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# Inhibition of DNA Helicase, ATPase and DNA-Binding Activities of *E. coli* RecQ Helicase by Chemotherapeutic Agents

**Bo Zhang[1], Ai-hua Zhang[1], Lei Chen[2] and Xu Guang Xi[1,2,*]**

*[1]CNRS, UMR 2027, Institut Curie – Section de Recherche, Centre Universitaire, Bâtiment 110, F-91405 Orsay, France; and [2]Sino-French Research Center for Life Sciences and Genomics Rui Jin Hospital, School of Medicine, Jiao Tong University, 197 Rui Jin Er Road, Shanghai, 200025, China*

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**RecQ helicases play an essential role in maintaining genetic integrity in all organisms from *Escherichia coli* to humans. Defects to these enzymes are responsible for three distinct human diseases: Werner syndrome, Bloom syndrome and Rothmund–Thomson syndrome. All three diseases are characterized by a predisposition to cancer due to increased genomic instability. Previous studies on the effects of non-covalent DNA modifications on the catalytic activity of purified Werner and Bloom DNA helicases have shown that both enzymes have similar sensitivity profiles to these DNA-binding agents and are most strongly inhibited by the minor groove binder distamycin A. In this study, we show that the sensitivity profiles of *E. coli* RecQ to a number of DNA-binding ligands are different to those observed for WRN and Bloom helicases. These observations may give insights into the differences in molecular mechanisms underlying efficient motor function of RecQ helicases.**

**Key words: helicase, human diseases, DNA modification, minor groove binder, molecular motor.**

Abbreviations: dsDNA, double stranded DNA; SSB, single-stranded DNA binding protein; hRPA, human replication protein A.

Helicases are ubiquitous enzymes that are involved in most aspects of various nucleic acid metabolic pathways, such as DNA replication, DNA repair, recombination, transcription, RNA processing and protein translation (*1*). Abnormalities in three members of the RecQ helicase family have been shown to cause three different human diseases that are associated with inherent genomic instability: Bloom syndrome, Werner syndrome and Rothmund–Thomson syndrome (*2*). All RecQ helicases have seven highly conserved motifs that contain an ATP-binding sequence (Walker A and B box) and the DEXH box, which is characteristic of the RecQ helicase family. Also, *Escherichia coli* and Bloom RecQ helicases have a zinc finger motif that is highly conserved structurally and functionally. This motif plays an essential role in DNA binding and DNA substrate discrimination (*3*, *4*). In living cells, helicases co-ordinately interact with others proteins to perform their different functions. It has been reported that *E. coli* RecQ helicase unwinding activity is greatly enhanced by the single-stranded DNA-binding protein (SSB) (*5*, *6*). Recently, *E. coli* RecQ helicase has been shown to physically and functionally interact with SSB (*7*). Although the mechanism of helicase-mediated unwinding is still poorly understood, the biochemical properties of numerous helicases from various organisms are well known. Helicases are molecular motors that convert the chemical energy derived from nucleoside triphosphate hydrolysis to the mechanical energy to unwind double-stranded (ds) DNA and to translocate along DNA. Recent studies have revealed that the roles of helicases extend beyond simply separating double stranded DNA (dsDNA). Several helicases have been shown to dislodge proteins bound to DNA and streptavidin bound to biotinylated oligonucleotides (*8*, *9*). The *E. coli* RecQ helicase is 609-amino acids long, whereas most eukaryotic RecQ helicases are more than 1 000-amino acids long, with long N-terminal and C-terminal regions on either side of the helicase domain. These structural differences may confer additional functions to eukaryotic RecQ helicases. Indeed, the N-terminal sequence of the WRN protein, which has been shown to be similar to the N-terminal sequence of DNase D, possesses an intrinsic exonuclease activity (*10*, *11*). We wondered whether the structural differences between helicases from prokaryotes and eukaryotes might influence their reactivity with chemotherapeutic drugs used as antibiotic and anti-tumour agents. Previous studies have shown that both Werner and Bloom helicases are potently inhibited by drugs that bind the DNA minor groove (*12*). Here, we report the effect of various DNA-binding chemotherapeutic agents on the DNA binding, ATPase activity and helicase activity of *E. coli* RecQ helicase. Our results show that the sensitivity profiles of this enzyme to some DNA-binding ligands are different to those observed for WRN and Bloom helicases. These observations may give insights into the different molecular mechanisms underlying efficient motor function of RecQ helicase family members.

*To whom correspondence should be addressed. Tel: +33-1-69-86-31-81, Fax: +33-1-69-86-94-29,
E-mail: xu-guang.xi@curie.u-psud.fr

 

## MATERIALS AND METHODS

*Oligonucleotides*—PAGE-purified, unlabelled and fluoresein-labelled synthetic oligonucleotides were purchased from Proligo (France). As some of the compounds used in this study preferentially bind to DNA duplex tracts containing a 4–5 A-T tract, we used the following DNA sequences: Oligo A:5′-ATGCTGATGCAAATCCA ATCGCAAGACA$(T)_{20}$-3′ and Oligo B: 5′-TGTCTTGC GATTGGATTTGCATCAGCAT-Fluorescein-3′. Double-stranded oligonucleotides were prepared in a 20 mM Tris–HCl buffer, pH 7.2, containing 100 mM NaCl. The mixture was heated to 85°C for 5 min and annealed by slow cooling to room temperature.

*Preparation of DNA-Binding Compounds*—All chemicals were obtained from Sigma and were prepared as previously described (*12*). The concentrations of the DNA-binding compounds were determined spectrophotometrically according to published extinction coefficients (*12*).

*Enzyme*—His-6-tagged *E. coli* RecQ helicase was expressed from the pET-15b plasmid in *E. coli* strain BL21 (DE3) and purified as previously described (*13*). Briefly, the over-expressed protein was purified under native conditions using chromatography on $Ni^{2+}$-nitrilotriacetic acid columns (Qiagen, Valencia, CA, USA), followed by FPLC size exclusion chromatography (Superdex 200, Pharmacia) and ion-exchange chromatography (DEAE Sephadex A-50). The purity of the RecQ preparation was determined as >95% based on Sypro Orange-stained SDS–PAGE and electrospray mass spectrometry analyses.

*Helicase Assay*—We carried out an unwinding assay using a Beacon 2000 polarization instrument, according to Xu *et al*. (*14*). An appropriate quantity of fluorescein-labelled duplex oligonucleotide (1 nM) was added to the helicase unwinding buffer containing 25 mM Tris–HCl (pH 8.0), 30 mM NaCl, 3 mM $(CH_3CO_2)_2Mg$, 0.1 mM DTT (150 μl total) in a temperature-controlled cuvette at 25°C. The anisotropy was continuously measured until it stabilized. Helicases were then added to the cuvette. When the anisotropy again became stable, the unwinding reaction was started by the rapid addition of ATP solution to a final concentration of 1 mM. The anisotropy decrease was recorded every 8 s until it stabilized. We determined the apparent catalytic constants from the equation: $A_t = A \exp(-k_{obs}t)$, where $A_t$ is the anisotropy at time $t$, and $k_{obs}$ is the observed rate constant. For some experiments, Helicase activity was also confirmed by radiometric assay (*4*).

DNA unwinding activity in the presence of distamycin A was also measured by a radiometric assay. The reaction was carried out in a reaction mixture containing 25 mM HEPES-NaOH, pH 7.5, 25 mM NaOAc, 7.5 mM $Mg(OAc)_2$, 2 mM ATP, 1 mM DTT, 0.1 mg/ml BSA, $^{32}$P-labelled partial DNA duplex substrate (10 fmol, 3 000 c.p.m/fmol) and the indicated inhibitor concentrations. The partial DNA duplex substrate is the same as that used for the fluorimetric assay, except that the oligo B was $^{32}$P-radiolabelled at the 5′ position, rather than fluorescein labelled at the 3′ position. The reaction was initiated by addition of 80 nM RecQ helicase at 37°C for 20 min. The reaction was terminated by the addition of 5 μl of 5× loading buffer (50 mM EDTA, 0.5% SDS, 0.1% xylene cyanol, 0.1% bromophenol blue and 50% glycerol). The reaction products were resolved on a 12% (w/v) PAGE run in a TBE buffer (90 mM Tris, 90 mM boric acid, pH 8.3 and 1 mM EDTA) at 100 V for 2 h at 4°C.

*ATPase Assay*—ATPase activity was measured from the amount of radioactive $\gamma^{-32}$P liberated during hydrolysis (*15*). Briefly, the experiment was carried out at 37°C in a reaction mixture containing 1.5 μM of nucleotide and heat-denatured *Hind*III-cut pGEM-7Zf linear DNA at the same concentration as ATP. The reactions were started by the addition of RecQ helicase into 100 μl of reaction mixture. Every 30-s aliquot (80 μl) was pipetted from one of the reaction mixtures into a hydrochloric solution of ammonium molybdate. The liberated radioactive $^{32}$P was extracted with a solution of 2-butanol-benzene-acetone-ammonium molybdate (150 μM) (750:750:15:1). An aliquot of the organic phase was counted in 6 ml of Aquasol.

*DNA-Binding Assays*—DNA-binding assays were carried out using a Beacon 2000 polarization instrument (PanVera Corp.) (*16*). An appropriate quantity of fluorescein-labelled dsDNA was added to a standard titration buffer (150 μl total volume) in a temperature-controlled cuvette at 25°C. The anisotropy of the fluorescein-labelled DNA was measured continuously in the presence of different concentrations of the compounds until it stabilized. An appropriate quantity of RecQ helicase was then added. After each addition of RecQ helicase the anisotropy was measured continuously until a stable plateau was reached. The relative DNA-binding activity was determined as: $(A_n/A_0) \times 100\%$, where $A_n$ is the fluorescence anisotropy at a given concentration of different compounds and $A_0$ is the fluorescence anisotropy in the absence of the compounds.

## RESULTS AND DISCUSSION

Based on their chemical structure, the DNA-binding agents used in this study can be divided into three kinds: DNA intercalators (ethidium bromide, mitoxantrone, m-AMSA and actinomycin D), minor groove binders (Hoechst 33258, DAPI and distamycin A) and topoisomerase inhibitors (camptothecin, 10-hydrocamptothecin and VP16) (Fig. 1). Here we have used our previously developed rapid, real-time helicase assay (*14*) to determine the effect of the DNA-binding agents on the helicase activity. This assay is based on the observation that fluorescein-labelled free oligonucleotides tumble more rapidly due to their very rapid rotational diffusion, than helicase–oligonucleotide complexes. Therefore, the DNA unwinding reaction can be followed in real time by measuring the change in fluorescence polarization. This approach has been used successfully in a number of studies and the obtained data were comparable to the data acquired with the conventional radiometric assay (*13*). Here we have determined the apparent catalytic constant of *E. coli* RecQ helicase in the presence of increasing concentrations of the different agents by measuring the fluorescence anisotropy of the reaction mixtures. The apparent unwinding constant can be derived from fitting an exponential function to the kinetic curves. An example for mitoxantrone is shown

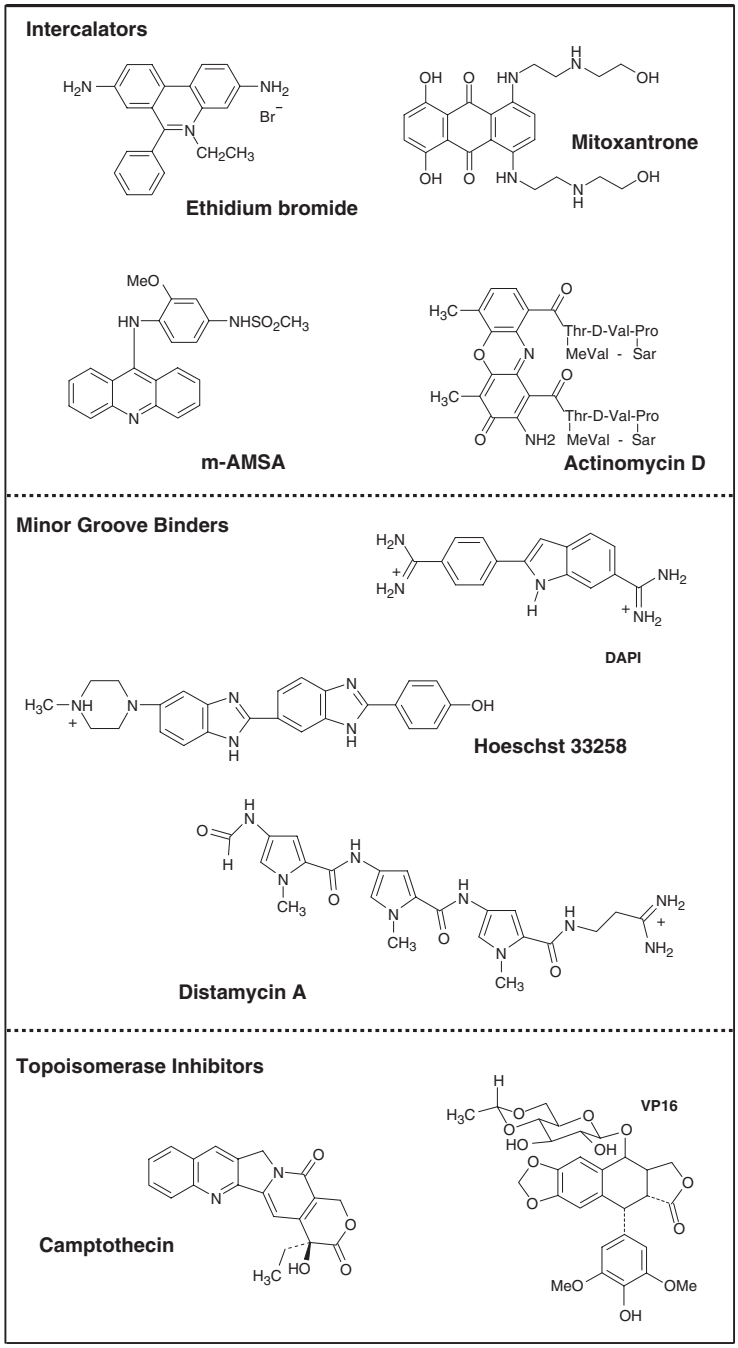


Fig. 1. **Chemical structures of DNA binding compounds.**

in Fig. 2A. We also systematically determined the DNA binding and ATPase activities under the same experimental conditions in order to better understand the mechanism of action of these agents.

*The RecQ Helicase Sensitivity Profiles to DNA-Intercalators*—The curves for the relative DNA unwinding rate constants of *E. coli* RecQ helicase in the presence of four different DNA-intercalators showed that both ethidium bromide and mitoxantrone inhibited unwinding more strongly than actinomycin D and m-AMSA (Fig. 2B), as a 50% unwinding was achieved with 2–5 μM ethidium bromide or mitoxantrone *versus* 40 μM m-AMSA or 50 μM actinomycin D. We then examined the ATPase activity as a function of increasing concentrations of the DNA-binding agents to study their mechanism of enzyme inhibition. Consistent with the previous results, ethidium bromide and mitoxantrone inhibited ATPase activity by 50% at a concentration of <30 μM, whereas at least 200 μM were required to inhibit the ATPase activity by 10% for m-AMSA or 60% for actinomycin D (Fig. 2C). As RecQ helicase is a DNA-stimulated ATPase and an ATP-dependent helicase,

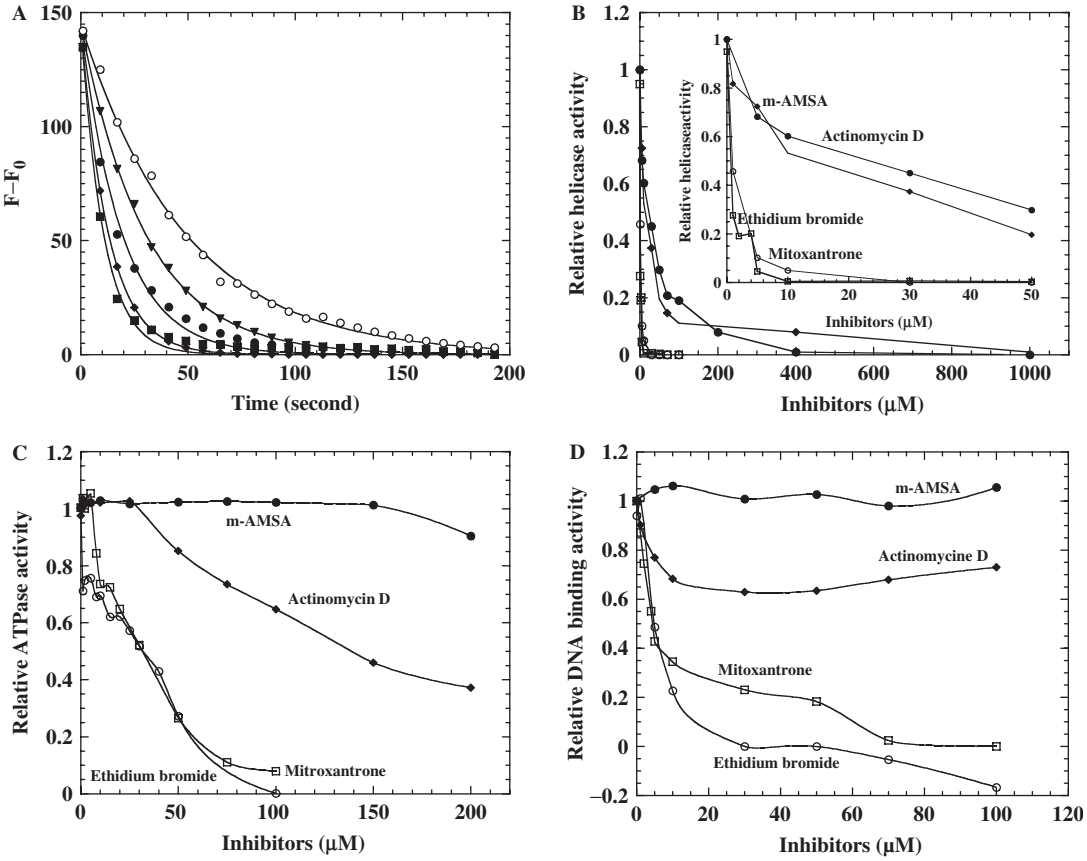


Fig. 2. **Effect of the intercalators used to study DNA unwinding, ATPase and DNA-binding activities of *E. coli* RecQ helicase.** (A) The kinetic unwinding curve of *E. coli* RecQ helicase in the absence (square) and presence of 0.5 μM (diamond), 1 μM (closed circle), 3 μM (triangle) and 5 μM (open circle) mitoxantrone. DNA and the protein concentrations were 1 nM of 3′-fluorecent-labelled partial duplex DNA and 50 nM helicase. The apparent catalytic constants were determined according to the equation: $A_t = A \exp(-k_{obs}t)$, where $A_t$ is the anisotropy amplitude at time $t$, and $k_{obs}$ is the observed rate constant. (B) RecQ helicase activity was determined as in Fig. 2A in the presence of different compounds at different concentrations. All experiments were carried as per the conditions indicated in MATERIALS AND METHODS section. DNA and the protein concentrations were 1 nM 3′-fluorescent-labelled duplex DNA and 50 nM helicase. (C), Experiments were carried out as indicated in MATERIALS AND METHODS section. Helicase concentrations used were 50 nM. (D) DNA and the protein concentrations were 1 nM 3′-fluorescent-labelled duplex DNA and 50 nM RecQ helicase. The experimental conditions were as described in MATERIALS AND METHODS section.

DNA binding is essential for regulating ATPase and helicase activities. Therefore, we determined the DNA-binding activities of RecQ helicase in the presence of the DNA-binding agents, at concentrations identical to those used for the ATPase and helicase assays. The DNA-binding activity of the enzyme was completely inhibited by 50 μM ethidium bromide or mitoxantrone (Fig. 2D), with apparent $K_i$ values of 10 μM for ethidium bromide and 12 μM for mitoxantrone. In contrast, AMSA had no detectable inhibitory effect up to a concentration of 150 μM, and at the same concentration actinomycin D only inhibited by about 20% the DNA-binding activity. These results suggested that ethidium bromide and mitoxantrone inhibit DNA binding, which in turn affects the ATPase and helicase activities. These observations are consistent with the crystal structure of the ethidium bromide–DNA complex (*17*, *18*). Ethidium bromide is a potent inhibitor of DNA synthesis and may bend and distort the DNA structure through electrostatic binding to the phosphodiester backbone of DNA. It is possible that *E. coli* RecQ helicase is extremely sensitive to this modification of the DNA structure and therefore fails to bind to DNA. Also, the sensitivity profiles of the enzyme to ethidium bromide and mitoxantrone are almost identical, a result also seen for the WRN and BLM proteins (*12*).

*The Sensitivity Profiles to Minor Groove Binders*—When the minor groove binders were studied, (Fig. 3), we found that the most effective unwinding inhibitors were DAPI and H33258, with apparent $K_i$ of 15 and 20 μM, respectively (these and the other results are given in Table 1). Although, the apparent $K_i$ value for distamycin A was estimated to be about 200 μM, the maximum inhibition for the enzyme unwinding activity reached only 70% of the control and the inhibition curve never reached an asymptotic minimum even at concentrations of 500 μM. These observations were further confirmed by radiometric assay (Fig. 3A, insert). Thus, the sensitivity of *E. coli* RecQ helicase to distamycin A is similar to that reported for UvrD helicase (*19*), but is completely

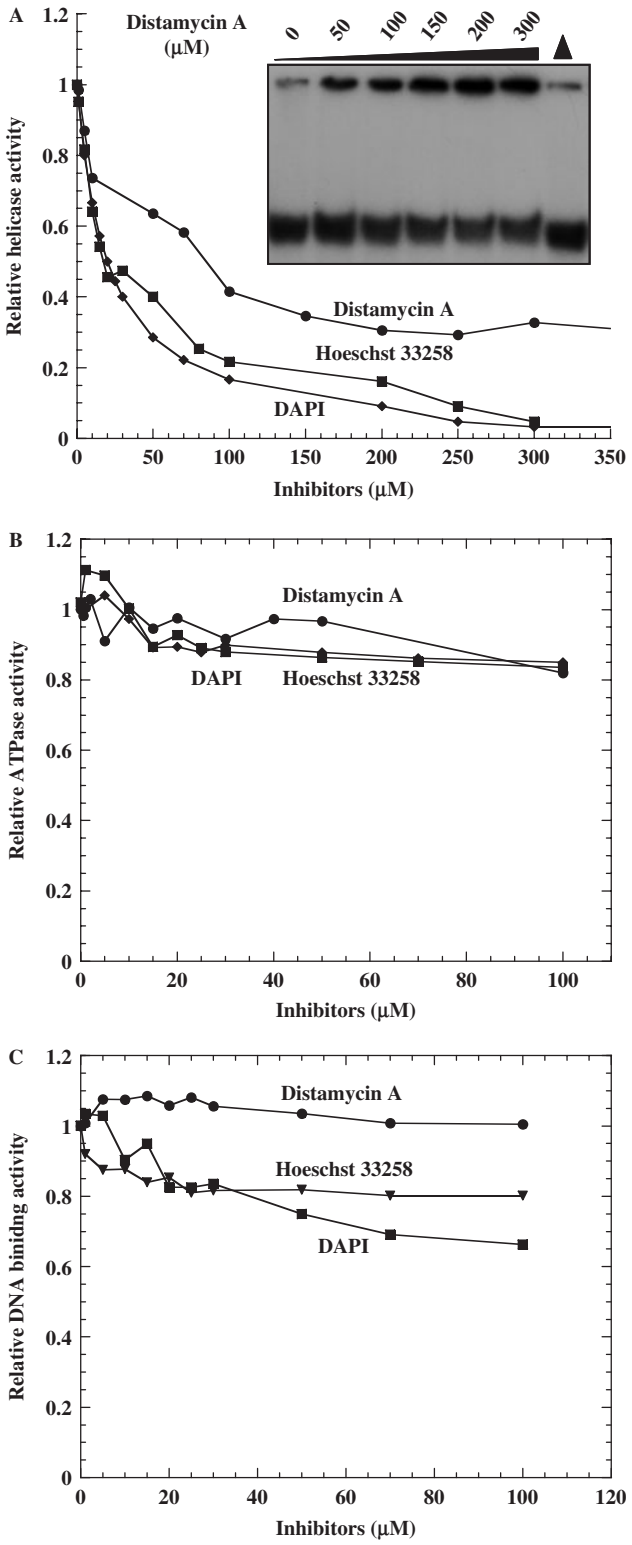


Fig. 3. **Effects of minor groove compounds on the helicase (A), ATPase (B) and DNA-binding activities (C) of *E. coli* helicase.** The reaction conditions and the enzyme quantities are identical to those in Fig. 2. Insert in Fig. 3A is the radiometric assay of RecQ helicase unwinding activity and the function of increasing distamycin A concentration. The detailed experimental conditions for radiometric assay were indicated in the MATERIALS AND METHODS section.

Table 1. **Comparison of DNA-binding compounds on the inhibition of DNA unwinding by RecQ helicase family from human and *E. coli*.**

| Drug | Apparent Ki (μM) | | |
|---|---|---|---|
| | WRN[a] | BLM[a] | *E. coli* RecQ |
| Intercalator | | | |
| Ethidium bromide | 10 | 10 | 2 |
| Mitoxantrone | 10 | 10 | 5 |
| m-AMSA | 100 | 100 | 40 |
| Actinomycin | 100 | 100 | 50 |
| Minor groove | | | |
| DAPI | 10 | 10–10 | 15 |
| Hoescht 33258 | 10 | 10–10 | 20 |
| Distamycin A | 0.5–2.5 | 0.5–2.5 | ≥200 |
| Topoisomerase inhibitors | | | |
| Camptothecin | 100 | 100 | ND |
| 10-Hydrocamptothecin | | | ND |
| VP16 (Etoposide) | 100 | 100 | ND |

ND, non detectable.
[a]Werner and Bloom helicases (*12*).

different from that measured for the WRN and BLM helicases (*12*). In this latter study, distamycin A potently inhibited both WRN and BLM helicase activity at a concentration ranging between 0.5 and 1 μM. These three minor groove binders, which inhibit helicase activity by different extents, all inhibit the ATPase activity by similar amounts, about 70% (Fig. 3B), suggesting that these compounds do not markedly inhibit DNA binding. Indeed, the binding studies showed that distamycin A had no detectable inhibition effect on DNA binding (Fig. 3C), whereas both H33258 and DAPI had similar inhibition profiles to that for ATPase activity. These results suggested that distamycin A does not prevent the helicase from binding to the DNA substrate, consistent with a previous study (*12*), showing that distamycin A efficiently inhibits the helicase activity of WRN and BLM while having no detectable effect on ATPase activity. It is possible that the WRN and BLM helicases are more sensitive to distamycin A than the *E. coli* RecQ helicase despite the fact that these enzymes belong to the same RecQ helicase family and have high sequence similarities. *Escherichia coli* RecQ helicase and human RecQ helicase are however considerably different with respect to their structure and enzymatic activity. The N-terminal and C-terminal extension sequences of the WRN and BLM helicases certainly confer different enzymatic activities to these helicases compared to *E. coli* helicase. More importantly, the ATPase and helicase activities of *E. coli* RecQ helicase are higher than those of WRN and BLM (*20–23*). As shown in Table 2, *E. coli* RecQ helicase efficiently catalyses the hydrolysis of ATP than its homologues eukaryotic RecQ family helicases. The binding of distamycin A to DNA may physically block the continued translocation of WRN and BLM helicases. *Escherichia coli* RecQ helicase may overcome this inhibitory effect through its highly efficient ATP hydrolysis and high helicase activity, whereas the low ATPase activities of the human RecQ helicases (WRN and BLM) do not provide enough energy to overcome this obstacle.

Table 2. **Comparison of the $k_{cat}$ ($min^{-1}$) for ATP hydrolysis of RecQ family helicases.**

| Helicases | $k_{cat}$ ($min^{-1}$) | References |
|---|---|---|
| *hs*RECQL | 126.3 ± 3.4 | (*20*) |
| *hs*BLM | 1163 ± 358 | (*12*) |
| hsWRN | 58.4 ± 17 | (*22*) |
| *dm*RECQ5 | 900 ± 3.4 | (*23*) |
| *E. coli* RECQ | 1452-2000 | (*6*) |

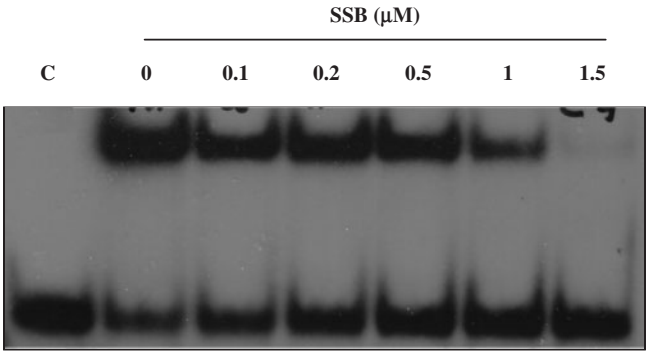


Fig. 4. ***Escherichia coli* RecQ helicase–mediated DNA unwinding activity in the presence of distamycin A and SSB protein.** RecQ protein (50 nM) was incubated with 10 fmol (3 000 c.p.m./fmol) $^{32}$P-labelled partial duplex DNA substrate and 150 μM distamycin A in the presence of increasing concentration of SSB protein, as indicated in the figure. Helicase reaction was conducted as described in MATERIALS AND METHODS section. Column c represents the boiled DNA substrate.

Previous studies have shown that SSB stimulates RecQ DNA unwinding (*5*, *6*). In order to determine whether RecQ helicase could overcome the partial inhibitory effect of distamycin A in the presence of the SSB protein, we have measured the helicase activity of *E. coli* RecQ in the presence of both distamycin and SSB. As shown in Fig. 4, the residual inhibitory effect of distamycin A is completely overcome by RecQ helicase in the presence of SSB protein. In the study by Brosh *et al.* (*12*) the overall pattern of distamycin inhibition for WRN or BLM helicases was the same in the presence or absence of human replication protein A (hRPA), suggesting that hRPA does not alleviate the potent inhibition of WRN or BLM unwinding activity by the minor groove binder distamycin A. The different inhibitory effects of distamycin A to the same RecQ family helicases indicate that *E. coli* RecQ helicase is a powerful molecular motor when works co-ordinately with SSB protein.

*The Sensitivity Profiles to Topoisomerase Inhibitors*—VP16, camptothecin and hydro-camptothecin did not change the DNA helicase, ATPase and DNA-binding activities (Table 1). These observations are consistent with previous results obtained with WRN, BLM and UvrD helicases (*12*, *19*), suggesting that these topoisomerase inhibitors cannot bind and modulate *E. coli* RecQ helicase.

## CONCLUSION

RecQ helicases play an essential role in genome integrity. Unlike the RecQ helicases of prokaryotes, the RecQ helicases of eukaryotes usually have extended C-terminals or N-terminals. We compared the behaviour of these enzymes after exposure to chemotherapeutic agents, comparing the inhibition profiles of *E. coli* RecQ helicase to the human RecQ helicases, WRN and BLM. The results of DNA-binding inhibition for *E. coli* and human RecQ helicases are summarized in Table 1. Both *E. coli* and the human RecQ helicases exhibit similar inhibition profiles for the DNA intercalator compounds and are consistent with previous studies on WRN and BLM helicases. However, *E. coli* RecQ helicase is less sensitive to the minor groove binders, despite the fact that distamycin A strongly inhibited both WRN and BLM unwinding activity. Even at very high concentrations of distamycin A, the inhibition of *E. coli* RecQ helicase reached only 70% inhibition. The different effects of distamycin A on *E. coli* and human DNA helicases may reveal mechano-chemical properties of these helicases to DNA-binding agents. Although helicases are mainly known for their ability to unzip DNA or RNA, it has become increasingly clear that helicases can use the energy derived from ATP hydrolysis to disrupt any obstacle when helicases encountered on DNA lattice. The collision between a helicase and an obstacle can lead to stalling of the motor or displacement of the DNA-binding agents, depending on the force imparted by the molecular motor. Considering the fact that *E. coli* RecQ helicase possesses higher ATPase activity than BLM and WRN helicases it is evident that *E. coli* RecQ helicase is a stronger molecular motor than the human helicases BLM and WRN, and is able to overcome the physical obstacle of distamycin A to unwind DNA. More importantly, in the presence of SSB protein, *E. coli* RecQ helicase can completely overcome distamycin inhibitory effect. Taken together, these findings may give insights into the difference in molecular mechanisms underlying efficient motor function of RecQ helicase family.

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