabeled drug ([^{3}H]VP-16, 40 μM or [^{14}C]AD41, 25 μM) was incubated with 10 μg of kDNA for 30 min at 37° in 100 μl total volume. The kDNA was then sedimented with the microcentrifuge, and 90 μl of supernatant were removed and counted. The remaining buffer containing kDNA was then diluted 50-fold using PMD buffer. After varying time periods ranging from 0.5 to 30 min at 37°, the kDNA was again sedimented, and 450 μl of the supernatant was removed and counted. The remaining pellet was diluted with 450 μl of PMD and counted to determine bound drug. The kDNA recovery in the final pellet was greater than 90%.

Filter binding assay. Each sample containing 2–5 pmol of plasmid (11–27 nmol of DNA phosphate) and different concentrations of radiolabeled drug in PMD or PD (PMD without $MgCl_2$) buffer were incubated at 37° for 1 hr. Longer periods of incubation did not affect the results. The 25-μl reaction mixture was then spotted onto DE81 paper (Whatman, Clifton, NJ), and the paper was washed three times

with PMD or PD buffer. Filters were then dried, and both filter and wash were counted. Drug bound to DNA was calculated by subtracting the radioactivity of control filters containing drug but no DNA. Background filter radioactivity was always less than 5% of the samples containing DNA.

Equilibrium dialysis. Confirmation of drug binding values obtained by the above-cited methods was obtained by equilibrium dialysis in a 1.5-ml Teflon dialysis cell or a dot-blot apparatus (Bio-Rad) with SpectraPor-2 membranes (Spectrum Medical Industries, Los Angeles, CA). Both chambers of the filled Teflon assembly or the lower chamber of the dot blotter were agitated at room temperature. The time required for achievement of equilibrium was approximately 12 hr. Binding experiments were carried out at 5 and 10 μM [^{3}H]VP-16 and 0.56–2.8 μM plasmid DNA. In different experiments, drug was added to either of the dialysis chambers with identical values observed at equilibrium. Limited drug availability precluded performing equilibrium dialysis experiments with AD41 and AD32.

Unwinding Assay. Supercoiled pUC18 was relaxed in a 20-μl reaction mixture containing 40 mM Tris, pH 7.4, 85 mM KCl, 5 mM $MgCl_2$, 4 mM dithiothreitol, 0.5 mM EDTA, 30 μg/ml bovine serum albumin, 0.1–1 unit of calf thymus topoisomerase I (Bethesda Research Laboratories), and various amounts of drug (14). Reactions were carried out at 37° for 1 hr and then terminated by addition of 2 μl of 1% sodium dodecyl sulfate. After phenol extraction and ethanol precipitation, the DNA was resuspended in 20 μl of loading buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 5% glycerol, and 0.03% bromophenol blue). Half of the volume was loaded directly onto a 1% agarose gel in 2× TBE (180 mM Tris-borate, pH 8.3, 2 mM EDTA). The other half, after addition of 1 μl of 100 μg/ml EtBr, was loaded onto 1% agarose gel containing 50 nM EtBr. No EtBr was in the running buffer (2× TBE). Electrophoresis was done at room temperature at 50 V for 3 hr.

Results

The binding of adriamycin, VP-16, and the *N*-acyl anthracyclines AD32 and AD41 to kDNA is shown in Fig. 2. As expected, there are many more DNA binding sites for adriamycin than for the other compounds. Indeed, no saturation is observed in the dose range examined. The calculated K_d (based on a Scatchard analysis) for adriamycin is 2.4×10^{-6} M, similar to previously published values. Significantly, however, saturation of DNA binding is observed for the *N*-acyl anthracyclines and VP-16. There appear to be approximately 75, 30, and 10 drug binding sites per kDNA minicircle under saturating conditions for AD41, VP-16, and AD32, respectively. Therefore, based on an average kDNA minicircle size of 2500 base pairs, there are approximately 1.2×10^{-2} drug molecules bound per base pair under saturating conditions for VP-16 and 3×10^{-2} drug molecules per base pair for AD41. Equilibrium dialysis experiments performed using kDNA and VP-16 yielded similar values (data not shown).

The reversibility of drug binding is shown in Table 1. For AD41, there is a rapid but only partial reversal of binding in the first minute after dilution. Even 30 min after dilution, significant amounts of drug are bound. In contrast, VP-16 is completely reversed within 1 min after drug dilution. In the case of both drugs, the association of drug with DNA was complete within 1 min under conditions of temperature and drug concentration similar to the dissociation experiments (data not shown).

The binding of VP-16 to DNA was examined in greater detail using supercoiled pUC18 plasmid in the presence or absence of magnesium (Fig. 3). The filter binding method was used in these experiments. There is a rapid increase in DNA binding at low drug concentrations. However, an initial plateau is reached at approximately three drug binding events per plasmid molecule. As the drug concentration is increased, a second binding phase is observed. This binding curve is remarkably similar to that observed for nalidixic acid analogs (11). In the absence of magnesium, binding is significantly reduced. The magnesium effect was also observed using the kDNA centrifugation method for DNA binding (data not shown). In order to assess the possibility that loosely binding drug might be removed during the filter washes, these experiments were repeated at selected drug concentrations using the equilibrium dialysis method, and similar values were obtained (data not

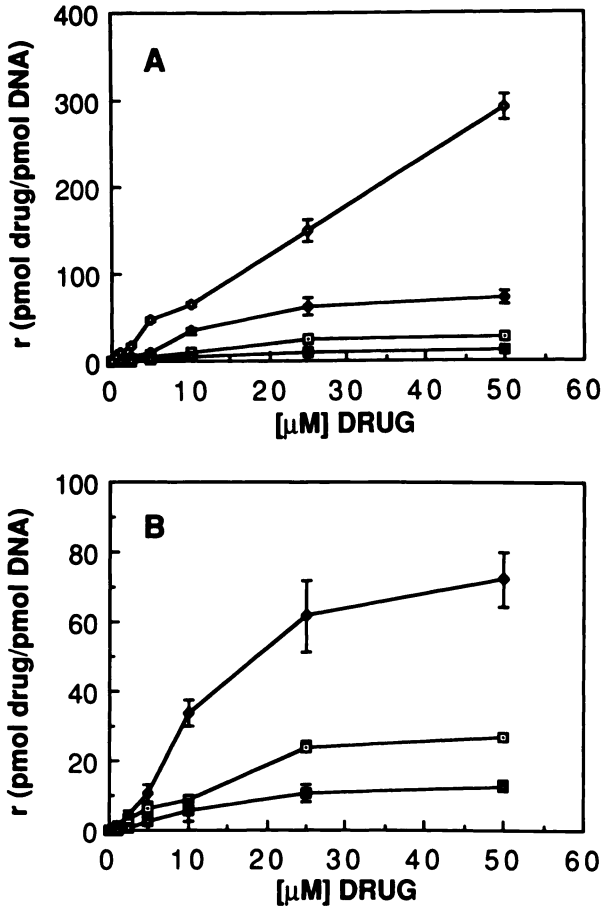

Fig. 2. Drug binding to kDNA. A, Binding of AD32 (□), etoposide (⊡), AD41 (♦), and adriamycin (◇) to kDNA as a function of drug concentration. Each *point* represents mean ± the standard deviation. A pmole of DNA is defined in Methods. B, The data are the same as in A except that the *ordinate* scale has been altered to allow a more detailed examination of binding by AD41 (♦), VP-16 (⊡) and AD32 (□).

TABLE 1

Dissociation of AD41 and VP-16 from DNA

Radiolabeled drug was incubated with 10 μg of kDNA for 30 min then removed by centrifugation of DNA and dilution in drug-free buffer. After various periods, the kDNA was again sedimented and counted. Values are mean ± standard error.

Time	AD41	VP-16
min	*pmol of drug/pmol of kDNA*	
0	74.8 ± 16	22.7 ± 11.2
0.5	36.7 ± 12.2	3.7 ± 2.7
1.0	27.3 ± 5.3	0.7 ± 0.5
5.0	33.5 ± 9.2	0.3 ± 0.2
10	21.7 ± 4.2	0.7 ± 0.7
30	21.7 ± 3.7	0.1 ± 0.1

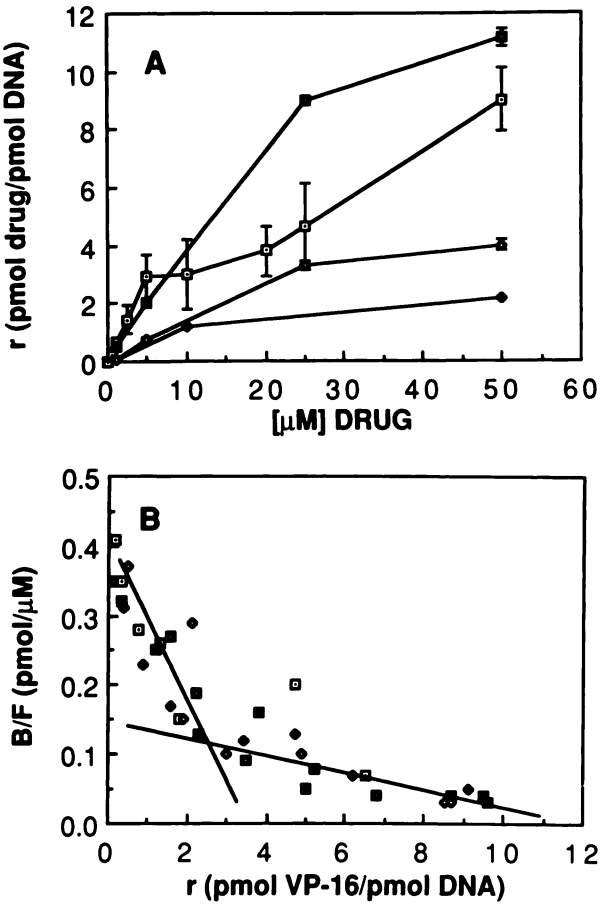

Fig. 3. Drug binding to plasmid DNA. A, Form I pUC18 plasmid DNA was incubated with AD41 (□), AD32 (◇), and VP-16 in the presence (⊡) or absence (♦) of 5 mM $MgCl_2$. Binding was assayed by the filter finding method. Each *point* represents the mean ± the standard deviation. B, Scatchard analysis of DNA binding by VP-16. Individual data points for etoposide binding to pUC18 DNA in the presence of magnesium were analyzed by the Scatchard method. The estimated K_d for the high and low affinity sites are 1.1×10^{-5} M and 5.8×10^{-5} M, respectively. The calculated B_{max} for these sites are 3.5 and 12 sites per plasmid molecule. Different *symbols* represent individual experiments.

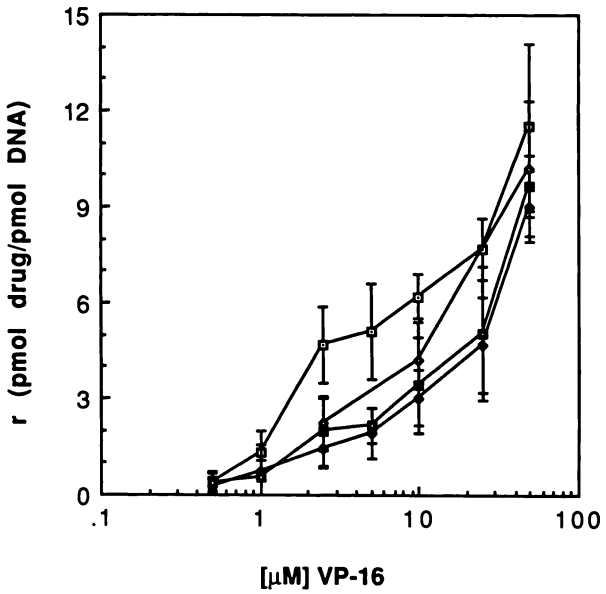

Fig. 4. Etoposide preferentially binds to single-stranded DNA. Binding experiments were performed as in Fig. 3A, except that the DNA was either single-stranded (⊡), linearized with a four-base stagger at the termini (◇), linearized with blunt ends (□), or supercoiled (♦). All experiments were performed in the presence of magnesium. Each *point* represents the mean ± the standard error.

shown). When the VP-16 binding data shown in Fig. 3A are analyzed using the Scatchard method (15), there are two distinct binding sites of different affinities observed (Fig. 3B). The first is a relatively high affinity site with a calculated K_d of 1.1×10^{-5} M and a total of 3.5 binding sites per plasmid molecule. The low affinity binding sites exhibit a K_d of 5.8×10^{-5} M, and there are approximately 12 binding sites per plasmid. In other experiments using this method, the drug concentration has been held constant, and the DNA concentration (12–560 nM) varied. The amount of drug bound in these experiments varies stoichiometrically with the amount of DNA present in the experiment (data not shown). As shown in Fig. 4, the binding of VP-16 to heat-denatured pUC18 DNA is significantly greater than that for native linear or supercoiled DNA. Linear DNA cut with *Sma*I and thus bearing blunt ends binds to drug less well than does linear DNA cut with *Hind*III, having ends bearing a four-base stagger. There is no difference between blunt-ended linear DNA and supercoiled DNA. Similar effects were observed when AD41 was used instead of etoposide (data not shown).

The *N*-acyl anthracycline AD41 not only binds to DNA but it also unwinds topologically restrained DNA, suggesting that, like its congener adriamycin, it intercalates between base pairs (Fig. 5). In the experiment shown in Fig. 5A, negatively supercoiled pUC18 DNA is incubated with various concentrations of AD41 and relaxed with topoisomerase I in the presence of drug. The drug is then removed by phenol extraction, and the topological state of the DNA is analyzed by gel electrophoresis. Under these experimental conditions, an intercalating agent will progressively unwind DNA, resulting in a topological progression from negatively supercoiled DNA to the form that is relaxed and finally to a positively supercoiled topoisomer. The action of topoisomerase I relaxes the DNA in the presence of drug. After drug removal, the final state of supercoiling depends on the number of negative supercoils removed before relaxation. Fig. 5A demonstrates that the topology of DNA relaxes by topoisomerase I is clearly altered in the presence of AD41 over the drug concentration range of 2–10 μM. The final state of the DNA appears to be virtually completely relaxed, suggesting that intercalation by AD41 produced just enough positive supercoiling that, upon relaxation and drug extraction, the final product is a highly relaxed topoisomer. As the concentration of AD41 was increased from 10 to 20 μM (*lanes 5* and *6*), the drug created more positive supercoiling which, upon DNA relaxation and drug extraction, resulted in net negative supercoiling of the DNA. The distinction between the negatively supercoiled DNA observed in *lanes 6–8* as compared with the distribution of relaxed DNA found in *lane 2* was made by electrophoresing the DNA in the presence of the intercalator EtBr. Under these conditions, the relaxed DNA (*lane 2*) becomes positively supercoiled under the influence of the intercalator and exhibits a higher mobility in the gel. Negatively supercoiled DNA is unwound, yielding a topoisomer with a lower gel mobility (*lane 1*). Such a phenomenon is also observed in *lanes 5–8*, confirming that the presence of AD41 has altered the topology of the topoisomerase I reaction product. It is of note that there is

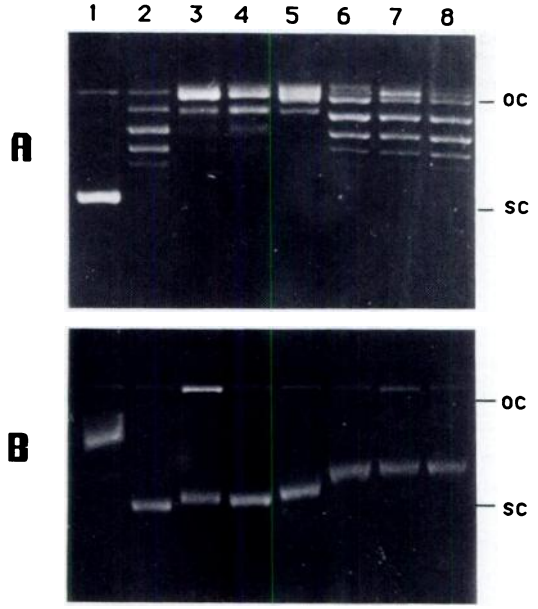

Fig. 5. Evidence for DNA intercalation by AD41. Form I pUC18 DNA was relaxed using purified topoisomerase I in the absence (*lane 2*) or presence (*lanes 3–8*) of various concentrations of AD41. The DNA was then extracted with phenol to remove drug and electrophoresed in a 1% agarose gel in the absence (A) or presence (B) of 5 nM ethidium bromide. *Lane 1* represents control DNA in the absence of enzyme or drug. The concentrations of AD41 used were 2, 5, 10, 20, 50, and 100 μM (*lanes 3–8*, respectively). The positions of open circle DNA forms (*OC*) and supercoiled forms (*SC*) of DNA are noted.

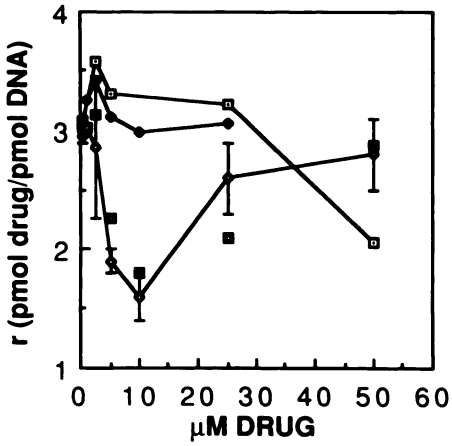

Fig. 6. Competition for VP-16 binding sites by VM-26 (□), VP-16 (◇), adriamycin (⊡), and ethidium bromide (♦). Form I pUC18 DNA was incubated with 10 μM [^{14}C]VP-16 simultaneously with various concentrations of the unlabeled drugs. After a 1-hr incubation, the binding of labeled VP-16 was determined using the filter binding technique. VM-26 and unlabeled VP-16 were equally effective in competing for binding sites up to a concentration of 10 μM.

little change in the superhelicity exhibited in *lanes 6–8* despite the 4-fold increase in drug concentration. This most likely relates to saturation of drug binding sites as shown in Fig. 1 for kDNA. (Some DNA in *lane 3B* did not enter the gel. This technical artifact was not observed in duplicate experiments.) Experiments to detect intercalation were also performed using VP-16 (5–200 μM) and AD32 (0.5–50 μM), and no evidence of DNA unwinding was observed in either case.

In order to rule out the possibility that the result shown in Fig. 5 simply results from inhibition of topoisomerase I by AD41, we have performed similar experiments using fully relaxed but covalent closed plasmid DNA as the initial substrate. As expected, the intercalation of AD41 followed by topoisomerase I-mediated relaxation and then drug extraction resulted in a final product that was negatively supercoiled (data not shown).

It was of interest to determine whether other topoisomerase II-active drugs could compete for VP-16 binding sites. In Fig. 6, the filter binding method was employed to address this issue. Radiolabeled VP-16 (10 μM) was incubated with pUC18 DNA simultaneously with various concentrations of unlabeled VP-16, VM-26, EtBr, or adriamycin. Up to a concentration of 10 μM, unlabeled VP-16 effectively competed for the binding sites of labeled drug. Above this concentration, however, binding again increased. Interestingly, VM-26, a VP-16 congener with approximately 10-fold higher potency as a topoisomerase II inhibitor, was no more potent as a binding competitor than unlabeled VP-16. Both of the intercalating agents increased apparent VP-16 binding at low concentrations. Despite the much higher affinity and number of binding sites of these intercalators for DNA, there is little reduction in VP-16 binding at intercalator concentrations of less than 25 μM.

Unexpectedly, there is increased binding of labeled VP-16 as the concentration of the unlabeled competitor exceeds approximately 10 μM. This altered pattern is suggested by Fig. 3A as well and suggests that the structure of the DNA may be altered in such a way as to result in new binding sites being created.

Discussion

Contrary to previously published evidence, data presented herein clearly establish that each of the major categories of anticancer agents that stimulate DNA cleavage by topoisomerase II also binds to DNA. Furthermore, strong evidence for DNA intercalation is provided for one of the drugs, AD41. Careful examination of our results makes it clear why DNA binding was not observed for these drugs by previous investigators. Silber *et al.* (9) compared adriamycin, AD32, and AD41 for DNA binding affinity by determining anthracycline absorbance in the presence or absence of DNA and were unable to demonstrate binding by AD32 or AD41. The limited sensitivity of the absorbance assay very likely precluded detection of drug binding to the highly restricted number of sites available. Our laboratory previously reported that VP-16 did not bind to DNA, based on equilibrium dialysis studies using calf thymus DNA at a concentration of 0.9 mM phosphate and 0.03 μM VP-16 with a specific activity of 400 mCi/mmol (7). Although the DNA concentration is comparable to that in the current experiments, inspection of data in Figs. 2 and 3 reveals that no DNA binding would be observed at this low drug concentration. In summary, the principal reason why DNA binding was not observed for these agents in previous studies was the limited number of total binding sites available and the fact that the experimental conditions used were more appropriate for drugs such as classical intercalating agents with a higher affinity and large number of binding sites. Our data would also suggest that the frequency of binding sites varies, depending on the nature of the DNA substrate. Over a similar drug concentration range, fewer binding sites were observed using plasmid DNA as a substrate than kDNA. Because saturation was not achieved using the plasmid DNA, it is possible that this reflects decreased affinity of DNA for drug rather than a reduced total

number of sites. Whether the observed differences between the two forms of DNA can be ascribed to nucleotide sequence *per se* or higher orders of structure cannot be ascertained at this time.

The DNA binding characteristics of VP-16 share a number of features with the DNA gyrase inhibitors (11). Each occupies only a limited number of total binding sites. Furthermore, in each case there appear to be high and low affinity sites. In the case of norfloxacin, the high affinity sites saturate at approximately three to five total sites per plasmid, a number that is similar to that seen in the Scatchard analysis of VP-16 (Fig. 3B). Both the gyrase inhibitors and the epipodophyllotoxins demonstrate greater binding to single-stranded than double-stranded DNA, and binding to supercoiled DNA is equal to that to the linear form. The K_d value for VP-16 is almost identical to that of the structurally related oxolinic acid and significantly lower than that of nalidixic acid (11). Two features of DNA binding by epipodophyllotoxins are different from those reported for the binding of gyrase inhibitors. The first is that, in the case of the latter compounds, magnesium had no effect on DNA binding. Perhaps more significantly, however, Shen and Pernet (11) reported a strong correlation between DNA binding affinity and potency as gyrase inhibitors. In contrast, the competitive binding experiment shown in Fig. 6 indicates that the epipodophyllotoxin VM-26 is similar to VP-16 with respect to DNA binding, even though it has 10-fold greater potency as a topoisomerase II inhibitor. The significance of this is discussed below.

Silber *et al.* (9) have previously reported that AD41, like adriamycin, stimulates DNA cleavage by topoisomerase II. The esterified derivative AD32 did not have this effect. The site specificity of DNA cleavage for adriamycin and AD41 were different. Although their data are not presented in a quantitative fashion, it would appear that AD41 is less potent than adriamycin in stimulating topoisomerase II. Furthermore, adriamycin exhibits the phenomenon of autoinhibition whereas AD41 does not. Based on the fact that, like adriamycin, AD41 intercalates into DNA, we would propose that the two compounds share a common mechanism of action. It is tempting to speculate that the differences in site specificity for cleavage are based on differences in sites of drug binding. This will require experimental verification. The fact that topoisomerase-mediated DNA cleavage becomes inhibited as the drug concentration is increased for adriamycin but not for AD41 very likely relates to the fact that only a limited number of binding sites are available for the latter compound. Adriamycin, on the other hand, can bind at many sites and may thereby distort the conformation of the helix beyond recognition by topoisomerase II. The reason for the failure of AD32 to stimulate DNA cleavage by topoisomerase II is unclear. The drug seems to bind to DNA less well than does AD41, and we found no evidence for intercalation (see below). It is also possible that the valerate group interferes with the drug's interaction with enzyme as well as DNA. Further work will be required to establish this point.

Although we believe that DNA binding is important for the effect of drug on topoisomerase function, it is not possible at present to draw conclusions about the importance of the intercalative mode of binding. The vast majority of compounds that stimulate DNA cleavage by topoisomerase II intercalate between base pairs. Our data confirm that this is also true of AD41, although we found no evidence for intercalation by etoposide or AD32. We cannot exclude the possibility that, given the limited number of binding sites and the fact that these drugs could exhibit a small unwinding angle of intercalation, our assay may not have been adequate to detect intercalation.

Based on our currently available evidence, we believe the most satisfying model to account for the effects of epipodophyllotoxins and intercalating agents is one in which each drug molecule is composed of two functional domains. The first is necessary to allow the drug to bind to DNA. This is the predominant and most obvious domain for the intercalating agents and is represented by the planar chromophore in each of these drugs. The importance of the second domain is primarily in its interaction with enzyme. The existence of such a domain is supported by the differences in potency for etoposide and teniposide with respect to topoisomerase inhibition but not DNA binding. Presumably, the thienyl group present on teniposide contributes an order of magnitude of potency by virtue of hydrophobic interaction with enzyme. Computer modeling studies using structures based on crystallographic data indicate striking structural similarities between intercalating agents, such as adriamycin and *m*-AMSA, and the epipodophyllotoxins (2). If the analogy is correct, it predicts that the DNA binding region of the epipodophyllotoxins is the partially aromatic polycyclic array. Perhaps not coincidentally, this is the structural region that is related to oxolinic acid. The predicted positions of the sugar and the pendant ring in epipodophyllotoxins provide a chirality that is complementary to the right handed DNA helix. This allows binding along the minor groove of DNA as has been demonstrated for the daunosamine sugar of the anthracyclines (16) and the methanesulfon-*m*-ansidine ring of *m*-AMSA (17, 18). Here, they would be available for interaction with a DNA-binding enzyme such as topoisomerase II. Although such a model must be considered hypothetical at present, it may be of value in providing a basis for future experimental approaches to the problem, including synthesis of drug congeners.

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