Biochemical Pharmacology, Vol. 55, pp. 1025–1034, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00617-5

Anthracycline Antibiotic Blockade of SV40 T Antigen Helicase Action

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ABSTRACT. We previously showed that anthracycline antibiotics potently block SV40 large T antigen helicase; in the present study, we describe the kinetics and the structure–activity characteristics of this process. The concentration vs effect data for helicase blockade were fitted by the Hill equation to yield nearly parallel log-concentration effect curves for a series of active anthracycline antibiotics. The effective concentration for 50% helicase blockade (EC_{50}) values ranged from 0.34 μM for daunorubicin to 40.8 μM for 3′-deaminodaunorubicin. Clinically inactive 3′-*N*-acyl anthracyclines produced no blockade. The Hill constants for the blockade ranged from 1.1 to 1.6 for the entire series of active anthracyclines, indicating no positive cooperativity and suggesting that a single molecule of bound drug is sufficient to block helicase action. The EC_{50} values for several clinically effective anthracyclines showed a relationship to the average DNA binding constants for these drugs, and Lineweaver–Burk analysis of the blockade kinetics indicated non-competitive inhibition. The kinetics of the blockade indicated that the anthracycline, DNA, and helicase form a ternary complex that is irreversible under the reaction conditions. This mechanism may be central to the cytotoxic and anti-cancer activities of anthracycline antibiotics and may be useful in understanding the enzymatic mechanism of DNA helicase action. BIOCHEM PHARMACOL **55**;7:1025–1034, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. anthracyclines; daunorubicin; doxorubicin; T antigen; DNA helicase

Helicases are essential for the biochemical processing of ds† DNA and RNA because of their enzymatic action of separating hydrogen-bonded, ds oligonucleotides [1]. This enzymatic action of disrupting the hydrogen bonds that stabilize and hold two base-complementary strands of ds polynucleotides together is coupled with the consumption of energy provided by a nucleoside 5′-triphosphate. Unlike classic, dissociating enzyme–substrate catalytic action, most helicases bind to the substrate ds polynucleotides and catalyze progressive vectorial separation of the DNA strands without dissociating from the substrate [1]. We and others have reported that several classes of DNA-binding antibiotics are potent blockers of several DNA helicases [2–8]. We have proposed that the anti-helicase action of these antibiotics may be involved in their cytotoxic action [2, 4].

Among the potent helicase blockers are the anthracycline antibiotics, important anti-cancer agents that have been used clinically for three decades. Although the anthracycline antibiotics bind to ds DNA and are well-documented inhibitors of DNA and RNA syntheses, the precise mechanisms of cytotoxicity of these agents remain unclear, despite their poisoning and inhibition of topoisomerase II [9], DNA ligase inhibition [10], helicase blockade [2–4, 7, 8], and their free radical production [11, 12] and membrane binding [13].

In our previous studies, we found that anthracycline antibiotics were potent blockers of eukaryotic helicases from HeLa and mouse MF3A cells as well as virally induced SV40 large T antigen helicase [2]. We described a correlation between the ability of an anthracycline to bind to DNA and its interference with helicase action, and that the change in DNA melting temperatures that was associated with anthracycline binding to ds DNA only partially correlated with the blockade of the helicase activity. By studying the anthracycline blockade of T antigen helicase in more detail and depth, we now demonstrate a direct relationship of the binding affinity of several anthracyclines to ds DNA and their ability to block T antigen helicase. From kinetic analyses, we found that a single molecule of anthracycline bound to ds DNA was sufficient to block helicase action. This T antigen helicase blockade by anthracyclines involved a drug–DNA–helicase complex that was irreversible under our experimental conditions. These findings support this new mechanism of action for anthracycline antibiotics as being potentially involved in the anti-cancer action of these drugs.

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† *Abbreviations:* ds, double-strand; and ss, single-strand.

Received 9 July 1997; accepted 29 September 1997.

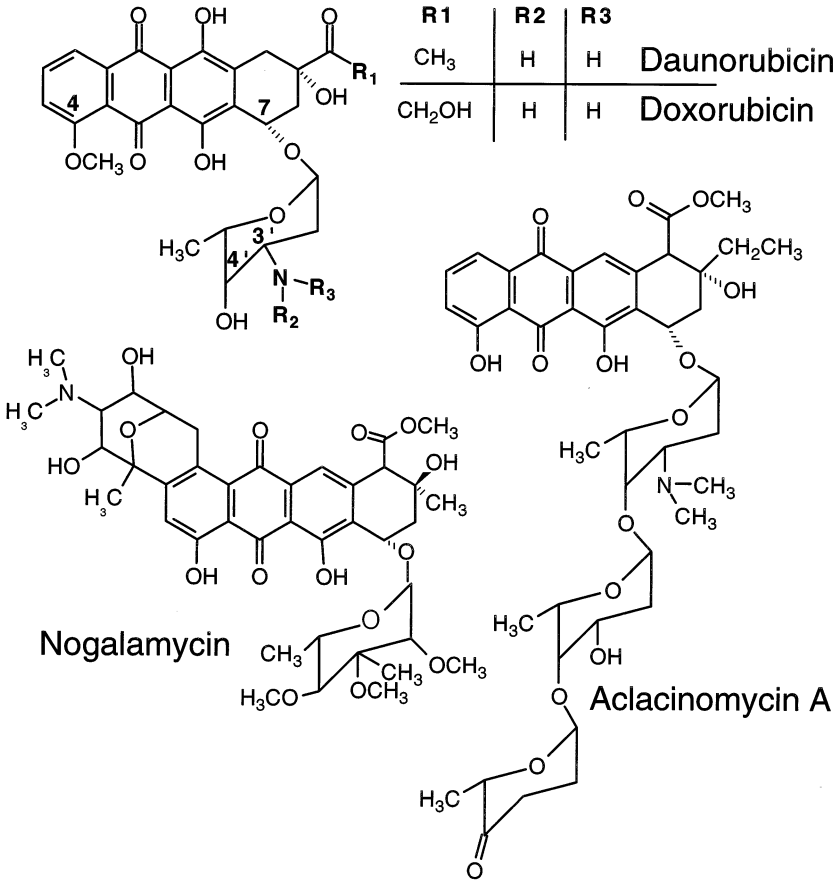

FIG. 1. Structures of anthracycline antibiotics used in helicase blockade.

MATERIALS AND METHODS

Selection of Anthracycline Antibiotics

The natural anthracycline antibiotics and synthetic analogs (Fig. 1) were obtained from several sources. Doxorubicin, daunorubicin, and 4′-epidoxorubicin were supplied by Farmitalia. Adria Laboratories provided 4-demethoxydaunorubicin. Other compounds were supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute.

Anthracycline antibiotic stock solutions (10^{-2} M) were made in DMSO and were stored at −20°. Dilutions of the stock solutions were made in 10 mM of Tris–HCl, pH 7.4, or water, before adding the test compounds to the helicase reaction mix. DMSO at concentrations to 0.028 M after drug dilution did not affect the helicase reactions. Preparation of SV40 large T antigen was as described by Simanis and Lane [14] and previously used in our studies [2].

Helicase substrate preparation of circular ss phage M13 MP19(+) DNA annealed to a radioactively labeled 17-mer oligonucleotide (5′-TCATGGTCATAGCTGTT) was described previously [2]. Bluescript plasmid was obtained from the Stratagene Cloning System, and calf thymus ds DNA was purchased from the Sigma Chemical Co.

Helicase Assay

The helicase assay was a modification of a previously described method [15]. The final reaction mixture (total 20 μL) contained 20 mM of Tris–HCl, pH 7.4, 5 mM of dithiothreitol, 2 mM of ATP, 50 ng of bovine serum albumin, 2 mM of $MgCl_2$, 50 mM of NaCl, 1 fmol of ^{32}P end-labeled partial ds DNA substrate, and T antigen helicase preparation. Because T antigen preparations varied in helicase activity, the amount of T antigen helicase used in each reaction was titrated to achieve linear reaction rates over the time of the experiment. The ^{32}P end-labeled partial ds DNA substrate was diluted in 20 mM of Tris–HCl, pH 7.4, 2 mM of EDTA, and 200 mM of NaCl. This reaction was run at 37° for 30 min unless otherwise stated.

ATPase Assay

ATPase activities were analyzed by the method of Hübscher and Stalder [16] with [^{3}H]ATP as the substrate. Following the reaction, the product [^{3}H]ADP and [^{3}H]AMP were separated by thin-layer chromatography and quantified by scintillation counting. The reaction conditions were identical to those described for the helicase assay, except that [^{3}H]ATP (0.1 mCi) at a final concentration of 1.25 mM was substituted for 2 mM of ATP.

Hill Analysis

The modified Hill equation [17, 18], currently used for analyzing data, is

$$\frac{\text{Blockade}}{\text{Blockade}_{max}} = \frac{[C]^n}{[EC_{50}]^n + [C]^n}$$

The observed value for Blockade_{max} is 1.0 for all the drugs fitted with this model. C is the drug concentration; EC_{50} is the effective concentration at which 50% helicase blockade occurred; and n, the Hill constant, is a measure of cooperativity. These values were determined for each drug with the ADAPT II program and the measured helicase blockade values produced by a range of drug concentrations [19].

RESULTS

Effects of Anthracycline Antibiotics on SV40 T Antigen Helicase Action

We evaluated the effects of a series of anthracycline antibiotic analogs (Fig. 1) on SV40 T antigen helicase activity. Blockade of the helicase action by the DNA-intercalating anthracyclines was plotted in a log concentration vs effect format (Fig. 2A). The clinically useful doxorubicin yielded a sigmoidal plot for helicase blockade. Plotting the helicase blocking effects of a series of anthracycline analogs, we obtained a family of nearly parallel log concentration effect curves (Fig. 2B), suggesting similar effects of these DNA intercalating binders on helicase blockade.

Analyzing these blockade data by the Hill equation, we obtained EC_{50} values ranging from 0.34 μM for daunorubicin to 40.8 μM for 3′-deaminodaunorubicin (Table 1). The clinically utilized anthracyclines, daunorubicin, 4-demethoxydaunorubicin, doxorubicin, and 4′-epidoxorubicin, were all potent blockers of the SV40 T antigen helicase activity.

Because chemical modifications of the primary amine of the daunosamine sugar of daunorubicin and doxorubicin have a dramatic effect on their anti-cancer activities [20, 21], we examined two series of analogs that were either N-alkylated or N-acylated (Table 1). The N-acyl compounds, which were inactive as anti-cancer drugs, did not block SV40 T antigen helicase activity at concentrations up to 50 μM, which was near their limit of solubility in our experimental conditions. Conversely, N-alkylated daunorubicins such as *N*-isopropyl, *N*,*N*-dimethyl, *N*,*N*-dibenzyl, and *N*-morpholino blocked the helicase activity potently. Anthracyclines of other structural types, nogalamycin, 7-*O*-methylnogarol and aclacinomycin, which have cytotoxic and anti-cancer activities, all blocked the T antigen helicase action.

Analysis of the helicase blockade data by the Hill equation yielded Hill constants ranging from 1.1 to 1.6 for the entire series of active anthracyclines (Table 1). This indicates no positive or negative cooperativity and that a single molecule of bound anthracycline was sufficient to block the helicase action.

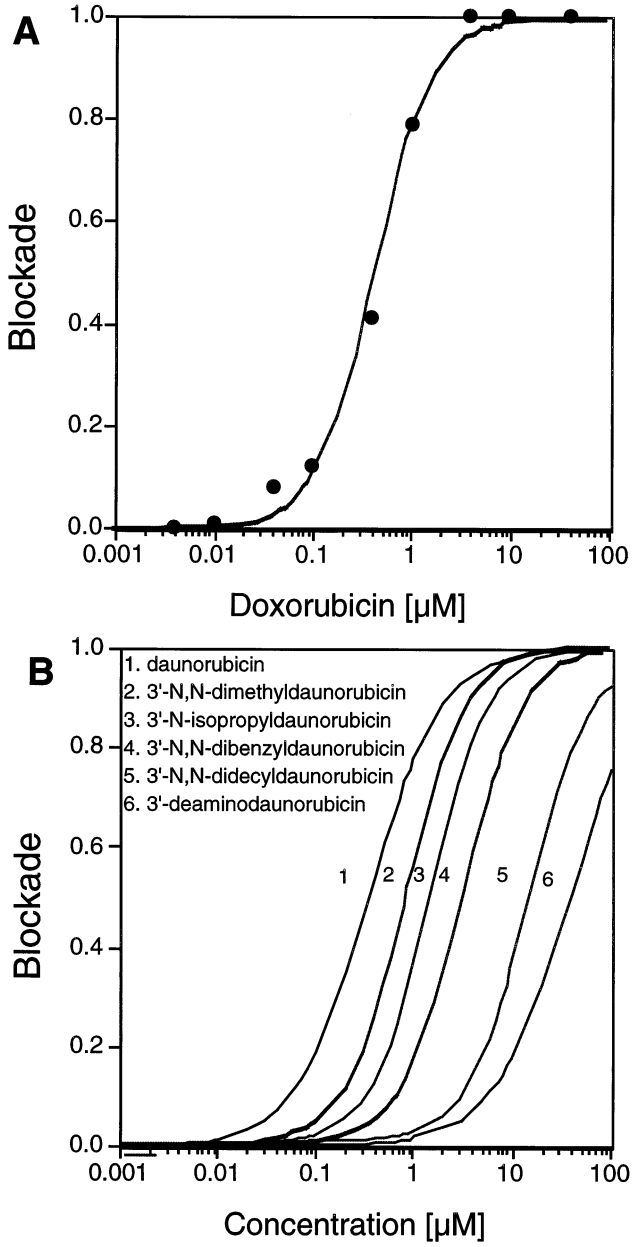

FIG. 2. SV40 T antigen helicase blockade by anthracycline antibiotics. The partial ds (M13/17-mer) DNA substrates were preincubated with increasing concentrations of drugs for 15 min at room temperature. Helicase activity was determined by measuring the percentage of 17-mer DNA dissociated in 30 min at 37°. In the control reactions, sufficient T antigen helicase was added to achieve total dissociation of the ^{32}P end-labeled 17-mer. The helicase blockade, fraction of helicase activity inhibition, is shown as a function of concentration of anthracycline. The log-concentration response curves were fitted with the observed data to the Hill model described in Materials and Methods. Panel A: doxorubicin. Panel B: daunorubicin and several analogs. Helicase blockade for (1) daunorubicin, (2) 3′-*N*,*N*-dimethyldaunorubicin, (3) 3′-*N*-isopropyldaunorubicin, (4) 3′-*N*,*N*-dibenzyldaunorubicin, (5) 3′-*N*,*N*-didecyldaunorubicin, and (6) 3′-deaminodaunorubicin are shown as a function of drug concentration.

TABLE 1. EC_{50} **values and Hill constants for anthracycline antibiotic blockade of SV40 T antigen helicase**

Anthracycline antibiotics	EC_{50} [μM]	(C.I. 95%) [μM]	Hill constant
Free amino anthracyclines			
Daunorubicin	0.34	(0.19–0.50)	1.2
Doxorubicin	0.46	(0.39–0.53)	1.5
4-Demethoxydaunorubicin	1.51	(1.09–1.94)	1.5
4′-Epidoxorubicin	1.20	(0.71–1.68)	1.1
3′-*N*-Acyl anthracyclines			
N-Formyldaunorubicin	>40		NA
N-Acetyldaunorubicin	>40		NA
N-Propionyldaunorubicin	>40		NA
N-Butyryldaunorubicin	>40		NA
N-Trifluoroacetyldoxorubicin (AD-41)	>50		NA
N-Trifluoroacetyldoxorubicin 14-*O*-valerate (AD-32)	>50		NA
3′-*N*-Alkyl anthracyclines			
N-Isopropyldaunorubicin	1.55	(1.31–1.79)	1.5
N,*N*-Dimethyldaunorubicin	0.86	(0.73–1.00)	1.4
N,*N*-Diethyldaunorubicin	1.23	(0.91–1.56)	1.2
N,*N*-Diethyldoxorubicin	1.04	(0.87–1.19)	1.6
N,*N*-Pentamethylene daunorubicin	0.98	(0.83–1.14)	1.4
N-Benzyldaunorubicin	1.35	(0.86–1.84)	1.6
N,*N*-Dibenzyldaunorubicin	3.88	(2.57–5.20)	1.4
N,*N*-Didecyldaunorubicin	15.48	(12.59–18.38)	1.4
N-Morpholinodoxorubicin	0.70	(0.53–0.86)	1.4
N,*N*,*N*-Triethyl (Cl^-) daunorubicin	0.81	(0.69–0.93)	1.3
Deamino anthracyclines			
3′-Deaminodaunorubicin	40.81	(20.76–60.86)	1.1
Other anthracyclines			
Nogalamycin	0.36	(0.21–0.50)	1.3
7-*O*-Methylnogarol	5.1	(3.3–6.9)	1.4
Aclacinomycin	2.3	(0.8–3.7)	1.1

The EC_{50} values were derived from the experimental data analyzed by the Hill equation. A minimum of three full concentration range sets for helicase blockade were obtained for each anthracycline compound. The EC_{50} values, 95% confidence intervals (C.I. 95%), and the Hill constants were determined by the ADAPT II Program with generalized least-squares weighting. NA indicates data are not available.

Relationship of T Antigen Helicase Blockade and Anthracycline DNA Binding Constant

We compared published DNA-binding constants of several of the anthracyclines [22–28] to their corresponding helicase EC_{50} values. When the data were plotted as anthracycline DNA binding $1/K_{app}$ vs EC_{50}, we obtained a straight line relationship, implying that the average binding constant of these anthracyclines to calf thymus ds DNA had a direct relationship to the drug blockade of T antigen helicase activity (Fig. 3). This linear fit had an R^2 of 0.957.

Anthracycline Concentration Effects on Helicase Kinetics

By examining the effects of various anthracycline concentrations on helicase reaction kinetics, we found distinctive characteristics of helicase blockade. Extending the time of the reaction (Fig. 4) demonstrated a persistent, overall reduction of product formation proportional to the concentration of the daunorubicin, with no evidence of reversal of the blockade. The prolonged control reaction also showed that the dissociated DNA strands do not reanneal readily after strand dissociation. In addition, changes in the initial rates of the strand dissociation were indicated in this set of reactions. To evaluate the early effects of the daunorubicin blockade, we shortened the time of the kinetics measurements (Fig. 5A). In these studies, a distinct lag phase at the start of the helicase reaction was seen as well as the change

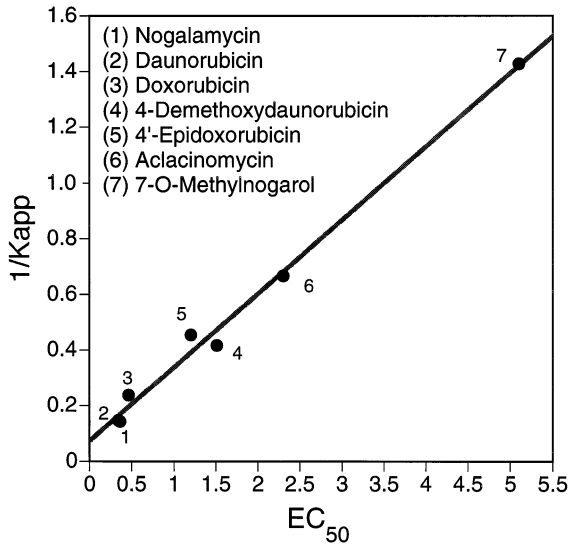

FIG. 3. Relationship between helicase blockade and DNA binding affinity. The $1/K_{app}$ values are shown as a function of T antigen helicase derived EC_{50} values. K_{app} values were obtained from Refs. 22–28.

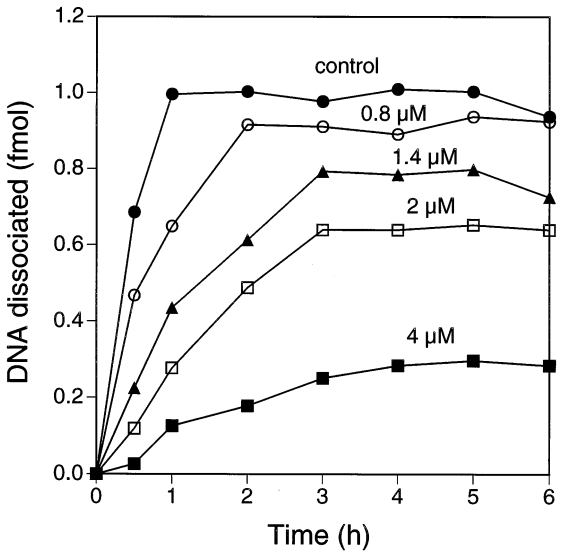

FIG. 4. T antigen helicase blockade kinetics: Effects of various daunorubicin concentrations on T antigen helicase kinetics. The helicase reaction was described in Materials and Methods with the addition of increasing concentrations of daunorubicin (0.8 to 4 μM).

in the initial rates. These effects on kinetics indicated binding of the antibiotic molecules to the DNA substrate. It is possible that when an antibiotic molecule binds to the DNA substrate, this antibiotic–DNA complex is not suitable or available as helicase substrate. This can account for the stoichiometric reduction of product formation.

Lineweaver–Burk Analysis of Anthracycline Effects

The linear helicase reaction rates were measured after the initial lag period caused by daunorubicin (Fig. 5A). A Lineweaver–Burk analysis of the T antigen helicase kinetics with daunorubicin blockade yielded a plot indicating noncompetitive inhibition of the helicase (Fig. 5B).

Irreversibility of SV40 T Antigen–Anthracycline–DNA Complex

Because of the stability of the helicase blockade by anthracyclines, we questioned if the SV40 T antigen formed a ternary complex with the anthracycline–DNA substrate complex and if such a ternary complex were reversible. If SV40 T antigen helicase formed a ternary complex with modified anthracycline–DNA substrate, then bound helicase might be sequestered from the reaction. The addition of excess helicase might then overcome the established helicase blockade if the helicase were limited in the reaction. However, the addition of excess (up to 10-fold) T antigen helicase did not overcome the helicase blockade established by 1 μM of daunorubicin (data not shown). As a control, 1000 ng of added bovine serum albumin protein did not affect the helicase blockade either.

Because the binding of anthracyclines to ds DNA is a

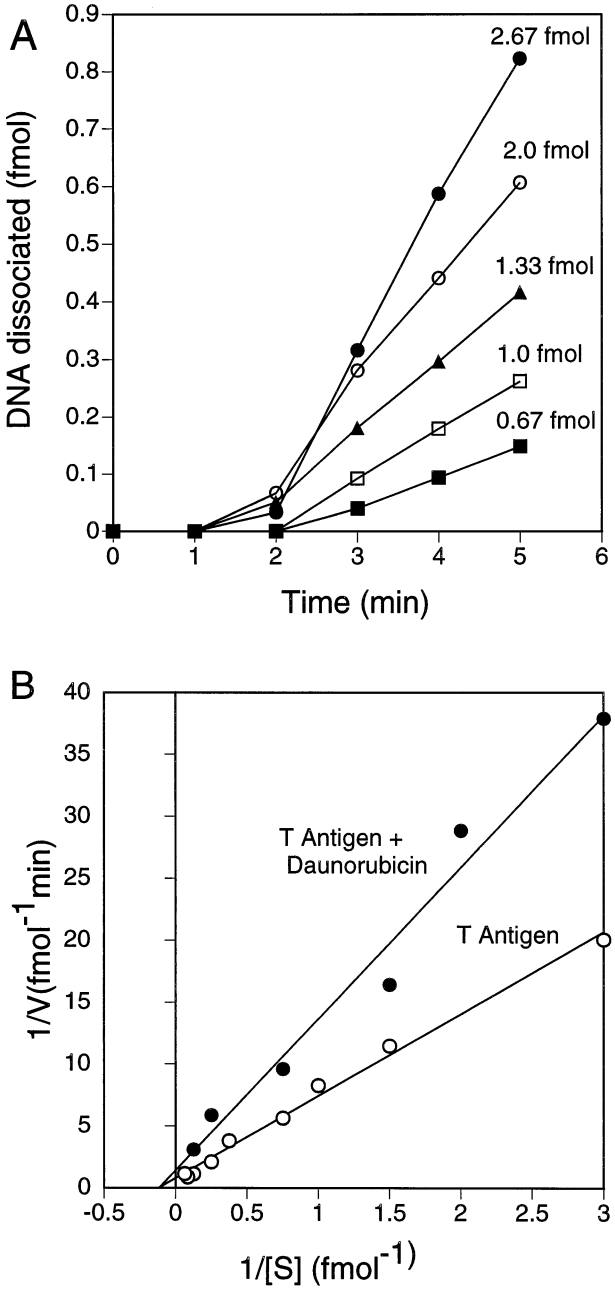

FIG. 5. Initial rate analysis of T antigen helicase activity inhibition by daunorubicin. (A) The helicase reaction was described in Materials and Methods with the addition of increasing amounts of DNA substrate (0.67 to 2.67 fmol). (B) The linear T antigen helicase reaction rates after the "lag-phase" caused by daunorubicin were used to determine a Lineweaver–Burk analysis of T antigen helicase kinetics with daunorubicin (1 μM) and without daunorubicin.

reversible process, we sought to reverse the blockade by providing competing DNA binding sites to pull drug from the DNA substrate. Once the anthracycline blockade was established at 1 and 5 μM of daunorubicin, the addition of 800 ng (320-fold excess) of calf thymus ds DNA was unable to reverse the blockade and did not alter the kinetics of the helicase blockade. Anthracycline blockade by 1 μM of daunorubicin gave 84% of control activity and 87% when

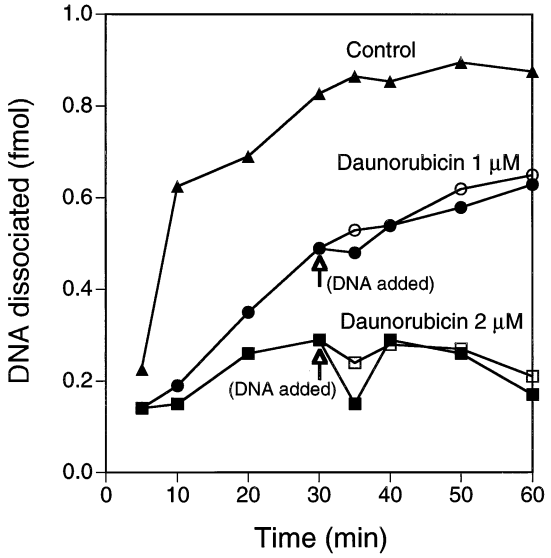

FIG. 6. Effects of competitive binding with excess ds DNA plasmid on T antigen helicase blockade by daunorubicin. The helicase reaction was described in Materials and Methods with the addition of SV40 T antigen helicase (4000 ng) and partial ds (M13/17-mer) DNA substrate alone (▲), with 1 μM of daunorubicin (○) and with 2 μM of daunorubicin (□), for 1 hr in a 200-μL reaction mixture. Unlabeled ds (bluescript) DNA substrate (4000 ng) was added to the helicase assay at 30 min with 1 μM (●) or 2 μM of daunorubicin (■), and then the reactions were allowed to proceed for an additional 30 min. At the times indicated, samples (20 μL) of the reaction mixture were removed to determine helicase activity.

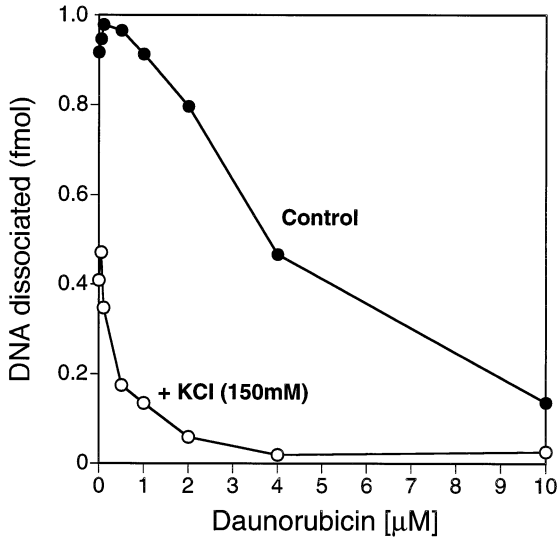

FIG. 7. Effects of KCl concentration on T antigen helicase activity with daunorubicin. The helicase reaction was described in Materials and Methods with the addition of increasing concentrations of daunorubicin, without KCl and with KCl (150 mM). The partial ds (M13/17-mer) DNA substrate was preincubated with KCl and daunorubicin for 15 min at room temperature.

excess ds DNA was added. With 5 μM of daunorubicin, the helicase activity was 23% of control and 22% after excess DNA (800 ng) was added. The large excess of ds DNA did not change the final levels of drug-established helicase blockade over time.

Calf thymus DNA might contain ss regions that could also compete for T antigen helicase binding. Therefore, we repeated the experiments with circular plasmid ds DNA, which did not provide binding sites for T antigen helicase but did compete for anthracycline binding. The addition of 400 ng (160-fold excess) of plasmid DNA did not affect the blockade pattern of helicase reaction established by daunorubicin at 1 and 2 μM (Fig. 6). The total DNA dissociation never recovered to the control levels seen in the absence of drug. Similarly, with 2 μg (800-fold excess) plasmid DNA supplementation, helicase activity blocked by 1 μM of daunorubicin plateaued after 1 hr at 64% of control activity after plasmid DNA was added, compared with 61% when no DNA was added.

Ionic Effects

Because both SV40 T antigen helicase action [15] and anthracycline binding to DNA [29] were known to be affected by salt concentration, we assessed physiological ionic conditions on helicase kinetics and anthracycline blockade. Ionic conditions also change the overall properties of DNA substrate [30], which might affect helicase activity. Helicase activity decreased to 75 and 66% of control with added KCl (140 mM) or NaCl (140 mM), respectively. Our findings are consistent with earlier reports that increasing ionic concentration decreases T antigen helicase activity [15]. The addition of 150 mM of KCl also altered the daunorubicin blockade of helicase dramatically (Fig. 7). The overall effect of SV40 T antigen helicase blockade by daunorubicin was increased with increased ionic strength, such that the EC_{50} of daunorubicin decreased from 3.8 μM at no added KCl to 0.25 μM at 150 mM of KCl. This indicated that helicase blockade by anthracycline antibiotics was substantial under physiological salt conditions.

ATPase Activity

Helicases have inherent nucleotide triphosphatase activity as part of their helicase function, and ATP is required for the dissociation of ds DNA substrate by SV40 T antigen helicase [15]. To examine the effects of anthracycline antibiotics, we determined the ATPase characteristics of helicase during the blockade process. ATPase activities of the free T antigen helicase, with added daunorubicin, with added DNA substrate, and with the modified anthracycline–DNA substrate were measured (Fig. 8A).

SV40 T antigen protein has intrinsic ATPase activity that is non-DNA dependent [15], and this activity was not affected by daunorubicin (Fig. 8A). These results indicate that daunorubicin does not inhibit the ATPase activity directly since there was no effect on the ATPase activity of the free enzyme even at 10 μM of daunorubicin. The

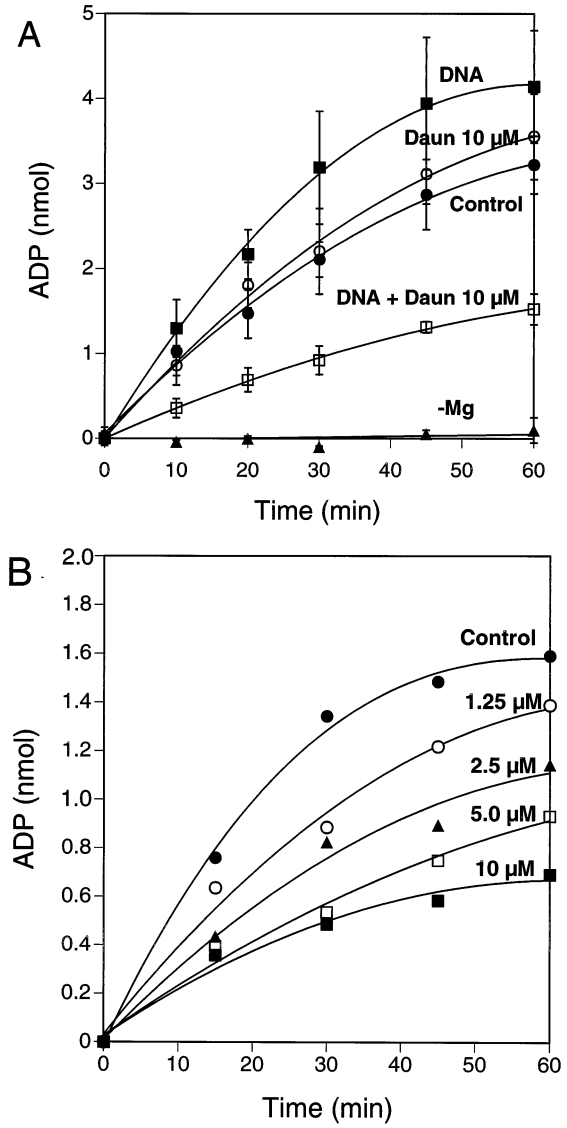

FIG. 8. Effects of daunorubicin on ATPase activity of T antigen helicase. (A) SV40 T antigen helicase (4.5 mg) was incubated alone (●), without magnesium chloride (▲), with DNA substrate (0.5 μg) (■), with daunorubicin (10 μM) (○), or with both DNA substrate and daunorubicin (□) in a 20-μL reaction mixture containing [^{3}H]ATP and ATP (1.25 mM). Error bars denote standard error of the mean, N = 3. (B) SV40 T antigen helicase (1.8 μg) was incubated with DNA substrate (1 fmol) and with increasing concentrations of daunorubicin: (○) 1.25 μM, (▲) 2.5 μM, (□) 5.0 μM, and (■) 10 μM. The closed circle (●) indicates the control. At the times indicated, aliquots (2 μL) of reaction mixture were removed to measure ATPase activity described under Materials and Methods. Values are the averages of duplicate determinations.

addition of ds DNA substrate to the T antigen helicase stimulated the ATPase activity. However, the addition of the ds DNA + 10 μM daunorubicin inhibited the ATPase activity even below the intrinsic ATPase activity level of the free enzyme. This implies that the SV40 T antigen helicase was modulated by the modified anthracycline–DNA substrate.

In light of these results, we questioned if a concentration dependence of ATPase activity inhibition by daunorubicin could be seen. The controls were treated the same way as in the standard helicase assay without daunorubicin present. The data indicated that there was a concentration-dependent effect on the inhibition of ATPase activity (Fig. 8B).

DISCUSSION

Our helicase blockade data correlated with previous observations concerning the effects of anthracycline structure on DNA binding and cytotoxicity. N-Acylated anthracyclines such as *N*-formyl, *N*-acetyl, and *N*-propionyl daunorubicin do not bind well to ds DNA [22], and we found they were not helicase blockers. These compounds are not useful as anti-cancer agents.

In contrast, N-alkylated anthracyclines, which retain the positive charge on the nitrogen of the daunosamine sugar, retain cytotoxic activity [21], as well as helicase blockade activity. As the *N*-alkyl substitution was increased in size and complexity, there was a reduction in the ability of the substituted antibiotic to interfere with the helicase reaction, and, presumably, to bind to the DNA. However, even quite large substitutions such as *N*,*N*-dibenzyl or *N*,*N*-didecyl daunorubicins retained strong anti-helicase activities. When the amino group was a quaternary amino group and retained a positive charge as in *N*,*N*,*N*-triethyl daunorubicin, the compound had significant antihelicase activity and presumably bound to the DNA readily. If the amino group was removed, as with 3′-deaminodaunorubicin (EC_{50} = 40.81 μM), the effect on helicase decreased over 100-fold less than that of the parent daunorubicin (EC_{50} = 0.34 μM).

Other anthracyclines, nogalamycin, 7-*O*-methylnogarol and aclacinomycin, all of which have positively charged amino groups as part of their structure, blocked T antigen helicase at low antibiotic concentrations and apparently bound readily to the ds DNA.

It is interesting that the *N*-trifluoroacetyl doxorubicins, AD41 and AD32, are cytotoxic agents and anti-cancer drugs. These compounds are different from N-alkylated anthracyclines, because they have little apparent binding to ds DNA and do not interfere with helicases or with topoisomerase [31]. Therefore, their action must be through a different mechanism, as has been suggested [31].

In our analysis of the relationship of T antigen helicase blockade to the published average binding constants of several anthracyclines, we found a linear relationship between these characteristics. From this limited sampling, a very important characteristic for helicase blockade was shown to be the binding affinity of the antibiotic to average ds DNA substrate. Some parts of the anthracycline molecule, important for other effects, had little effect on the helicase blockade characteristics for the T antigen helicase. For example, anthracyclines with such diverse structures (Fig. 1) as daunorubicin (MW 527.5), which contains a single sugar group, compared with nogalamycin (MW 801.8), which contains two separated sugar groups, and

thirdly aclacinomycin (MW 811.88), which contains a trisaccharide of considerable bulk, all bound to the ds DNA 17-mer; and their blockade of T antigen helicase correlated more with their binding affinity to the DNA rather than the complexity or bulk of their sugar structure.

In our assessment of the structure–activity relationships of a series of anthracycline antibiotic analogs and their blockade of SV40 T antigen helicase, we applied the Hill equation to the log concentration effect plotting of the data, and found a correlation of fit for this process. In the mechanism of this blockade, the anthracyclines bound to helicase substrate, the ds DNA 17-mer. We originally selected this 17-mer as substrate because it contains the base sequence AGCT, which offered selective binding for daunorubicin [32]. However, other reports [33–35] have indicated other DNA base sequence selectivities for daunorubicin, doxorubicin, and other anthracyclines. From our analysis of the helicase blockade data, using the Hill equation, we found no evidence of cooperativity or anti-cooperativity occurring in the case of multiple drug molecules binding to the substrate. Instead, we have determined Hill constants ranging from 1.1 to 1.6, which indicated a blockade of the helicase by single molecular interactions between drug and the 17-mer ds DNA.

In kinetic studies, increasing anthracycline concentrations reduced ds DNA substrate availability quantitatively; and the binding of the antibiotic to the DNA was the source of the blockade, rather than there being a direct effect of antibiotic on the helicase enzyme. These studies also showed an anthracycline-induced lag effect before the early linear phase of strand dissociation. This lag effect is a typical characteristic of non-competitive inhibition and reflects inhibitor binding to substrate [36].

One question that arose in our study was whether the DNA-bound helicase was capable of releasing from a blocked DNA substrate or whether the enzyme remained bound in an irreversible, ternary complex. Because of the processive action and binding characteristics of the SV40 T antigen helicase, once the enzyme was bound to a DNA substrate fork, it no longer interacted rapidly with other substrates [37]. Although drugs bound to ds DNA are in equilibrium with free drug and may separate from the DNA, this was not the case when T antigen helicase was also bound to the DNA–drug complex and produced a ternary complex of drug–DNA–helicase.

To determine the reversibility of the blockade process, we conducted a series of competition experiments. During the time required for the complete helicase dissociation of the DNA strands, we added back to the blocked reactions excess quantities of unlabeled ds DNA substrate, with the presumption that this large excess of DNA would compete for the bound drug that was blocking the helicase on the 17-mer duplex region. If the bound drug were removed, the unblocked helicase would resume activity to complete the reaction. We did not see resumption of helicase activity; rather, the blockade was not affected by added excess DNA. Therefore, the T antigen helicase, anthracycline antibiotic, and ds DNA were linked as a stable ternary complex that was irreversible under our experimental conditions. Even the addition of excess helicase to this reaction was unable to overcome the blockade. We conclude that the formed ternary complex [T antigen helicase–ds DNA–anthracycline] stopped the helicase action and physically trapped the enzyme and drug on the DNA.

It is well documented that SV40 T antigen helicase activity is adversely affected by salt [15]. For that reason, and the fact that potassium is the predominant cation in cells, our experiments focused on the effects of potassium on helicase activities. The reasons for the changes in shape of the helicase kinetic curve in the presence of 150 mM KCl cannot be attributed solely to direct effects of potassium on the enzyme itself or to indirect interactions between the potassium and the DNA substrate. In either case, increases in salt concentration increased the effectiveness of daunorubicin as a helicase blocker, despite the reduction in the drug's binding constant caused by the interaction of the salt ions and the DNA [38, 39].

Our finding of increased helicase blockade by daunorubicin with increased salt concentration may be explained by our original hypothesis of increased ds DNA stability induced by intercalating antibiotics and salt. Salt greatly influences the internal stability of the DNA as measured by the melting temperature [30]. However, the reduction of the binding affinity of the drug to DNA by increased ionic strength arises from the competition of drug and cation binding to DNA [29]. Although this puzzling aspect of the ionic strength dependence of daunorubicin binding to DNA remains unexplained, one may wonder if salt increases base-sequence binding specificity. Because daunorubicin has preferential binding to GC base-paired regions of ds DNA, the combination of the increased ds DNA stability induced by intercalating antibiotics and the increased DNA stability induced by potassium ions may yield an even greater effect on helicase action.

An inherent component of T antigen helicase activity is a DNA-stimulated ATPase. Daunorubicin did not affect the intrinsic or free enzyme ATPase of SV40 T antigen. However, daunorubicin significantly inhibited the ATPase in the presence of ds DNA substrates. This suggests that DNA-bound helicase has a drug-sensitive ATPase, but there is a residual ATPase action that is not inhibited by daunorubicin even when DNA dissociation is blocked completely. This also may indicate unbound helicase enzyme in the reaction mixture because the unbound, free enzyme may continue to hydrolyze ATP.

We have shown that both ATPase and helicase activities of T antigen yield concentration-dependent inhibition by daunorubicin. This suggests that the inhibition of ATPase reflects the coupling of ATP hydrolysis to the translocation of the SV40 T antigen helicase and the dissociation of the duplex regions. The reduction of ATPase can also be explained by the enzyme being sequestered by the modified anthracycline–DNA substrate. Thus, the results of the

competitive binding experiments along with those examining ATPase support the existence of a ternary complex.

The mechanisms of the anti-cancer action of anthracycline antibiotics are an important, yet incompletely resolved question. Of the various proposed mechanisms, the poisoning and inhibition of topoisomerase II are widely held to be the primary cytotoxic mechanisms [9]. However, experimental observations indicate that poisoning and inhibition of topoisomerase II are not the only mechanisms of anthracycline antibiotic cytotoxic action. Although some anthracyclines poison topoisomerase II, several cytotoxic anthracycline antibiotics do not affect topoisomerase II action [31]. Several studies of anthracyclines and topoisomerase II report that anthracyclines affect DNA strand breaks and cytotoxicity disproportionately and suggest yet unknown mechanisms to be responsible for cytotoxicity [39–42]. It is, therefore, reasonable to propose that helicase blockade is one component of several mechanisms of anthracycline action that contribute to cytotoxicity.

DNA helicase blockade is a logical mechanism of action for anthracycline antibiotics and is a mechanism that may answer unresolved questions of anthracycline antibiotic actions. Eukaryotic cells have multiple DNA helicases [1] that have specificity for replication, transcription, and repair. Because of the number and variety of DNA helicases, differential actions of anthracyclines on these different DNA helicases may explain the variety of actions that anthracyclines have on cells. Similarly, anthracycline antibiotics show complete blockade of DNA helicases at high antibiotic concentrations with no "self-inhibition," as seen with topoisomerase II [43]. It is possible that the irreversible ternary complex [anthracycline–DNA–helicase] that we demonstrate is responsible for DNA damage and protein-associated DNA cleavage that can be associated with anthracycline cytotoxicity. It is important to determine the exact causes and effects of anthracycline induced-DNA strand breakage and their relationships to cell cytotoxicity and other effects, such as the inhibition of DNA synthesis, which may be the product of combined mechanisms.

While we have characterized the anthracycline blockade of T antigen helicases substantially, and related anthracycline structure to this mechanism of action, we are presently studying human DNA helicases, the DNA sequence specificity of binding, and the kinetics of blockade by anthracyclines and other DNA binders. These relationships may prove useful in understanding the mechanism of DNA helicase enzymatic action and provide new targets for drug development.

This research was supported by the American Cancer Society, Grant DHP-106 (to N. R. B.), and a Bressler Grant from the University of Maryland (to N. R. B.).

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