THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 267, No. 15, Issue of May 25, pp. 10683-10689, 1992
Printed in U.S.A.

Inhibition of DNA Helicase II Unwinding and ATPase Activities by DNA-interacting Ligands

KINETICS AND SPECIFICITY*

(Received for publication, October 11, 1991)

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Although DNA helicases play important roles in the processing of DNA, little is known about the effects of DNA-interacting ligands on these helicases. Therefore, the effects of a wide variety of DNA-binding ligands on the unwinding and ATPase reactions catalyzed by *Escherichia coli* DNA helicase II were examined. DNA minor groove binders and simple DNA intercalators did not inhibit helicase II. However, DNA intercalators, such as mitoxantrone and nogalamycin, which position functionalities in the major groove upon binding duplex DNA, were potent inhibitors of helicase II. To determine the mechanism by which mitoxantrone inhibited helicase II, the unwinding and DNA-dependent ATPase activities of helicase II were measured using a spectrum of double- and single-stranded DNA substrates. Using either a 71-base pair (bp) M13mp7 partially duplexed DNA substrate or a 245-bp blunt-ended, fully duplexed DNA substrate, the apparent K_i value for inhibition by mitoxantrone of both the unwinding and ATPase reactions was ~1 μM for both substrates, suggesting that the mechanism of inhibition of helicase II by mitoxantrone is the same for both substrates and requires the presence of double-stranded structure. To strengthen this conclusion, the ability of mitoxantrone to inhibit the DNA-dependent ATPase activity of helicase II was determined using two single-stranded substrates, poly(dT) and the 245-bp substrate after heat denaturation. Using either substrate, mitoxantrone inhibited the ATPase activity of helicase II far less effectively. Thus, these results indicate that the intercalation of mitoxantrone into double-stranded DNA, with accompanying placement of functionalities in the major groove, generates a complex that impedes helicase II, resulting in both inhibition of ATP hydrolysis and unwinding activity. Furthermore, we report here that DNA-binding ligands inhibit the unwinding activity of helicases I and IV and Rep protein from *E. coli*, demonstrating that the inhibition observed for helicase II is not unique to this enzyme.

Ligands that bind DNA have generated great interest in the last 30 years due in part to their antibiotic and antitumor properties (1, 2). These drugs can be characterized by their mechanisms of physical interaction with DNA. DNA intercalators are generally planar polycyclic compounds that bind to double-stranded DNA (dsDNA)[1] by inserting between base pairs, resulting in the partial unwinding of dsDNA around the intercalation site (3–5). Additional structural features of intercalators may include a cationically charged functionality on the chromophore and structures that reside in the major or minor groove of dsDNA. In some cases, functionalities reside in both grooves of DNA. Other antibiotics have been shown to bind to the minor groove of dsDNA. These minor groove binders are highly AT base pair (bp)-specific and require at least 3 consecutive AT bp to bind (6, 7).

Many DNA-binding compounds have been shown to inhibit DNA and RNA metabolism by binding to the nucleic acid and disrupting the enzymatic machinery that interacts with it. For example, daunomycin has been shown to inhibit DNA-directed RNA synthesis by inhibiting *Escherichia coli* RNA polymerase *in vitro* (8). DNA topoisomerases, which are involved in all facets of DNA and RNA metabolism (9–12), can also be inhibited by a number of these DNA-binding ligands. The mechanism by which DNA intercalators inhibit topoisomerases has been intensively studied. Effective intercalators trap the cleaved intermediate in the reaction, generating a ternary complex containing a covalent topoisomerase-DNA complex bound with the intercalator (13). This appears to be a common mechanism of inhibition for type II topoisomerases (14). The precise mechanism by which the generation of the cleavable complex causes cell death is unknown, but it appears to be the primary requirement for cytotoxicity of these compounds (15).

Until now, no work has been performed to consider the effects of these DNA-interacting ligands on the DNA helicases. DNA helicases are DNA-dependent nucleoside 5′-triphosphatases that catalyze the unwinding of dsDNA (16). This important class of enzymes functions in DNA replication, repair, and recombination. In *E. coli*, at least 10 different helicases have been described. Helicases have also been described in bacteriophage, viral, and eukaryotic systems (17). *E. coli* helicase II (*uvrD* gene product) was chosen as the focus of the studies reported here because it is one of the most extensively characterized helicases at present. The enzyme is a DNA-dependent ATPase that translocates unidirectionally on single-stranded DNA (ssDNA) by a processive mechanism (18). The unwinding of dsDNA has been shown to occur in the 3′ to 5′ direction with respect to the strand on which the enzyme initially binds. At low concentrations of enzyme, unwinding occurs by a concentration-dependent mechanism

* This work was supported in part by National Institutes of Health Grant GM 33476 (to S. W. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Recipient of an American Cancer Society faculty research award.

[1] The abbreviations used are: dsDNA, double-stranded DNA; bp, base pair(s); ssDNA, single-stranded DNA; DAPI, 4,6-diamidino-2-phenylindole; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide.

in a reaction that requires a ssDNA-binding site for initial binding of the protein (19). At higher helicase II concentrations, unwinding of duplex DNA can be measured using helicase substrates lacking ssDNA (20). Genetic and biochemical studies have revealed roles for helicase II in UvrABC excision repair and methyl-directed mismatch repair (17, 21). The enzyme may have additional roles in DNA replication, recombination, and transposon excision based on known phenotypes of *uvrD* mutants (17, 21).

Here we report studies designed to determine the effect of various DNA-binding compounds on the helicase and ATPase activities of helicase II. The most potent inhibitors of helicase II were intercalators that position functionalities in the major groove of dsDNA. In addition, an analysis of the effect of several of these drugs on helicases I and IV and Rep protein from *E. coli* demonstrates that the inhibition observed for helicase II is not unique to this enzyme. Furthermore, the spectrum of sensitivities to the compounds is enzyme-specific. The inhibition of helicases by these ligands may be important in explaining some of the properties exhibited by these compounds *in vivo*.

EXPERIMENTAL PROCEDURES

Materials

DNA and Nucleotides—Bacteriophage M13mp7 replicative form I DNA and ssDNA were prepared as described (22). All unlabeled nucleotides were from U. S. Biochemical Corp. [α-^{32}P]dCTP and [γ-^{32}P]ATP were from ICN Radiochemicals or Amersham Corp. Poly(dT) was purchased from P-L Biochemicals. Concentrations of DNA and nucleotides were determined by UV spectrophotometry using published extinction coefficients.

Enzymes—Restriction endonucleases, DNA polymerase I (large fragment), bacteriophage T4 polynucleotide kinase, and bacterial alkaline phosphatase were purchased from New England BioLabs, Inc., or U. S. Biochemical Corp. The reaction conditions used were those suggested by the supplier. Proteinase K was purchased from Boehringer Mannheim. *E. coli* helicase II, Rep protein, and helicases I and IV were purified as previously described (18, 23–25).

DNA-binding Ligands—The compounds used in this study were the generous gift of Glaxo Inc. The compounds were dissolved into dimethyl sulfoxide at 2 mM and stored at 4 °C in the dark. Concentrations were checked by spectrophotometry using published extinction coefficients.

Methods

Helicase Substrate Preparation—The 71-bp M13mp7 partially duplexed helicase substrate was constructed as described (19). The 245-bp blunt-ended helicase substrate was constructed by digesting 33 μg of M13mp7 replicative form I DNA to completion with *Hae*III. After digestion, bacterial alkaline phosphatase was added to remove the 5′-phosphoryl groups. The reaction was terminated by the addition of 100 μg/ml proteinase K and 0.1% sodium dodecyl sulfate and incubated at 37 °C for 30 min. The DNA was subsequently applied to a nondenaturing polyacrylamide gel to resolve the products. The 245-bp DNA fragment was electroeluted, precipitated with isopropyl alcohol, and suspended in polynucleotide kinase buffer. The resuspended DNA was labeled using phage T4 polynucleotide kinase and [γ-^{32}P]ATP as described (26). To ensure that all 5′-ends were phosphorylated, the reaction was chased with 100 μM ATP. After phenol extraction, the [^{32}P]DNA was separated from unincorporated ATP by filtration through an agarose A-5m column (1.5 ml) equilibrated with 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 100 mM NaCl. The void volume containing the labeled DNA was collected and used directly as helicase substrate.

Helicase Assays—The helicase assay measures either the displacement of a labeled DNA fragment from a partial duplex DNA molecule or the denaturation of a fully duplex [^{32}P]DNA fragment by native polyacrylamide gel electrophoresis. The reaction mixture (20 μl) contained 40 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, 5% dimethyl sulfoxide, 10 mM NaCl, 2 or 4 mM rATP, [^{32}P]DNA substrate, and the indicated amount of helicase. The concentration of the 71-bp partial duplex substrate in the reaction mixture was ~2 μM (DNA phosphate). The concentration of the 245-bp substrate in the assay was 188 nM (DNA phosphate). Unless otherwise specified, all DNA-binding compounds were added to the helicase reaction prior to the addition of the enzyme. Reactions were incubated at 37 °C for 10 min and terminated by the addition of 10 μl of 50 mM EDTA, 40% glycerol, 0.5% sodium dodecyl sulfate, 0.1% bromphenol blue, and 0.1% xylene cyanol. The products of the helicase reaction using the 71-bp partial duplex substrate were resolved on an 8% nondenaturing polyacrylamide gel. Electrophoresis was at 5–15 V/cm for 14 h. The products of the helicase reaction using the 245-bp fully duplex helicase substrate were separated on a 5% nondenaturing gel containing 0.1% sodium dodecyl sulfate. Electrophoresis was at 9 V/cm for 14 h. Polyacrylamide gels were analyzed by film autoradiography or by slicing the gel into 1-cm sections and counting in a liquid scintillation counter.

ATPase Assays—The hydrolysis of ATP catalyzed by helicase II was assayed by measuring the formation of [^{3}H]ADP from [^{3}H]ATP as described (27). The reaction conditions were identical to those described for the helicase reaction, except that [^{3}H]ATP (3.0 cpm/pmol) was substituted for ATP at a concentration of 4 mM.

RESULTS

Inhibition of Helicase II by DNA-binding Compounds—The effect of various DNA-binding compounds on the helicase reaction catalyzed by *E. coli* DNA helicase II was measured using the 71-bp partial duplex substrate (Table I). Initially, each compound was assayed for inhibitory activity at final concentrations of 1, 10, and 100 μM in an effort to obtain an approximate K_i. The minor groove binders H33258, distamycin, and Netropsin did not inhibit helicase II at concentrations up to 100 μM. DAPI and Berenil were the only minor groove-binding drugs used in this study that were active at 10 and 100 μM, respectively. Novobiocin, camptothecin, and VM-26, all effective topoisomerase inhibitors, failed to inhibit helicase II at all the concentrations tested.

By far the most interesting ligands tested were the DNA intercalators. Amiloride, genistein, acridine, actinomycin D, and *m*-AMSA were not effective inhibitors, displaying appar-

TABLE I

Effects of DNA-binding compounds on the unwinding activity of helicase II

Helicase reactions were as described under "Experimental Procedures" using 40 ng of helicase II. Preincubations of compounds with the helicase substrate were carried out for 5 min at room temperature prior to addition of helicase II. In control reactions, helicase II catalyzed the displacement of ~50% of the 71-nucleotide fragment. The data represent the average of at least three independent determinations.

Compound	Apparent K_i
	μM
Intercalators	
Nogalamycin	<1
Mitoxantrone	~1
Daunomycin	~4
Ellipticine	~10
Ethidium	~10
Actinomycin D	>100
Acridine	>100
Amiloride	>100
m-AMSA	>100
Genistein	>100
Minor groove binders	
DAPI	>10
Berenil	~100
H33258	>100
Distamycin	>100
Netropsin	>100
Others	
VM-26	>100
Camptothecin	>100
Novobiocin	>100

ent K_i values >100 μM. Ellipticine and ethidium inhibited helicase II with an apparent K_i of ~10 μM, whereas daunomycin inhibited helicase II with an apparent K_i of ~4 μM. Of note, however, mitoxantrone and nogalamycin inhibited the unwinding activity of helicase II with an apparent K_i of 1 μM or less.

Mechanism of Inhibition—In an effort to determine the mechanism by which mitoxantrone inhibited the helicase II unwinding reaction, the effects of mitoxantrone on both the unwinding and ATPase activities of helicase II were measured simultaneously using the 71-bp partial duplex substrate (Fig. 1). The apparent K_i value for inhibition of both the unwinding reaction and the ATPase activity of helicase II by mitoxantrone was 0.9 μM.

Inhibition of both the ATPase and unwinding activities of helicase II by mitoxantrone using the 71-bp partial duplex substrate may be explained by one of several mechanisms. First, mitoxantrone may inhibit both activities by binding to ssDNA and disrupting the unidirectional translocation of helicase II along ssDNA. This would prevent helicase II from reaching the duplex region and thus would inhibit the unwinding reaction indirectly. Second, mitoxantrone may inhibit helicase II by intercalating into the duplex DNA present on the substrate. Duplex DNA in this case includes the 71-bp region used to measure unwinding activity and duplex regions of DNA present as hairpins in M13mp7 ssDNA (30). Evidence for binding of mitoxantrone to both ssDNA and dsDNA has been well documented (28, 29). Third, mitoxantrone may bind directly to helicase II and inhibit both activities by preventing an interaction between helicase II and the DNA substrate.

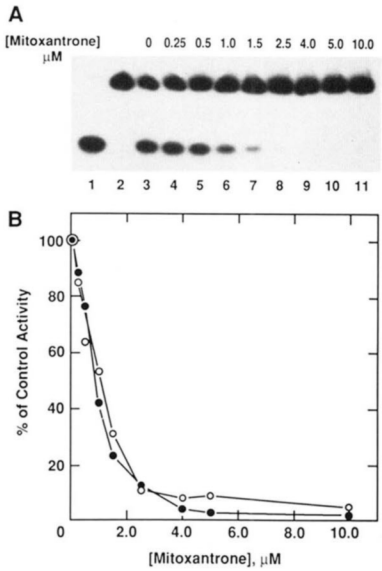

FIG. 1. **Effect of mitoxantrone on helicase and ATPase activities of helicase II utilizing 71-bp partial duplex DNA as substrate.** The 71-bp partial duplex substrate was preincubated with increasing concentrations of mitoxantrone for 5 min at room temperature in helicase II reaction mixtures containing 4 mM [^{3}H]rATP. Helicase II (32 ng) was added, and the reactions were incubated at 37 °C for 10 min. Reaction mixtures (20 μl) were analyzed for the production of [^{3}H]rADP (5 μl) and the displacement of the 71-bp DNA fragment (15 μl). *A*, typical autoradiograph of a helicase assay. *Lane 1*, heat-denatured control; *lane 2*, no enzyme control; *lanes 3–11*, mitoxantrone concentrations as indicated. *B*, data for both helicase (●) and ATPase (○) activities shown as percent control activity as a function of the micromolar concentration of mitoxantrone. In control reactions, helicase II catalyzed the displacement of ~50% of the 71-nucleotide fragment and hydrolyzed ~5.2 nmol of ATP (out of 80 nmol present in the reaction). All data points are the average of at least three independent determinations.

To determine whether mitoxantrone inhibits the interaction between helicase II and ssDNA, the effect of mitoxantrone on the DNA-dependent ATPase reaction was tested using either poly(dT) or M13mp7 ssDNA (without the 71-bp annealed fragment) as effectors of the ATPase reaction. Poly(dT) DNA lacks the secondary structure that exists in the M13mp7 ssDNA, making intercalation into duplex regions unlikely. As shown in Fig. 2, mitoxantrone did not effectively inhibit the ATPase activity of helicase II at concentrations up to 2.5 μM when poly(dT) DNA was the ssDNA effector. (The inhibition of ATPase activity between 2.5 and 4.0 μM mitoxantrone may result from a condensation of poly(dT) DNA caused by high concentrations of mitoxantrone as described by Kapuscinski and Darzynkiewicz (28) (see "Discussion").) In contrast, the results obtained using M13 ssDNA without the 71-bp annealed fragment compared favorably with those obtained with the 71-bp helicase substrate. The apparent K_i for inhibition of this ATPase reaction was 2.0 μM. Thus, the apparent K_i for mitoxantrone as an inhibitor of the ATPase reaction using M13 ssDNA was close to that observed for the 71-bp partial duplex helicase substrate. These results strongly suggest that mitoxantrone, when present at low concentrations, does not inhibit the ATPase reaction catalyzed by helicase II when ssDNA is provided as the effector of the reaction. These results also suggest that mitoxantrone does not inhibit the ATPase activity by binding directly to helicase II since there was little effect on ATP hydrolysis, at low mitoxantrone concentrations, when poly(dT) was used as the DNA effector.

In light of the results presented above, it was necessary to determine what effect mitoxantrone had on the helicase reaction when the drug was preincubated with helicase II. Helicase II was preincubated with 2 μM mitoxantrone under normal reaction conditions in the presence or absence of ATP. After preincubation, the drug/helicase II mixture was diluted to a final concentration of 0.05 or 0.1 μM mitoxantrone, and helicase activity was measured. Controls were treated the same way without mitoxantrone present. The results of this study are presented in Table II. There was little or no effect of preincubating helicase II with mitoxantrone. The results

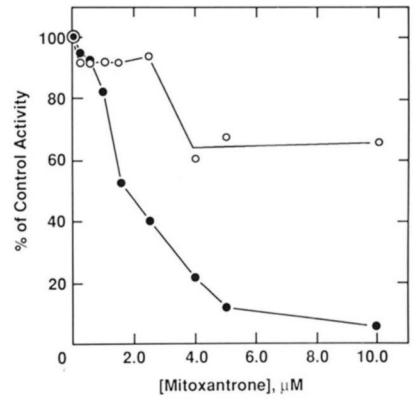

FIG. 2. **Mitoxantrone inhibits ATPase activity of helicase II on M13 ssDNA, but not on poly(dT) DNA.** Either M13 ssDNA or poly(dT) ssDNA (2 μM nucleotide) was preincubated with increasing concentrations of mitoxantrone at room temperature for 5 min in helicase II reaction mixtures containing 4 mM [^{3}H]rATP. Helicase II (32 ng) was added, and the reactions were incubated for 10 min at 37 °C. Reaction mixtures (20 μl) were analyzed by measuring the production of [^{3}H]rADP in a 5-μl aliquot. Data for M13mp7 ssDNA (●) and poly(dT) ssDNA (○) are shown as percent control activity as a function of the micromolar concentration of mitoxantrone. In control reactions, helicase II catalyzed the hydrolysis of ~28 nmol of ATP (out of 80 nmol present in the reaction). All data points represent the average of at least three independent determinations.

TABLE II

Preincubations of helicase II and mitoxantrone

Preincubation mixtures (10 μl) containing helicase II (320 μg) and 2 μM mitoxantrone were incubated in helicase II reaction mixtures with or without 4 mM rATP for 5 min at room temperature. After preincubation, the mixture was diluted to reduce the mitoxantrone concentration. Helicase activity was measured using the 71-bp partial duplex DNA substrate. Controls were treated in the same way without the addition of mitoxantrone. The final concentrations of mitoxantrone in the helicase reaction containing 16 and 32 ng of helicase II were 0.05 and 0.1 μM, respectively. Helicase reactions (20 μl) were performed as described under "Experimental Procedures." In control reactions, helicase II catalyzed the displacement of 35% (16 ng) and 55% (32 ng) of the 71-nucleotide fragment.

	Displacement
	% control activity
No rATP	
16 ng helicase II	93
32 ng helicase II	100
4 mM rATP	
16 ng helicase II	84
32 ng helicase II	98

from the preincubation performed in the presence of rATP were virtually identical to those without rATP. These results support the hypothesis that mitoxantrone does not interact directly with helicase II, but interacts with the DNA to inhibit helicase II. These results, together with those presented in Fig. 2, suggest that helicase II is inhibited when mitoxantrone intercalates into dsDNA.

To directly test the effect of mitoxantrone on the unwinding reaction in the absence of any ssDNA, we took advantage of the fact that helicase II will unwind blunt-ended, fully duplexed DNA fragments (20). Unwinding of a 245-bp fully duplex DNA fragment was effectively inhibited by mitoxantrone with an apparent K_i of 0.9 μM (Fig. 3). This K_i is identical to the apparent K_i measured using the 71-bp partial duplex substrate. These results clearly suggest that the inhibition observed for both substrates is likely accomplished by the same mechanism of inhibition. Additionally, the effect of mitoxantrone on the ATPase reaction catalyzed by helicase II was measured using the 245-bp blunt-ended fragment (Fig. 3). The apparent K_i for mitoxantrone in this reaction was 1.4 μM. Again, this K_i is almost identical to the apparent K_i measured using the 71-bp partial duplex substrate. If, however, the 245-bp DNA substrate was denatured prior to the addition of mitoxantrone and helicase II, mitoxantrone inhibited the ATPase reaction less well, with an apparent K_i of 6 μM. These data suggest that the mechanism of inhibition of helicase II is through the intercalation of mitoxantrone into the dsDNA substrate. The decrease in unwinding activity is the direct result of the inability of helicase II to move through duplex regions when mitoxantrone is bound. Most of the decrease in ATPase activity observed when mitoxantrone is complexed with the native substrate occurs for the following reasons. First, the hydrolysis of ATP is required to unwind the duplex DNA. Second, the ssDNA generated as a result of the unwinding reaction can serve as an effector DNA for further hydrolysis of ATP by helicase II.

Inhibition of Other DNA Helicases by DNA-binding Compounds—To determine if the inhibition of helicase II by DNA intercalators was unique to this enzyme, the effect of mitoxantrone, nogalamycin, *m*-AMSA, and H33258 on the helicase activity of *E. coli* Rep protein and helicases I and IV was determined utilizing the 71-bp partial duplex helicase substrate (Table III). These four drugs were selected to represent both potent inhibitors (mitoxantrone and nogalamycin) and inhibitors that showed no effect on helicase II at concentrations up to 10 μM (*m*-AMSA and H33258). Nogalamycin inhibited all three helicases dramatically. Helicase IV was inhibited completely at 1 μM nogalamycin, whereas Rep protein and helicase I were inhibited to <50% of control activity at 1 μM and 10 μM nogalamycin, respectively. Mitoxantrone inhibited all three helicases with apparent K_i values of between 1 and 10 μM. In contrast, *m*-AMSA did not inhibit Rep protein or helicase I or IV at 10 μM. Likewise, H33258 did not

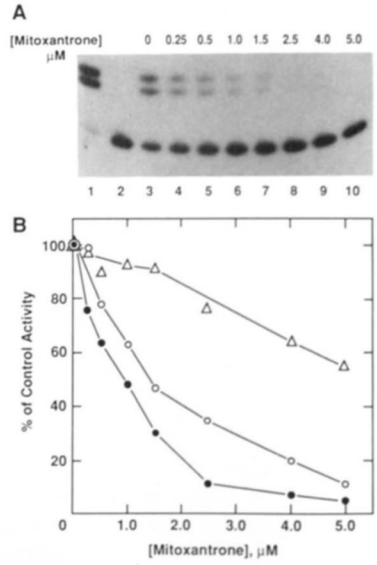

FIG. 3. **Mitoxantrone inhibits helicase II when 245-bp blunt-ended duplex DNA is utilized as substrate.** The 245-bp blunt-ended duplex DNA was preincubated with increasing concentrations of mitoxantrone in helicase II reaction mixtures containing 4 mM [^{3}H]rATP for 5 min at room temperature. Helicase II (160 ng) was added, and the reactions were incubated for 10 min at 37 °C. Reaction mixtures (20 μl) were analyzed for the production of [^{3}H] rADP using a 5-μl aliquot and unwinding of the duplex DNA using a 15-μl aliquot. *A*, typical autoradiograph of a helicase assay. *Lane 1*, heat-denatured control; *lane 2*, no enzyme control; *lanes 3–10*, mitoxantrone concentrations as indicated. *B*, data for both helicase (●) and ATPase (○) activities shown as percent control activity as a function of the micromolar concentration of mitoxantrone. In a separate reaction, the 245-bp DNA substrate was heat-denatured at 95 °C for 5 min prior to preincubation with mitoxantrone. ATPase reactions were carried out as described above. Data for ATPase activity (△) utilizing the heat-denatured substrate are presented as described above. In control reactions, helicase II catalyzed the unwinding of ~50% of the 245-bp substrate and hydrolyzed ~12 nmol of ATP (out of 80 nmol present in the reaction). All data points represent the average of at least three independent determinations.

TABLE III

Inhibition of Rep protein and helicases I and IV by DNA-binding ligands

Helicase reactions were as described under "Experimental Procedures" using helicase I (14 ng), Rep protein (75 ng), or helicase IV (4 ng) plus 2 mM rATP and the 71-bp partial duplex helicase substrate. Comparisons were made to zero ligand controls, which catalyzed the displacement of ~50% of the 71-nucleotide fragment. Preincubations of DNA-binding compounds with the helicase substrate were for 5 min at room temperature. Reactions were initiated with helicase and incubated at 37 °C for 10 min. The data represent the average of at least three independent determinations.

Compound	Apparent K_i		
	Helicase I	Rep protein	Helicase IV
		μM	
Nogalamycin	<10	<1	<1
Mitoxantrone	<10	<10	<10
m-AMSA	>10	>10	>10
H33258	>10	>10	<1

inhibit helicase I or Rep protein significantly at 10 μM. However, helicase IV was inhibited by H33258 at 1 μM to <50% of control activity. These data suggest that each enzyme has its own spectrum of sensitivities to DNA-binding ligands and that the inhibition of helicase II by these compounds is not unique.

DISCUSSION

The duplex DNA unwinding reaction and the associated ATPase reaction catalyzed by helicase II can be inhibited by several ligands that bind DNA. This inhibition has been shown to be highly specific with respect to the drug used. The topoisomerase inhibitors novobiocin, VM-26, and camptothecin, which do not bind DNA directly, showed no effect on the helicase reaction. In addition, drugs that bind to the minor groove in duplex DNA such as H33258, distamycin, Netropsin, DAPI, and Berenil also fail to inhibit the helicase II unwinding reaction at low concentrations. These ligands require 3 or more consecutive AT bp for binding (5, 31, 32, 34, 35), and there are four such sites in the 71-bp partial duplex substrate (36). Thus, failure of these drugs to bind the substrate is not a likely explanation for their inability to inhibit the unwinding reaction. It is interesting to note that DAPI, which falls into this broad class of drug, does inhibit the unwinding reaction slightly at a concentration of 10 μM. Since DAPI has been shown to intercalate at GC or mixed sequences (6), the inhibition of helicase II caused by DAPI may be due to intercalation of the compound into the DNA substrate. In general, ligands that bound dsDNA by intercalation were the most effective inhibitors of the helicase II unwinding reaction. These DNA-binding compounds varied both in their inhibition of the helicase II unwinding reaction and in their structure, as discussed below. The two most potent inhibitors of the unwinding reaction were nogalamycin and mitoxantrone.

Analysis of the effect of mitoxantrone on both the unwinding reaction and the ATPase reaction catalyzed by helicase II, using various DNA substrates, suggests that the mechanism by which the drug inhibits the reaction is through intercalation into the duplex DNA substrate. This presumably provides a physical block to continued translocation by the helicase, causing the unwinding reaction to be inhibited. Initial experiments demonstrated inhibition of both the DNA-dependent ATPase reaction and the helicase reaction on the 71-bp partial duplex substrate. The apparent K_i values for inhibition of both these reactions were essentially identical. The substrate contains ~7000 nucleotides of ssDNA in addition to the 71-bp duplex region. Inhibition of both the ATPase and helicase activities to the same extent at all drug concentrations tested suggested three possibilities. First, mitoxantrone could inhibit helicase II by binding directly to the enzyme and preventing its association with the DNA substrate. Second, mitoxantrone could bind to ssDNA and inhibit helicase II by preventing ATP hydrolysis-fueled translocation to the duplex region. Third, intercalation of mitoxantrone into dsDNA regions on the DNA substrate could disrupt both translocation to the duplex region and unwinding of the duplex region. With regard to this possibility, it is important to note that M13 ssDNA contains several regions capable of forming hairpin structures (30). Mitoxantrone can presumably intercalate into these regions of dsDNA.

To distinguish among these possibilities, the effect of mitoxantrone on the DNA-dependent ATPase activity of helicase II was measured using two different DNA effectors of the ATPase reaction: either M13 ssDNA, which contains secondary structure, or poly(dT), which lacks secondary structure. Mitoxantrone effectively inhibited the ATPase reaction using M13 ssDNA with an apparent K_i of 2 μM. In contrast, the drug had little effect on the ATPase reaction when poly(dT) was the DNA effector at mitoxantrone concentrations <2.5 μM. However, as the mitoxantrone concentration was increased to 4 μM, there was a decrease in the ATP hydrolysis reaction rate to ~60% of control activity. This inhibition of activity was most likely due to condensation of poly(dT) by mitoxantrone, which has been reported to occur at a concentration of ~2–3 μM (28). Nevertheless, the ATPase activity was not completely inhibited by mitoxantrone, perhaps due to the presence of short DNA fragments that are not removed in the condensation reaction. Inhibition of the ATPase reaction in the presence of M13 ssDNA was not a result of condensation of the DNA by mitoxantrone since the critical concentration of drug required for condensation of this DNA is ~6.6 μM, which is well above the K_i for inhibition. This suggests that the predominant mechanism of inhibition at low drug concentrations is through intercalation into the secondary structure present in M13 ssDNA. It is likely that helicase II either dissociates from the DNA molecule or becomes trapped within duplex regions as it encounters drug intercalated in dsDNA.

It is also unlikely that mitoxantrone binds directly to helicase II to inhibit the activities of the enzyme. This is evidenced by the fact that inhibition of the ATPase reaction at low drug concentrations is DNA structure-dependent. In addition, control experiments in which helicase II was incubated with inhibitory concentrations of mitoxantrone prior to dilution into an unwinding reaction demonstrated no inhibition of the enzyme's activities. Thus, we conclude that mitoxantrone and presumably other DNA intercalators inhibit the unwinding and ATPase reactions catalyzed by helicase II through the generation of a mitoxantrone-dsDNA complex that impedes movement of the enzyme.

To support the conclusion that specific DNA intercalators inhibit helicase II by intercalation into dsDNA regions, several experiments were performed using a 245-bp blunt-ended duplex DNA substrate. These experiments take advantage of the fact that helicase II is able to unwind a fully duplex DNA molecule by initiation of the unwinding reaction at the ends of the substrate (20). This substrate provides the added benefit of having no ssDNA to which the drug can bind by some other type of interaction. As expected, mitoxantrone inhibited both the unwinding reaction and the ATPase reaction catalyzed by helicase II when measured using the 245-bp duplex substrate. The apparent K_i for inhibition of the unwinding reaction was 0.9 μM, in excellent agreement with that observed using the 71-bp partial duplex substrate. These results suggest that the mechanism of inhibition was the same in each case, despite the considerable differences in the two substrates. The apparent K_i for inhibition of the ATPase reaction was 1.4 μM. When the duplex substrate was denatured prior to the addition of helicase II, the apparent K_i for inhibition was 6.0 μM mitoxantrone. Thus, the effect of mitoxantrone on the ATPase reaction was markedly reduced when the DNA effector was ssDNA. These results further support the conclusion that the inhibition of the helicase reaction is the result of an interaction between dsDNA and mitoxantrone. Moreover, the apparent K_i for inhibition of the helicase reaction is far below the critical concentration of mitoxantrone required for condensation of dsDNA (14.7 μM for calf thymus DNA) (28). Thus, it is apparent that the inhibition of helicase activity is due to intercalation of the drug into the duplex DNA substrate and that the inhibition of ATP hydrolysis reflects the coupling of ATP hydrolysis to unwinding of duplex regions. We conclude that intercalation of mitoxantrone into dsDNA gener-

ates a complex that impedes the progress of helicase II, resulting in either dissociation of helicase II from the substrate or physical trapping of the enzyme on the DNA. We cannot distinguish between these two possibilities at this time.

The DNA intercalators used in this study varied both in structure and efficiency of inhibition of helicase II. To begin defining properties of the intercalators that are important for the inhibition of helicase II, we examined the relationship of unwinding angle and DNA binding affinity to inhibitory activity (Table IV). Neither parameter correlates with inhibition of helicase II activity. For example, nogalamycin and mitoxantrone, the two most potent helicase II inhibitors, have unwinding angles of 18° and 23°, respectively. *m*-AMSA, which does not inhibit helicase II, has a measured unwinding angle of 21°.

Acridine, genistein, and amiloride are simple DNA intercalators without functional groups positioned in either groove of dsDNA. None of these compounds inhibited helicase II, suggesting that simple intercalation is not sufficient for inhibition. Both *m*-AMSA and actinomycin D also failed to inhibit helicase II. Structural studies of actinomycin D-DNA complexes (38) demonstrate selective binding to GC sites, with the two large cyclic peptides positioned in the minor groove of dsDNA (39). *m*-AMSA intercalates into DNA, with its acridine chromophore and the noncoplanar 9-anilino ring positioned in the minor groove (40). Since neither compound inhibited helicase II, intercalation coupled with a functionality placed in the minor groove may also not be adequate for inhibition. The results obtained with ethidium and ellipticine are more difficult to interpret. The ethidium-DNA complex has been well studied by x-ray diffraction techniques (41, 42). These studies suggest that the drug's ethyl and phenyl functionalities are positioned in the minor groove of dsDNA. The weak inhibition observed with ethidium may result from electrostatic binding to the phosphodiester backbone of ssDNA (43). The significance of the ellipticine inhibition cannot be evaluated until additional work is carried out to define the ellipticine-DNA complex.

Daunomycin, nogalamycin, and mitoxantrone (Fig. 4) were the most potent inhibitors of the helicase II unwinding reaction; and they were the only intercalators tested that position functionalities in the major groove when bound to dsDNA. The daunomycin-DNA complex has been well described (45, 46); ring D of the aglycon chromophore resides in the major groove when this ligand intercalates into dsDNA. Nogalamycin, an anthracycline antibiotic, intercalates into dsDNA and places the nogalose sugar in the minor groove; the charged dimethylamino group and the hydroxyl functionalities of the bicyclic sugar are positioned in the major groove (47–49). High-field ^{1}NMR studies of the mitoxantrone-DNA interaction suggest that the intercalation chromophore is positioned perpendicular to the base pairs, with the two side chains residing in the major groove (50). Thus, nogalamycin and mitoxantrone, the most potent inhibitors, position a positively charged amino group and two hydroxyl groups in the major groove.

TABLE IV

Unwinding angles and binding constants for DNA intercalation

Intercalator	Unwinding angle	K_d	Ref.
		μM	
Amiloride	5.9°		51
Ethidium	26°	1	2, 43
Actinomycin D	26°	0.2	2
m-AMSA	21°	1, 10	2, 33, 37, 40
Ellipticine	17° or 9°	6.6	2, 44
Daunomycin	11°	0.4	2, 46
Nogalamycin	18°	2.0	2, 48
Mitoxantrone	26.5°, 17.5°, 23°	0.2	29, 50, 53, 54

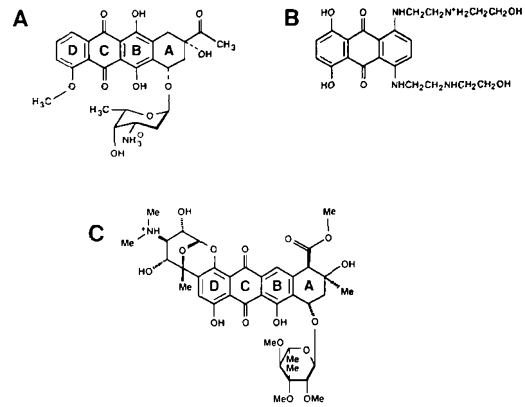

FIG. 4. **Chemical structures of mitoxantrone (*A*), nogalamycin (*B*), and daunamycin (*C*).**

Taken together, the data suggest the possibility that inhibitory intercalators must place a functionality in the major groove when bound to DNA or that the binding of a ligand in the major groove, in the absence of intercalation, may be sufficient for inhibition of helicase II. The data do not discriminate between these two possibilities. Although there are no small DNA-binding ligands that interact solely with the major groove of DNA, construction of DNA triplex substrates may provide further insight into this problem. Until further analysis is carried out, the exact requirements for inhibition of helicase activity must be considered speculative.

The ligands that were effective inhibitors of DNA helicase II exhibit both antitumor and mutagenic properties (for reviews, see Refs. 1 and 2). In addition, it has been demonstrated that inhibition of helicase activity by DNA-binding compounds is not restricted to helicase II. Three additional helicases, Rep protein and helicases I and IV, were also inhibited by a spectrum of the compounds tested in this study (Table III). In fact, a range of sensitivities was obtained that depended on both the compound and the helicase tested. These results may be important for understanding both the mechanism by which duplex DNA is unwound by a helicase and the mechanism by which these compounds inhibit cellular function. Thus, it may be possible that some of the cytotoxic properties exhibited by these drugs are due to an inhibition of specific helicase reactions in the cell, as helicases are known to be involved in all aspects of DNA metabolism. For example, although *m*-AMSA and mitoxantrone show a similar capacity to induce cleavable complexes in cellular DNA, mitoxantrone is far more cytotoxic than *m*-AMSA to human cells (52). Additional biochemical and genetic analysis will be required to test these ideas.

Acknowledgments—We thank Dr. Michael Corey for critical reading of this manuscript and Susan Whitfield for preparation of the artwork.

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