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# Helicase Inhibition by Anthracycline Anticancer Agents

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**SUMMARY**

Helicases are essential to both DNA replication and transcription because they separate double-stranded DNA, preparing the single strands for replication or transcription. Because the anticancer anthracycline antibiotics stabilize double-stranded DNA primarily by their intercalative binding, we expected the intercalated antibiotics to interfere with helicase action. We examined anthracycline antibiotic effects on SV40 large T antigen helicase activity, using a duplex DNA helicase substrate of $^{32}$P-labeled 17-mer annealed to complementary M13mp19(+) circular single-stranded DNA. The T antigen helicase activity was potently inhibited by the anthracycline antibiotics. The T antigen helicase $IC_{50}$ values for the anthracycline antibiotics were as follows: nogalamycin, $2 \times 10^{-7}$ M; daunorubicin, $4 \times 10^{-7}$ M; doxorubicin, $4 \times 10^{-7}$ M; idarubicin, $1.8 \times 10^{-6}$ M; 4′-epidoxorubicin, $2 \times 10^{-6}$ M; aclacinomycin, $4 \times 10^{-6}$ M; and menogaril, $6 \times 10^{-6}$ M. Partially purified helicases from HeLa cells and murine mammary carcinoma FM3A cells also were potently inhibited by doxorubicin, with $IC_{50}$ values of $4 \times 10^{-7}$ M and $9 \times 10^{-7}$ M, respectively. Because the abundance, specificities, and types of helicases vary in the cell, this site of action for anthracycline antibiotics may help explain anthracycline potency, drug specificity for DNA or RNA inhibition, and some types of cellular resistance to these drugs.

The anthracycline antibiotics have several well described biological actions, such as the inhibition of DNA polymerases (1, 2), RNA polymerases (3), topoisomerase II (4), and DNA repair enzymes (5), production of free radicals (6), modulation of membranes (7), and others that have been associated with the anticancer activities of this drug family. Although each of these actions may be correlated with cytotoxicity of some anthracycline antibiotic analogs, no one action entirely satisfies all the correlations. Whether this is because the anthracycline antibiotics have multiple mechanisms of action or the specific cytotoxic mechanism is not yet explained remains a question. Certainly, the interaction of anthracycline antibiotics and DNA is considered a most likely site for cytotoxic anticancer activity.

In experiments on the anthracycline antibiotic inhibition of T4 bacteriophage DNA polymerases (8), we found that anthracyclines inhibited the polymerases substantially, by two distinct modes, uncompetitive and competitive inhibition. At low anthracycline concentrations, these drugs caused the formation of a tightly bound complex of DNA, polymerase, and drug (enzyme-DNA-drug), which was not reversible under the conditions of the experiment. The inhibited enzyme could not be dissociated from the enzyme-DNA-drug complex to an active form. This lead us to postulate this ternary complex of enzyme-DNA-drug as a mechanistic model for drug action. In a somewhat similar fashion, other examples of enzyme-DNA-drug complex have been described for the anthracycline antibiotic interactions with DNA and topoisomerase II, to form an enzyme-DNA-drug complex that is correlated with DNA strand breaks (9). We feel that the specificity of action of the anthracycline antibiotics lies not only in their interaction with specific duplex DNA sequences but also in the additional specificity of a ternary protein-DNA-drug complex. These observations have prompted us to investigate other biochemical characteristics of anthracycline-stabilized duplex DNA.

To utilize DNA biochemically, the cell must separate the DNA duplex to single strands, so that each strand can be copied in the replication process or a single strand of DNA can be transcribed to mRNA. This energy-consuming process of separating the DNA double strands is catalyzed by DNA helicases (10). Because DNA-intercalating drugs such as anthracyclines increase the physicochemical stability (e.g., increase the melting temperature) of double-stranded DNA (11), we postulated that intercalated anthracyclines should inhibit DNA helicase action. For these reasons, we initiated this study, which is aimed at determining the effects of anthracycline-intercalating drugs on DNA strand dissociation by helicase. We find that the anthracycline antibiotics do indeed modulate helicase action and are potent inhibitors of the enzymatic separation of duplex DNA strands.

## Materials and Methods

Anthracycline antibiotics (Fig. 1) were obtained from several sources. Doxorubicin, 4-demethoxydaunorubicin, and 4′-epidoxorubicin were

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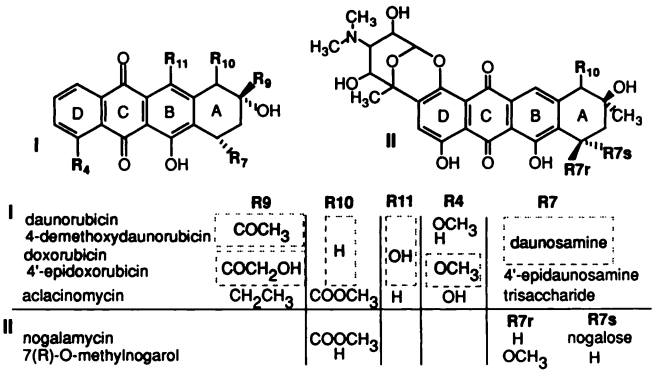


**Fig. 1.** Anthracycline antibiotic structures.

generously provided by Farmitalia (Milan, Italy). Nogalamycin and 7-*O*-methylnogarol were provided by the Upjohn Company, and aclacinomycin was obtained from the Drug Development Branch, National Cancer Institute. The M13 mp19(+) circular DNA and the 17-mer were purchased from Bethesda Research Laboratories. All other chemicals and reagents were of the highest grade commercially available, and all solutions were made in ultrapure deionized water except where noted otherwise.

**Helicase enzyme-SV40 large T antigen preparation.** We chose the SV40 large T antigen as our primary helicase for assay (12). SV40 T antigen helicase is prepared by a modification of the immunoaffinity purification procedure described by Simanis and Lane (13). This method is a simple and rapid procedure to purify and concentrate T antigen helicase and follows the steps of a detergent extraction of cells with a high speed centrifugation, after which the supernatant solution is applied to an immunoaffinity column of Protein A-Sepharose containing an immobilized anti-T antigen monoclonal antibody, PAB419. Comassie blue staining after sodium dodecyl sulfate-polyacrylamide electrophoresis of the SV40 large T antigen shows that only one protein component elutes from the affinity column.

**Helicase substrate preparation.** Our standard substrate for T antigen-catalyzed DNA dissociation is circular single-stranded phage M13 DNA carrying a hydrogen-bonded radioactively labeled stretch of cDNA. The bound cDNA is a 17-mer (sequence, 5′-TCATGGTCATAGCTGTT) with a 5′ end radioactive phosphate.

The 17-mer (60 ng; 109 pmol) is suspended in 10 μl of 10 mM Tris/1 mM EDTA buffer, pH 8.0, and the following additions are made: 22 μl of 3 units/μl polynucleotide kinase (66 units), 10 μl of polynucleotide kinase buffer (10×) (0.5 M Tris·HCl, pH 7.6, 100 mM $MgCl_2$, 100 mM 2-mercaptoethanol), and 58 μl of [$\gamma$-$^{32}$P]ATP (4500 Ci/mmol) (128.8 pmol), for a total of 100 μl. This reaction mixture is incubated at 37° for 45 min and is assayed to check the phosphorylation efficiency. After the reaction shows maximal phosphorylation (about 60%), the $^{32}$P-labeled 17-mer is separated from unincorporated [$\gamma$-$^{32}$P]ATP and unphosphorylated 17-mer by centrifugation through a Chroma spin TE-10 column (Clontech).

The radiolabeled 17-mer oligodeoxynucleotide is annealed to 5 ng of single-stranded circular M13 mp19 DNA in 20 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, by heating at 95° for 2 min, followed by immediate cooling to 70° for 2 min. The preparation is then allowed to cool slowly to room temperature. Nonhybridized oligodeoxynucleotide is removed by filtration through a Chroma spin +TE-400 column Clontech), with 10 mM Tris·HCl, pH 8.0, 1 mM EDTA.

**Helicase assay.** We measured the SV-40 T antigen helicase dissociating activity with the annealed radiolabeled 17-mer from the M13 DNA. The reaction mixture totals 10 μl, containing 20 mM Tris·HCl, pH 7.4, 5 mM dithiothreitol, 2 mM ATP, 50 μg bovine serum albumin, $^{32}$P-labeled helicase substrate (3000 cpm), and T antigen preparation at varying protein concentrations. This reaction is run at 37° for 30 min and terminated by addition of 2 μl of stock stop buffer, followed by heating at 37° for 5 min.

After termination of the dissociation reaction, the reaction mixture is run in a 16-cm × 18-cm 12% nondenaturing polyacrylamide gel (Bio-Rad protein gel), at constant 25 mA with 10–20 V/cm, with water cooling. The gel is dried under vacuum and exposed to Kodak RPXRP-5 film, with an intensifying screen, at −70°, for autoradiography. DNA helicase activity is quantified with a Betascope 603 (Betagen radioimaging instrument). Unreacted duplex substrate and product 17-mer are measured, and the radioactivity is expressed in fmol of product dissociated from the duplex DNA form.

Anthracycline antibiotic stock solutions are made in dimethylsulfoxide at about $10^{-2}$ M, and they are stored at −20°. Dilutions of the stock solutions are made in 10 mM Tris·HCl, pH 7.4, before mixing with the M13–17-mer substrate. The M13–17-mer DNA substrate (3000 cpm; ~1 fmol) is incubated with appropriate concentrations of anthracycline antibiotics or other drugs, in 10 mM Tris·HCl, pH 7.4, before proceeding to the helicase assay. Dimethylsulfoxide was tested up to 0.028 M and did not affect control helicase reactions.

**HeLa cell culture and helicase purification.** HeLa cells are grown in culture and helicase is isolated according to a modified method of Malkas *et al.* (14) by a combination of differential centrifugation The HeLa cell helicase is assayed as described above. Murine FM-3A cell helicase is isolated from FM-3A cells, a murine mammary cancer cell line, by a method identical to the HeLa cell helicase preparation described above.

## Results

The T antigen helicase dissociated the M13–17-mer duplex DNA substrate in a concentration-dependent manner, yielding a reaction rate that was readily quantifiable by measuring the amount of 17-mer that was dissociated from the duplex DNA. For our inhibition studies, we selected reaction conditions and concentrations of helicase and duplex DNA substrate that gave optimal duplex DNA strand dissociation in 30 min (Fig. 2).

We evaluated effects of anthracycline antibiotics on the helicase reaction by preincubating the duplex M13–17-mer DNA substrate with a range of antibiotic concentrations, from $10^{-8}$ M to $10^{-5}$ M, for up to 2 hr and then assaying the T antigen helicase reaction with the DNA-antibiotic complexes. Doxorubicin inhibited the T antigen helicase potently at the $10^{-7}$ M level, with an $IC_{50}$ of $4 \times 10^{-7}$ M (Fig. 3). The binding of doxorubicin to M13–17-mer duplex DNA occurred as quickly as 1 min before the start of the helicase dissociation reaction; this time was as effective as longer preincubation times. Preincubation of the enzyme with doxorubicin had no additional effect on the degree of inhibition.

In our appraisal of other anthracycline antibiotics, we tested a range of concentrations of each antibiotic in the standard duplex DNA dissociation reaction. All the anthracyclines were preincubated with M13–17-mer substrate for at least 10 min before the addition of the T antigen helicase and the start of the dissociation reaction. From these data, we determined an $IC_{50}$ for each compound (Table 1). All of the anthracycline antibiotics significantly inhibited the T antigen helicase activity. The most potent anthracycline tested, nogalamycin, had an $IC_{50}$ of $2 \times 10^{-7}$ M. Very close to this activity were the $IC_{50}$ values of doxorubicin ($4 \times 10^{-7}$ M) and daunorubicin ($4 \times 10^{-7}$ M). Other anthracycline compounds had $IC_{50}$ values as follows: 4-demethoxydaunorubicin, $1.8 \times 10^{-6}$ M; 4′-epidoxorubicin, $2 \times 10^{-6}$ M; aclacinomycin, $4 \times 10^{-6}$ M; and 7-*O*-methylnogarol, $6 \times 10^{-6}$ M.

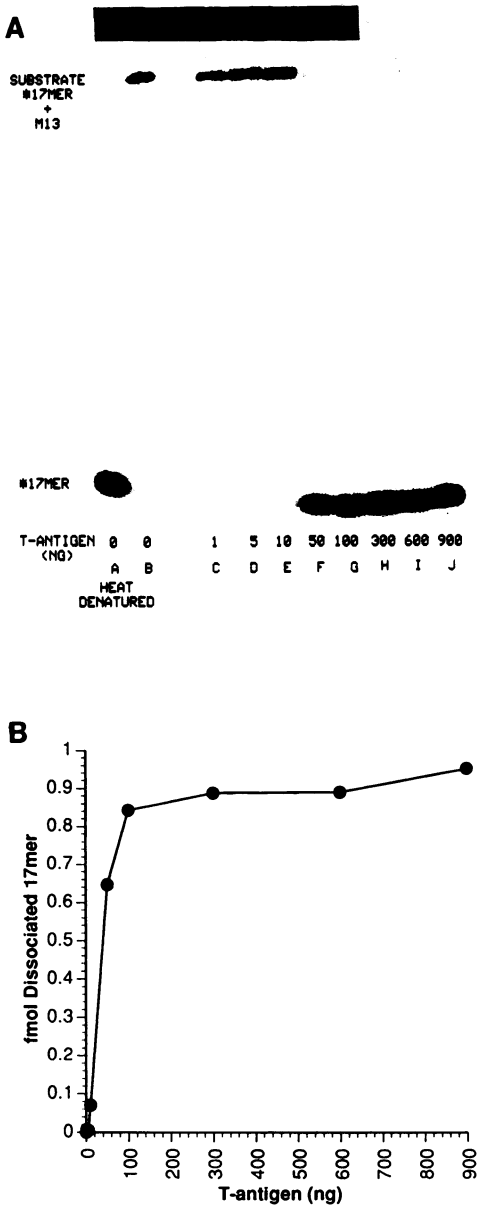


**Fig. 2.** A, Radioimage of T antigen helicase assay separation on polyacrylamide gel electrophoresis. Substrate duplex DNA is at the *top* and dissociated 17-mer is at the *bottom*. *Lane A*, control heat-dissociated duplex DNA; *lane B*, untreated duplex DNA. *Lanes C–J*, helicase reaction with increasing concentrations of SV40 T antigen. The reaction mixtures contain 20 mM Tris·HCl, pH 7.4, 2 mM ATP, 5 mM dithiothreitol, 50 μg of bovine serum albumin, 2 mM $MgCl_2$, and 40–400 μg of T antigen helicase protein. *B*, Quantitation of T antigen helicase reactivity.

As controls, we tested several other compounds that had disparate DNA-binding properties. Ethidium bromide, a potent DNA-intercalating binder, inhibited the helicase with an $IC_{50}$ of $4 \times 10^{-6}$ M. Methotrexate and vindesine, which do not intercalate into DNA, were not inhibitory at $10^{-4}$ M. Unactivated porfiromycin and cyclophosphamide, at $10^{-4}$ M, also did not inhibit the helicase reaction.

Because the SV40 T antigen is an induced viral helicase, we felt it important to evaluate a human helicase for the effects of the anthracycline anticancer drugs. We prepared a helicase from HeLa cells, which dissociated the M13–17-mer duplex DNA in our assay system (Fig. 4A). Under our standard assay

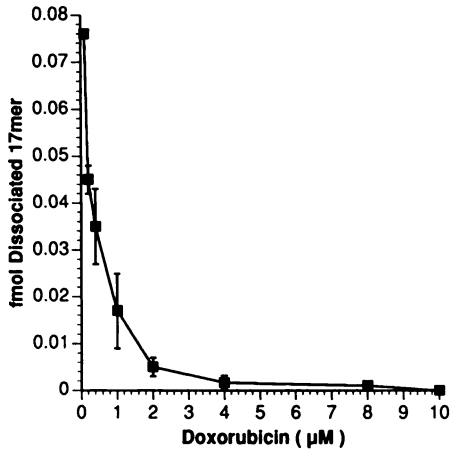


**Fig. 3.** Inhibition of T antigen helicase by doxorubicin. Increasing concentrations of doxorubicin produced this effect on SV40 T antigen helicase activity. This is the mean plot of five experiments, with standard deviations shown.

TABLE 1
**Anthracycline antibiotic effects**

| Compound | Helicase $IC_{50}$[a] | $\Delta T_m$ | Ref. |
|---|---|---|---|
| | μM | | |
| Nogalamycin | 0.2 ± 0.14 | 15°[b] | 15 |
| Daunorubicin | 0.4 ± 0.2 | 13.4° | 16 |
| Doxorubicin | 0.4 ± 0.2 | 14.8° | 16 |
| 4-Demethoxydaunorubicin | 1.8 ± 0.8 | 16.5° | 17 |
| 4′-Epidoxorubicin | 2.0 ± 2.3 | 12.5° | 18 |
| Aclacinomycin | 4.0 ± 2.8 | | |
| 7-*O*-Methylnogarol | 6.0 ± 3.0 | 0° | 15 |

[a] Inhibitory concentration of antibiotic to produce 50% inhibition of SV40 T antigen helicase activity. These values are the average ± standard deviation of three to five experiments.
[b] $\Delta T_m$ for nogalamycin was referenced against a $\Delta T_m$ of 18° for doxorubicin (15).

conditions, doxorubicin is a potent inhibitor of the HeLa cell helicase (Fig. 5). Other anthracycline antibiotics similarly inhibit the HeLa cell DNA helicase (data not shown).

To substantiate the anthracycline antibiotic inhibition of eukaryotic helicases, we purified a murine helicase from FM3A cells, which we assayed in our standard assay. This helicase also displayed concentration-dependent dissociating activity for our M13–17-mer DNA substrate (Fig. 4B). Doxorubicin inhibited the FM3A helicase (Fig. 5), as did other anthracycline antibiotics (data not shown).

Comparing the inhibitory action of doxorubicin for the three different helicases analyzed, we see an indication of different sensitivities of the helicases (Fig. 6). The estimated doxorubicin $IC_{50}$ value for FM3A helicase is $9 \times 10^{-7}$ M; this helicase is 2-fold less sensitive to the drug than is the HeLa cell helicase or the SV40 T antigen helicase, both of which have $IC_{50}$ values of $4 \times 10^{-7}$ M. These differences could be the basis of different sensitivities of cancer cells and normal cells to anthracycline antibiotics.

## Discussion

We find that anthracycline antibiotics potently inhibit viral and eukaryotic DNA helicases at drug concentrations that lie well within the pharmacological concentrations for these drugs. This is not unexpected, because the anthracycline antibiotics bind to DNA and inhibit other DNA-directed enzymatic activities, such as DNA polymerase and topoisomerase II, but we

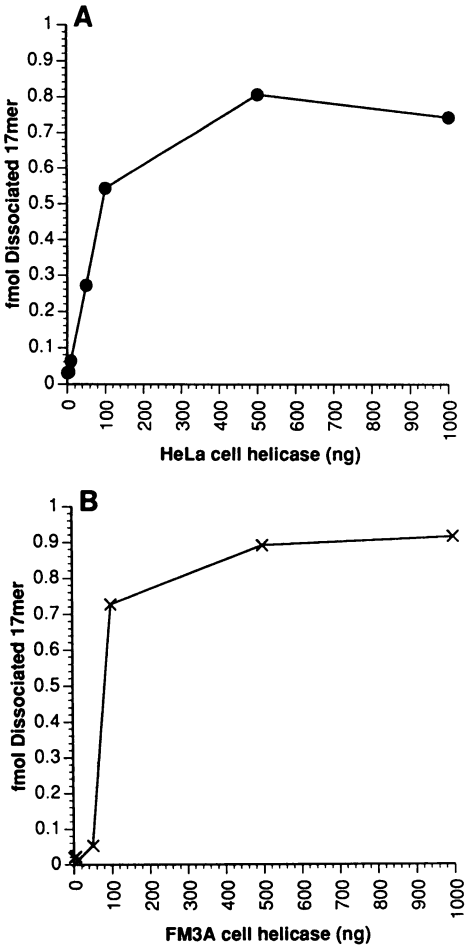


**Fig. 4.** A, HeLa cell P4 helicase activity. The effect of increasing concentrations of partially purified HeLa cell helicase on M13–17-mer DNA duplex. These reaction mixtures are the same as used for the T antigen helicase assay. B, FM3A cell helicase activity. The effect of increasing concentrations of partially purified FM3A cell helicase on M13–17 DNA duplex. These reaction mixtures are the same as used for the T antigen helicase assay.

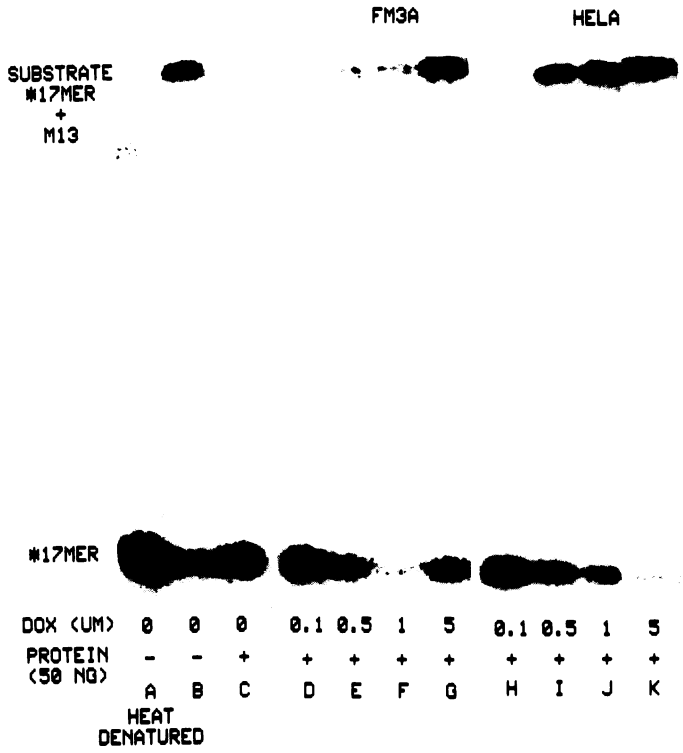


**Fig. 5.** Radiogram of HeLa and FM3A helicase activity and effects of doxorubicin (*DOX*). *Lanes A–C*, controls; *lanes D–G*, FM3A helicase (50 ng) with increasing doxorubicin concentrations; *lanes H–K*, HeLa helicase (50 ng) with increasing doxorubicin concentrations. Substrate duplex DNA is at the *top* and dissociated 17-mer is at the *bottom*.

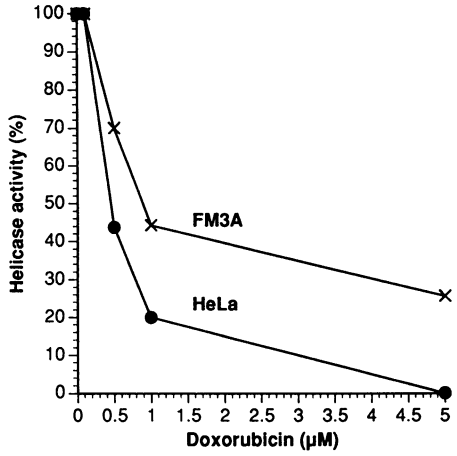


**Fig. 6.** Comparative inhibition of helicases by increasing concentrations of doxorubicin. Partially purified FM3A (100 ng) and HeLa (100 ng) helicases. Helicases were assayed in the standard helicase reaction, with increasing doxorubicin concentrations.

find the level of inhibition for helicase remarkable. Although our original idea was that the increased stability of the duplex DNA caused by intercalative binding of anthracyclines would be sufficient reason for helicase inhibition, our data indicate that other relationships exist. The $\Delta T_m$ values derived from other reports (Table 1) show no clear relationship to the anthracycline antibiotic $IC_{50}$ values we determined against the SV40 helicase.

Anthracycline antibiotics bind to duplex DNA primarily by intercalative binding (11) and modify the duplex DNA. Because the intercalated drug-DNA complex is much more stable and requires more energy to effect its physical dissociation to single strands, this effect alone could account for the inhibition of DNA helicase activity. In addition to increased stability, anthracycline intercalation also increases DNA helix rigidity and deforms, lengthens, and unwinds the DNA helix (11). Inhibition of helicase activity could result from any of these effects or a combination of them. In addition to these effects of intercalating agents on duplex DNA, other factors, such as DNA binding affinity, base sequence specificity, the duplex DNA base to drug ratio, and structural characteristics of the intercalating agents themselves, must be considered. Because the structural differences among the anthracycline analogs are varied and involve modifications and substitutions of the A-, B-, and D-rings and sugar modifications (Fig. 1), we feel that a more thorough examination of the kinetics of this inhibitory action with regard to structure-activity relationships is necessary.

Whereas nogalamycin is the most potent helicase inhibitor we tested, 7(*R*)-*O*-methylnogarol, an analog of nogalamycin, is about 30-fold less potent. This may correspond to the weaker binding of menogaril to duplex DNA, as indicated by the inability of this compound to increase $\Delta T_m$ (15), even though

investigators report evidence of DNA binding (19). Nogalamycin is a dumbell-shaped molecule that intercalates into the DNA helix with the nogalose in the minor groove and the cage-like bicycloamino sugar in the major groove (20–22). Nogalamycin intercalation distorts the DNA helix more than does daunorubicin and doxorubicin and unwinds the DNA helix differently than does daunorubicin-type intercalation (20). These differences may account for the higher helicase-inhibitory activity of nogalamycin, compared with doxorubicin and daunorubicin. However, the loss of inhibitory activity by 7(*R*)-*O*-methylnogarol is associated with the loss of the carboxymethyl group at C10 of the A-ring and the replacement of the nogalose with *R*-conformation *O*-methyl at C7. Whereas the carbonyl oxygen of the carboxymethyl group contributes to the DNA binding, the nogalose sugar is not implicated in the binding to DNA (21), but its steric size and position may directly affect the binding and translocation of the helicase on the duplex DNA. Certainly, the loss of the nogalose may contribute to the decreased inhibitory activity displayed by 7(*R*)-*O*-methylnogarol, through steric effects.

Daunorubicin and doxorubicin are both very potent inhibitors of the helicases, whereas their analogs 4-demethoxydaunorubicin and 4′-epidoxorubicin are 5-fold less active. The structural modifications related to these activity differences are on the C9 side chain, the C4 methoxy, and the 4′-daunosamine hydroxyl. In comparing doxorubicin and 4′-epidoxorubicin, we would not predict that a stereospecific change of the 4′-hydroxyl on the sugar daunosamine would matter, because the sugar lies in the minor groove of duplex DNA and may not bind to the DNA (23), although other evidence suggests that the daunosamine does interact with the DNA in the minor groove (24). The change of helicase-inhibitory potency caused by the loss of the 4-methoxy group in 4-demethoxydaunorubicin does not correlate. This compound produces the greatest increase in $\Delta T_m$ of the anthracyclines tested, yet it is almost 10-fold less inhibitory than nogalamycin and 5-fold less active than doxorubicin and daunorubicin.

A likely determinant for the inhibition by anthracyclines is base sequence specificity for DNA binding. The favored DNA binding sequences for daunorubicin are 5′-AGC (23) (25) or 5′-$\overset{A}{T}$CG or 5′-$\overset{A}{T}$GC (26). In our 17-mer-M13 duplex DNA substrate, one AGC sequence unit occurs. This sequence unit may favor the binding of daunorubicin and similar anthracyclines at very low drug concentrations. However, specific sequence preferences for the other anthracyclines are still controversial, and no relevance of the AGC sequence is apparent in the inhibitory properties of daunorubicin.

Another factor that may affect potency of inhibition is the number of drug molecules intercalated into the M13–17-mer duplex DNA. The reported maximum for daunorubicin and calf thymus DNA is one drug molecule intercalated per 2.6 base pairs (27), with similar values for other anthracyclines. Because anthracyclines intercalate in a 3-base pair region (25, 26), our 17-mer duplex can intercalate five drug molecules at saturation. However, we find significant inhibition at drug concentrations well below saturation. Whether a sequence preference for specific anthracyclines determines the first and best-fit binding of a drug molecule to the duplex, followed by cooperative binding of additional molecules (28), or whether the binding is less structured remains to be determined.

In addition to the fundamental process of drug intercalation, the secondary distortion of the drug and the duplex DNA structure after intercalation (29) can modify the fundamental intercalation process, to yield a highly distorted drug-duplex DNA complex. Finally, structural groups of the intercalating agent that have no apparent involvement in the intercalative binding process protrude into the major and minor grooves of the distorted DNA helix and may interfere directly with the helicase-catalyzed dissociation of duplex DNA.

The SV40 large T antigen binds to DNA in two modes, first, a highly specific binding to the replication origin of the SV40 genome and, second, a nonspecific binding to DNA (30). Because no origin sequence exists in our duplex DNA substrate, our helicase assay model is based on nonspecific binding of the enzyme and subsequent enzymatic translocation catalyzing duplex DNA dissociation. We are currently working to determine whether the anthracyclines interfere with the helicase binding step and/or the helicase translocation and whether the helicase-DNA-anthracycline complex is a reversible or nonreversible ternary complex, as was done with polymerase-DNA-anthracycline (9).

Anthracycline antibiotics show a preferential inhibition of DNA and RNA synthesis (11). The mechanism for this preference could reside with the type of helicase that is processing the DNA. The inhibition of DNA helicases would lead specifically to inhibition of DNA replication. Similarly, inhibition of a helicase involved in transcription would inhibit DNA transcription and the synthesis of new RNA. The SV40 large T antigen used in our analysis is a DNA helicase involved in replication. The anthracyclines that have higher RNA inhibition preference (i.e., nogalamycin and aclacinomycin) show no apparent pattern for DNA helicase inhibition, compared with anthracyclines with equal DNA or RNA inhibition (i.e., daunorubicin, doxorubicin, 4′-epidoxorubicin, and 7-*O*-methylnogarol), or greater DNA-inhibitory characteristics (i.e., 4-demethoxydaunorubicin). The association of preferential inhibition remains to be tested, comparing replication- and transcription-specific helicases.

With our findings concerning helicase inhibition by anthracycline antibiotics, we have documented another important action of these useful anticancer drugs. The helicase process for the dissociation of duplex DNA strands is a necessary step for DNA replication, DNA repair, and transcription. It is possible that the inhibition of helicase activity by the anthracyclines may be their most important pharmacological function against cancer cells.

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