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Antitumor bisdioxopiperazines inhibit yeast DNA topoisomerase II by trapping the enzyme in the form of a closed protein clamp

[enzyme mechanism/drug design/bis(2,6-dioxopiperazines)]

JOAQUIM ROCA*, RYOJI ISHIDA†, JAMES M. BERGER*, TOSHIWO ANDOH†, AND JAMES C. WANG*

*Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138; and †Laboratory of Biochemistry, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464, Japan

Contributed by James C. Wang, November 15, 1993

ABSTRACT **The mechanism of inhibition of eukaryotic DNA topoisomerase II [DNA topoisomerase (ATP-hydrolyzing), EC 5.99.1.3] by a member of the bisdioxopiperazine family of anticancer drugs, ICRF-193, was investigated by using purified yeast DNA topoisomerase II. In the absence of ATP, ICRF-193 has little effect on the binding of the enzyme to various forms of DNA. In the presence of ATP, the drug converts the enzyme to a form incapable of binding circular DNA. Incubation of a preformed circular DNA–enzyme complex with ICRF-193 and ATP converts the complex to a form stable in molar concentrations of salt. These results can be interpreted in terms of the ATP-modulated protein-clamp model of type II DNA topoisomerases [Roca, J. & Wang, J. C. (1992) *Cell* 71, 833–840]; ICRF-193 can bind to the closed-clamp form of the enzyme and prevents its conversion to the open-clamp form. This interpretation is further supported by the finding that whereas both ATP and the drug are needed to form the salt-stable circular DNA–enzyme complex, ATP is not needed for maintaining this complex; furthermore, a signature of the closed-clamp form of the enzyme, *Staphylococcus aureus* strain V8 endoproteinase cleavage site at Glu-680, is observed if the enzyme is incubated with both ATP and ICRF-193. Inhibition of interconversion between the open- and closed-clamp forms of type II DNA topoisomerases offers a new mechanism in the selection and design of therapeutics targeting this class of enzymes.**

Eukaryotic DNA topoisomerase II [DNA topoisomerase (ATP-hydrolyzing), EC 5.99.1.3] is a member of the type II DNA topoisomerase family, which catalyzes the ATP-dependent transport of one double-stranded DNA segment through another (1–3). In the past decade, a number of anticancer drugs have been found to target the enzyme (4–7). One class of these drugs, including doxorubicin, amsacrine, etoposide, and teniposide, was shown to interfere with the DNA-rejoining activity of the enzyme. Such a drug binds to an enzyme–DNA complex and stabilizes the covalent complex in which a pair of DNA phosphodiester bonds, one in each strand of the DNA duplex, are severed, and the 5′ phosphoryl group of each broken DNA strand is covalently linked to a protein tyrosyl residue—Tyr-805 in the case of human DNA topoisomerase IIα (8). Drugs that stabilize the covalent DNA–DNA topoisomerase reaction intermediates are often referred to as DNA topoisomerase poisons, as they act by converting these normal cellular enzymes into DNA-damaging agents (5, 9).

There are a number of DNA topoisomerase II-targeting drugs that apparently do not stabilize the covalent intermediate. Bisdioxopiperazine derivatives including ICRF-159 and ICRF-193 are among the newest members of this category (10, 11), which also includes fostriecin (12), merbarone (13), and aclarubicin (14). Other than their inhibition of eukaryotic DNA topoisomerase II-catalyzed topological transformations of DNA rings, there is little information on the mechanisms by which these drugs affect eukaryotic DNA topoisomerase II.

Recently, a protein clamp model has been proposed for eukaryotic DNA topoisomerase II (15). In this model, a homodimeric enzyme acts as an ATP-modulated protein clamp: ATP-binding closes the two jaws of the clamp, and hydrolysis of the bound ATP precedes the reopening of the clamp. As a DNA-bound protein clamp closes its jaws, it can capture a second DNA segment, termed the "T-segment," and transport it through an enzyme-mediated gate in the bound DNA, termed the "G-segment." By using a nonhydrolyzable β,γ-imido analog of ATP, adenosine 5′-[β,γ-imido]triphosphate (AMP-*P*[NH]*P*), it was shown that the binding of the analog to yeast *Saccharomyces cerevisiae* enzyme triggers a concerted conformational change of the enzyme, which is detectable by the switching of a *Staphylococcus aureus* strain V8 (SV8) endoproteinase-sensitive site at Glu-410 to a new site at Glu-680 (16, 17). Detailed studies on the interactions between yeast DNA topoisomerase II and various topological forms of DNA in the presence and absence of AMP-*P*[NH]*P* led Roca and Wang (15) to interpret the AMP-*P*[NH]*P*-triggered conformational change in terms of the closure of a protein clamp. Because all type II DNA topoisomerases are found to be evolutionarily and structurally conserved (for examples, see refs. 18 and 19), the protein-clamp model is most likely to be applicable to all type-II DNA topoisomerases. In the present communication, we show that the bis(2,6-dioxopiperazine) derivative ICRF-193 by itself has no effect on the binding of various forms of DNA to yeast DNA topoisomerase II. When both ICRF-193 and ATP are present, however, the enzyme is converted to the closed form, as evidenced by the SV8 endoproteinase cleavage pattern as well as the characteristics of the DNA–enzyme complexes. These results suggest that bis(2,6-dioxopiperazines) act by stabilizing eukaryotic DNA topoisomerase II in the closed-clamp form and preventing it from opening again.

MATERIALS AND METHODS

ICRF-193 was provided by Zenyaku Kogyo (Tokyo). Yeast DNA topoisomerase II was purified from *S. cerevisiae* cells overexpressing a plasmid-borne *TOP2* gene (8, 16), by a procedure using phosphocellulose and Q-Sepharose column chromatography (J.M.B. and J.C.W., unpublished data). Plasmid DNAs were purified by standard methods including equilibrium centrifugation in CsCl/EtdBr as the final step. All other materials were obtained from commercial sources as indicated below: ATP (Sigma), [α-^{32}P]ATP (New England

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Abbreviations: AMP-*P*[NH]*P*, adenosine 5′-[β, γ-imido]triphosphate; SV8, *Staphylococcus aureus* strain V8.

Nuclear), SV8 endoproteinase (Sigma), hexokinase (Worthington), and GF/C glass-fiber filters (Whatman).

Mapping of SV8 endoproteinase cleavage sites was done by the procedures described previously (16), and ATPase assays were done by thin-layer chromatography as described (20). All other methods have been described by Roca and Wang (15).

RESULTS

ICRF-193 by Itself Has Little Effect on DNA Binding by Yeast DNA Topoisomerase II. To determine the step or steps at which the bisdioxopiperazine anticancer drugs inhibit the action of eukaryotic DNA topoisomerase II, binding of DNA by the enzyme was measured in the presence and absence of one of the more potent members of this family, ICRF-193. The results of a filter-binding experiment (15) are shown in Fig. 1. Lane 1 contained the DNA by itself upon filtration through a glass-fiber filter. As expected, nearly all DNA, the bulk of which in the supercoiled form (denoted by S in the figure) and a small fraction in the nicked circular form (denoted by N), was found in the filtrate. Eluting the filter with a buffer containing 1 M NaCl showed little DNA in the wash (lane 2). In the presence of yeast DNA topoisomerase II, DNA retention on the filter was apparent; upon washing with 1 M NaCl, which dissociates the DNA from the filter-bound DNA–enzyme complex unless a circular DNA is topologically linked to an enzyme in its closed-clamp form (15), a large fraction of the DNA retained on the filter was found in the wash (lanes 3 and 4). The relative amounts of unbound nicked and supercoiled DNA in the filtrate shown in lane 3 indicate that the enzyme binds preferentially the supercoiled form, as has been observed (15, 21, 22). The addition of ICRF-193 to the DNA–enzyme mixture had little effect on the binding of the DNA to the enzyme (compare the patterns in Fig. 1 shown in lanes 5 and 6 to those shown in lanes 3 and 4).

Circular DNA and Yeast DNA Topoisomerase II Form a Salt-Stable Complex in the Presence of Both ATP and ICRF-193. When both ATP and ICRF-193 were added to the plasmid DNA–yeast DNA topoisomerase II mixture, an enzyme–DNA complex resistant to 1 M salt was formed. In the sample shown in lane 1 of Fig. 2, enzyme, ICRF-193, and supercoiled pBluescript DNA were first incubated for 10 min at 30°C in 50 mM Tris·HCl, pH 8/50 mM KCl/8 mM $MgCl_2$/7 mM 2-mercaptoethanol/100 μg of bovine serum albumin per ml. ATP was then added to 1 mM, and incubation was continued for 10 min. NaCl was then added to the mixture to

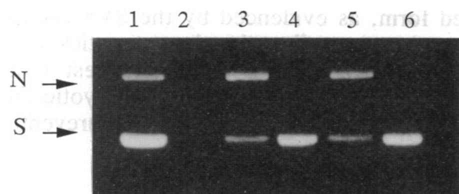

FIG. 1. Lack of effect of the bisdioxopiperazine ICRF-193 on the binding of yeast DNA topoisomerase II to circular DNA in the absence of ATP. A reaction mixture containing 200 ng of pBluescript DNA in 20 μl of 50 mM Tris·HCl, pH 8/50 mM KCl/8 mM $MgCl_2$/7 mM 2-mercaptoethanol/100 μg of bovine serum albumin per ml was first incubated at 30°C for 10 min and then filtered through a glass-fiber filter as described (15). The filter was then eluted with 1 M NaCl. DNA was recovered from the filtrate and eluent and was analyzed in lanes 1 and 2, respectively. A second reaction mixture, identical to the one described but containing in addition 200 ng of yeast DNA topoisomerase II, and a third reaction mixture, identical to the second one but containing in addition 100 μM ICRF-193, were similarly processed and analyzed in lanes 3–6. S, supercoiled DNA; N, nicked circular DNA.

1 M final concentration, and the mixture was passed through a glass-fiber filter. As shown in lanes 1 and 2 of Fig. 2, most of the DNA was retained on the filter, indicating that the protein–DNA complex that was formed under these conditions is stable in 1 M NaCl; as expected, the filter-bound DNA was released upon eluting the filter with 1% sodium dodecyl sulfate (SDS) (lane 2). No salt-stable complex was observed when either ATP or ICRF-193 was omitted in this experiment (Fig. 1 and ref. 16).

Reversing the order of incubation of the enzyme–DNA complex with ICRF-193 and ATP had no effect on the formation of the salt-stable complex; a 10-min incubation with ATP followed by a 10-min incubation with the drug (Fig. 2, lanes 5 and 6) yielded the same results described above (Fig. 2, lanes 1 and 2). When the enzyme was first incubated with both ATP and ICRF-193, however, the subsequent addition of plasmid DNA yielded no salt-stable complex: all DNA was found in the 1 M salt filtrate (lane 3) and none in the SDS wash (lane 4). Furthermore, whereas in the previous two cases the DNA was converted to the relaxed form and thus migrated as a ladder of bands (Fig. 2, lanes 1, 2, 5, and 6), in the last sample the DNA remained in the supercoiled form (Fig. 2, lane 3).

ICRF-193 Inhibits the ATPase Activity of Yeast DNA Topoisomerase II. Whereas the presence of both ATP and ICRF-193 is necessary for the formation of the salt-stable DNA–enzyme complex, the drug was found to inhibit the ATPase activity of the enzyme. Data from a series of measurements are shown in Fig. 3. In this experiment, four reaction mixtures, each with 5 μg of yeast DNA topoisomerase II in 50 μl of 50 mM Tris·HCl, pH 8/100 mM KCl/8 mM $MgCl_2$/7 mM 2-mercaptoethanol/100 μg of bovine serum

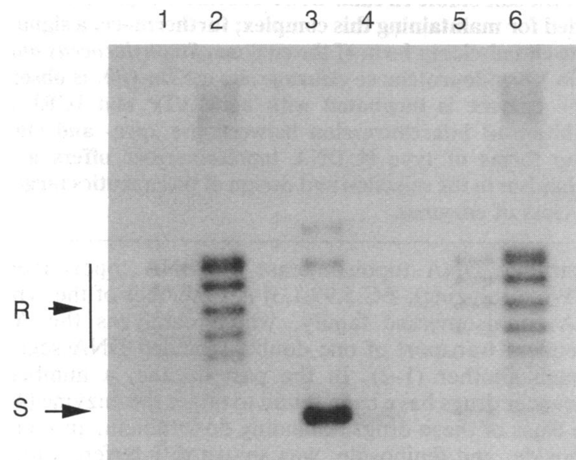

FIG. 2. Formation of a salt-stable yeast DNA topoisomerase II–plasmid DNA complex in the presence of both ATP and ICRF-193. Each reaction mixture contained 200 ng of yeast DNA topoisomerase II and 200 ng of pBluescript DNA in 20 μl of 50 mM Tris·HCl, pH 8/150 mM KCl/8 mM $MgCl_2$/7 mM 2-mercaptoethanol/100 μg of bovine serum albumin per ml/1 mM ATP/100 μM ICRF-193. In lanes 1 and 2, all components except ATP were first mixed and incubated for 10 min at 30°C and ATP was then added; after an additional 10 min at 30°C, NaCl was added to 1 M final concentration, and the solution was filtered through a glass-fiber filter, which was then eluted with 1% sodium dodecyl sulfate as described (15). DNA recovered from the filtrate and eluent was analyzed in lanes 1 and 2, respectively. In lanes 3 and 4, all components except DNA were incubated first, and incubation was continued after the addition of DNA; otherwise, the reaction mixture was processed as described above. DNA recovered from the filtrate and from the eluent was analyzed in lanes 3 and 4, respectively. Material in lanes 5 and 6 was the same as described for the other pairs of lanes, except that incubation was first carried out in the absence of ICRF-193 and then continued after its addition. R, relaxed circular DNA; S, supercoiled DNA.

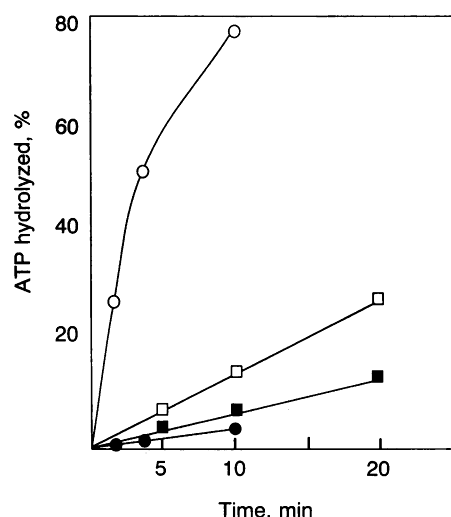

FIG. 3. Inhibition of the ATPase activity of yeast DNA topoisomerase II by ICRF-193. Each reaction mixture contained in a volume of 50 μl 50 mM Tris·HCl, pH 8/100 mM KCl/8 mM $MgCl_2$/7 mM 2-mercaptoethanol/100 μg of bovine serum albumin per ml/5 μg of yeast DNA topoisomerase II/1 mM [α-^{32}P]ATP (≈20 mCi/mmol), 0 or 5 μg of pBluescript DNA, and 0 or 100 μM ICRF-193. Because the stock solution of the drug contained dimethyl sulfoxide, the concentration of this solvent was also adjusted to 1% (vol/vol) in reaction mixtures with or without the drug. Aliquots were withdrawn at different times, and EDTA and sodium dodecyl sulfate were added to each aliquot to a final concentration of 50 mM and 0.5%, respectively, to terminate ATP hydrolysis catalyzed by the yeast enzyme. One microliter of each quenched sample was analyzed by thin-layer chromatography for the percentage of ATP hydrolyzed as described (20). ○, With DNA, no ICRF-193; □, without DNA, no ICRF-193; ●, with DNA and ICRF-193; ■, without DNA, with ICRF-193.

albumin per ml were supplemented with 0 or 5 μg of supercoiled pBluescript DNA and 0 or 100 μM ICRF-193. [α-^{32}P]ATP with a specific activity of ≈20 mCi/mmol (1 Ci = 37 GBq) was then added to each reaction mixture to a final concentration of 1 mM, and aliquots of the reaction mixtures incubated at 30°C were sampled at various times and quenched for the determination of the percentage of ATP hydrolyzed. As shown by data in Fig. 3, ICRF-193 inhibits the ATPase activity of yeast DNA topoisomerase II.

ATP Is Required for the Formation of the Salt-Stable Plasmid DNA–Yeast DNA Topoisomerase II Complex in the Presence of ICRF-193 but Is Not Required for the Maintenance of the Complex. The above experiments show that in the presence of ICRF-193, ATP is required to form the salt-stable enzyme–plasmid DNA complex; yet at the same time the presence of the drug inhibits the ATPase activity of the enzyme. These results suggest that in the presence of ICRF-193, ATP may be required for the formation of the salt-stable complex, but once the complex is formed, the nucleotide is not required for its maintenance. This interpretation was confirmed by an experiment in which ATP was removed enzymatically after the formation of the salt-stable complex. ATP was added to 1 mM final concentration to 100 μl of a reaction mixture containing 50 mM Tris·HCl/150 mM KCl/8 mM $MgCl_2$/7 mM 2-mercaptoethanol/100 mM glucose/100 μg of bovine serum albumin per ml/0.5 μg each of yeast DNA topoisomerase II and supercoiled pBluescript DNA, and 100 μM ICRF-193, to initiate the formation of the salt-stable enzyme–plasmid DNA complex. The reaction mixture was split into two equal portions and incubated at 30°C for 10 min. One unit of hexokinase, which phosphorylates glucose and converts ATP to ADP in the process, was added to one. After an additional 20 min at 30°C, NaCl was added to each reaction mixture to 1 M, and filtration through glass-fiber filters and elution with 1% SDS were then carried out as before.

In both the hexokinase-treated sample and the untreated control, the presence of the salt-stable complex was evident, as little DNA was found in the 1 M salt filtrate and most of the input DNA came off the filter in the SDS wash (results not shown). Analysis of the reaction mixtures with and without hexokinase showed that in the hexokinase-treated sample, ATP was converted to ADP and no detectable amount of ATP remained; in the sample without hexokinase, the bulk of the nucleotide remained as ATP and only a trace was present as ADP. To rule out a trivial interpretation of the hexokinase results that the presence of glucose or hexokinase might inhibit DNA-bound yeast DNA topoisomerase II and thus trap the complex in the salt-stable form, control experiments were carried out in which ICRF-193 and either hexokinase or glucose were omitted from the complete reaction mixture. The samples were processed as before, and a second supercoiled DNA 10 kb in size was added to the reaction mixtures after the second incubation. After a final 10-min incubation at 30°C, the samples were analyzed by agarose gel electrophoresis. In either case, the second supercoiled DNA was found to be relaxed, showing that yeast DNA topoisomerase II is not inactivated by hexokinase or glucose.

Yeast DNA Topoisomerase II Is Cleaved at the Same Site by SV8 Endoproteinase in the Presence of AMP-*P*[NH]*P* or a Combination of ATP and ICRF-193. The formation of a salt-stable complex between yeast DNA topoisomerase II and DNA in the presence of both ATP and ICRF-193 suggests that the protein clamp is in the closed or locked form (15) under these conditions. This interpretation is supported by the results shown in Fig. 4.

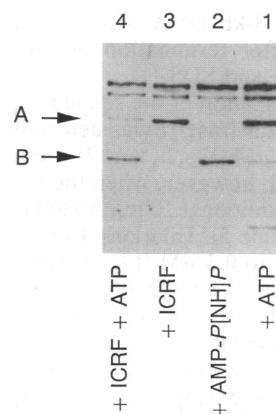

FIG. 4. Cleavage of immunotagged yeast DNA topoisomerase II by SV8 endoproteinase in the presence of 100 μM ICRF-193 and 1 mM ATP (lane 4), 100 μM ICRF-193 (lane 3), 1 mM AMP-*P*[NH]*P* (lane 2), and 1 mM ATP (lane 1). The immunotagged yeast enzyme was prepared from yeast cells overexpressing a plasmid-borne *top2* gene in which the first 1172 codons of yeast DNA topoisomerase II were followed by codons for a decapeptide human c-myc epitope and finally a stop codon. Cleavage by SV8 proteinase was carried out as follows. Reaction buffer (50 μl) containing 50 mM Tris · HCl, pH 8/8 mM $MgCl_2$/7 mM 2-mercaptoethanol/5 μg of bovine serum albumin/150 mM KCl, 10 μg of supercoiled DNA and 10 μg of immunotagged enzyme was supplemented with the drug or nucleotide as specified above and incubated at 30°C for 10 min. Two microliters of 1 M NH_4HCO_3 containing 0.1 μg of SV8 endoproteinase was added to each reaction mixture, and all samples were incubated at 30°C for 30 min. Proteolysis was stopped by the addition of an equal volume of a sodium dodecyl sulfate/polyacrylamide gel electrophoresis loading buffer. Samples were kept in a boiling water bath for 5 min before loading on the gel. Following electrophoresis, the resolved protein bands were blot-transferred to a nylon membrane and immunostained with a monoclonal antibody specific to the c-myc epitope (16). A and B, proteolytic cleavage at the carboxyl sides of Glu-410 and Glu-680, respectively.

Purified yeast DNA topoisomerase II immunotagged with a decapeptide epitope of the human c-myc protein after amino acid 1171, which is catalytically fully active (16, 23), was used in this experiment to facilitate the mapping of proteolytic cleavage sites (16). In the presence of ATP alone (Fig. 4, lane 1) or ICRF-193 alone (Fig. 4, lane 3), proteolytic cleavage by SV8 endoproteinase occurs mainly at site A, which has been mapped previously to the carboxyl side of Glu-410 (16). In the presence of a combination of ATP and ICRF-193 (Fig. 4, lane 4), however, the endopeptic cleavage site is predominantly at site B—the same as when AMP-*P*[NH]*P* was present (Fig. 4, lane 2). Previously, site B has been mapped to the carboxyl side of Glu-680 (16).

Inhibition of Yeast DNA Topoisomerase II by ICRF-193 Is Reversible. A series of dilution experiments was carried out to test the reversibility of the inhibition of the yeast enzyme by ICRF-193. Each reaction mixture (20 μl) contained 100 ng of a 10-kb plasmid DNA and 100 ng of yeast DNA topoisomerase II in 50 mM Tris·HCl, pH 8/150 mM KCl/8 mM $MgCl_2$/7 mM 2-mercaptoethanol/1 mM ATP/and 100 μg of bovine serum albumin per ml. ICRF-193 or the solvent dimethyl sulfoxide was added to the reaction mixtures to final concentrations of 0, 0.3, 1.0, 3.0, and 10 μM of the drug and 1% (vol/vol) of dimethyl sulfoxide. The reaction mixtures were incubated at 30°C for 30 min, and each was split into two 10-μl portions; 90 μl of the same reaction buffer without DNA or ICRF-193 were added to one to reduce the drug concentration by a factor of 10, and 90 μl of the same reaction buffer without DNA but with the same concentration of ICRF-193 as the sample were added to the other to maintain the initial drug concentration. One microgram of a 3-kb plasmid DNA was then added to each reaction mixture, and incubation of both sets of samples was continued for 10 min at 30°C. Relaxation of the 3-kb DNA was analyzed by agarose gel electrophoresis after termination of the reactions by the addition of EDTA and SDS.

Inhibition of relaxation of the second supercoiled DNA by yeast DNA topoisomerase II was detectable at an ICRF-193 concentration of 1 μM (Fig. 5, lane 4). Complete relaxation of the DNA occurred, however, when the reaction mixture was incubated for an additional 10 min following a 1:9 dilution of the drug (Fig. 5, lane 5). Dilution of the initial drug concentration from 3 μM to 0.3 μM (Fig. 5, lanes 6 and 7), or from 10 μM to 1 μM (Fig. 5, lanes 8 and 9), also showed that more supercoiled DNA became relaxed upon diluting the inhibitor. Total reversal of inhibition by the drug was not achieved in these experiments, however; some supercoiled DNA substrate remained, for example, in the sample first incubated with 3 μM ICRF-193 and then diluted to an ICRF-193 final concentration of 0.3 μM (Fig. 5, lane 7), whereas no supercoiled substrate was detectable if the drug was always present at 0.3 μM (Fig. 5, lane 2). A similar comparison can be made for the samples run in lanes 9 and 4.

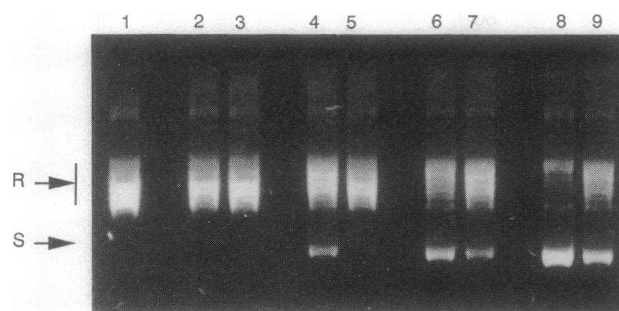

FIG. 5. Reversibility of inhibition of yeast DNA topoisomerase II by ICRF-193. Each reaction mixture (20 μl) contained 50 mM Tris·HCl, pH 8/150 mM KCl/8 mM $MgCl_2$/7 mM 2-mercaptoethanol/1 mM ATP/100 μg of bovine serum albumin per ml/100 ng of yeast DNA topoisomerase II, 100 ng of a 10-kb supercoiled plasmid DNA, and 0, 0.3, 1.0, 3.0, or 10 μM ICRF-193. After 30 min at 30°C, each of the samples other than the no-drug control was split into two 10-μl portions; 90 μl of the same buffer without enzyme and DNA but with the same concentrations of ICRF-193 as the original solutions was added to one, and 90 μl of the same buffer without enzyme, DNA, and ICRF-193 was added to the other to dilute the drug concentration by a factor of 10. One microgram of a second supercoiled plasmid DNA 3 kb in size was then added to each reaction mixture. After incubation for an additional 10 min at 30°C, the reactions were terminated and loaded in the sample wells of an agarose gel for electrophoresis. Lanes: 1, no drug control; 2, 0.3 μM ICRF-193; 3, 0.3 μM ICRF-193 initially and then diluted to 0.03 μM; 4, 1 μM drug initially; 5, 1 μM drug initially and then diluted to 0.1 μM drug; 6, 3 μM drug; 7, 3 μM drug diluted to 0.3 μM drug; 8, 10 μM drug; 9, 10 μM drug diluted to 1 μM. R, relaxed circular DNA; S, supercoiled DNA.

DISCUSSION

The results presented above can be interpreted in terms of the protein-clamp model previously proposed for type II DNA topoisomerases (15): ICRF-193 and its congeners can bind the closed or locked form of the clamp and prevent its reopening. ATP is required for the enzyme to go back and forth between the open and closed state; therefore, only in the presence of ATP can a drug of the ICRF-193 type access the closed form of the enzyme.

The rate of binding of ICRF-193 to the closed form of yeast DNA topoisomerase II appears to be relatively slow. When ATP was added to a preincubated mixture containing the enzyme, DNA, and the drug, relaxation of supercoiled DNA had apparently occurred before the DNA-bound enzyme became locked into the closed conformation by the drug: thus, the DNA in the final salt-stable complex was found to be in the relaxed rather than the original supercoiled form (Fig. 2, lanes 1 and 2).

Once the dioxopiperazine drug binds to the locked protein-clamp, however, ATP is apparently not needed to maintain the closed state of the clamp. Removal of ATP by hexokinase from the reaction mixture has no effect on the salt-stable yeast DNA topoisomerase II–plasmid DNA complex, in which the enzyme is believed to be in the closed-clamp form.

For anticancer drugs targeting eukaryotic DNA topoisomerase II, cytotoxicity can be a result of two different mechanisms. Drugs such as etoposide and amsacrine act by their trapping of the covalent enzyme–DNA complexes, and cytotoxicity of these drugs is closely related to breakage of double-stranded DNA, for example as a result of the collision between replication forks and DNA–enzyme–drug ternary complexes (5, 24–26); maximal cytotoxicity of drugs of this class occurs during the S phase (4–6). Drugs such as ICRF-193 and its congeners, on the other hand, apparently act by trapping the enzyme in the closed-clamp form: a DNA-bound enzyme molecule in the closed-clamp form would be prevented from transporting DNA segments, and a free enzyme molecule trapped in the closed-clamp form would be prevented from binding to DNA, as intracellular DNA has few free ends. Based on genetic analysis of *top2* mutants of the yeast *Saccharomyces pombe* and *S. cerevisiae* (27), trapping DNA topoisomerase II in the closed-clamp form is expected to affect chromosomal condensation and decondensation, and the segregation of multiply intertwined pairs of chromosomes during mitosis. Studies with mammalian cell lines show that treatment with ICRF-193 or its homologue inhibits cell cycle progression at G_2–M (10, 28–32) and prevents chromosome segregation during anaphase (33).

Bisdioxopiperazines including ICRF-193 are known to circumvent the cytotoxicity of etoposide (10). The trapping of the enzyme in the closed-clamp form by a bisdioxopiperazine is expected to prevent any free enzyme molecule from

binding to a DNA and thus prevent any etoposide-induced formation of covalent DNA topoisomerase II–DNA complexes. Further experiments are needed, however, to determine whether the bisdioxopiperazines interfere with the action of etoposide on a DNA-bound DNA topoisomerase II and what might be the mechanism if it does.

In the protein-clamp model for type II DNA topoisomerases, during each catalytic cycle an enzyme undergoes a major reversible transition. The results presented in this communication demonstrate that a drug that interferes with this transition is cytotoxic. Because of the intricacies of this structural transition, it is likely that diverse chemical classes of inhibitors that interfere with either the forward or the reverse reaction can be found or designed; this in turn implies that the full potential of type II DNA topoisomerases as targets of antimicrobial, antiparasitic, and anticancer therapeutics is yet to be explored.

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1. Wang, J. C. (1985) *Annu. Rev. Biochem.* **54**, 665–697.
2. Maxwell, A. & Gellert, M. (1986) *Adv. Protein Chem.* **38**, 69–107.
3. Wang, J. C. (1991) *J. Biol. Chem.* **266**, 6659–6662.
4. Drlica, K. & Franco, R. J. (1988) *Biochemistry* **27**, 2253–2259.
5. Liu, L. F. (1989) *Annu. Rev. Biochem.* **58**, 351–375.
6. Andoh, T., Ikeda, H. & Oguro, M., eds. (1993) *Molecular Biology of DNA Topoisomerases and Its Application to Chemotherapy*, Proceedings of the International Symposium on DNA Topoisomerases in Chemotherapy, Nagoya, Japan, 1991 (CRC, Boca Raton, FL), pp. 1–18.
7. Liu, L. F., ed. (1994) *DNA Topoisomerase and Their Applications in Pharmacology* (Academic, Orlando, FL), in press.
8. Worland, S. T. & Wang, J. C. (1989) *J. Biol. Chem.* **264**, 4412–4416.
9. Kreuzer, K. N. & Cozzarelli, N. R. (1979) *J. Bacteriol.* **140**, 424–435.
10. Ishida, R., Miki, T., Narita, T., Yui, R., Sato, M., Utsumi, K. R., Tanabe, K. & Andoh, T. (1991) *Cancer Res.* **51**, 4909–4916.
11. Tanabe, K., Ikegami, Y., Ishida, R. & Andoh, T. (1991) *Cancer Res.* **51**, 4903–4908.
12. Boritzki, T. T., Wolfard, T. S., Besserer, J. A., Jackson, R. C. & Fry, D. A. (1988) *Biochem. Pharmacol.* **37**, 4063–4068.
13. Drake, F. H., Hofman, G. A., Mong, S. M., Bartus, J. O., Hertzberg, R. P., Johnson, R. K., Mattern, M. R. & Mirabelli, C. K. (1989) *Cancer Res.* **49**, 2578–2583.
14. Jensen, P. B., Sørensen, B. S., Demant, E. J. F., Sehested, M., Jensen, P. S., Vindelov, L. & Hansen, H. H. (1990) *Cancer Res.* **50**, 3311–3316.
15. Roca, J. & Wang, J. C. (1992) *Cell* **71**, 833–840.
16. Lindsley, J. E. & Wang, J. C. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10485–10489.
17. Lindsley, J. E. & Wang, J. C. (1993) *Nature (London)* **361**, 749–750.
18. Caron, P. R. & Wang, J. C. (1993) in *Molecular Biology of DNA Topoisomerases and Its Application to Chemotherapy*, Proceedings of the International Symposium on DNA Topoisomerases in Chemotherapy, Nagoya, Japan, 1991, eds. Andoh, T., Ikeda, H. & Oguro, M. (CRC, Boca Raton, FL), pp. 1–18.
19. Caron, P. R. & Wang, J. C. (1994) in *DNA Topoisomerase and Their Applications in Pharmacology*, ed. Liu, L. (Academic, Orlando, FL), in press.
20. Lindsley, J. E. & Wang, J. C. (1993) *J. Biol. Chem.* **268**, 8096–8104.
21. Zechiedrich, E. L. & Osheroff, N. (1990) *EMBO J.* **9**, 4555–4562.
22. Pommier, Y., Kerrigan, D. & Kohn, K. (1989) *Biochemistry* **28**, 995–1002.
23. Crenshaw, D. G. & Hsieh, T. (1993) *J. Biol. Chem.* **268**, 21328–21334.
24. Holm, C., Covey, J. M., Kerrigan, D. & Pommier, Y. (1989) *Cancer Res.* **49**, 6365–6368.
25. D'Arpa, P., Beardmore, C. & Liu, L. F. (1990) *Cancer Res.* **50**, 6919–6924.
26. Shin, C.-G. & Snapka, R. M. (1990) *Biochemistry* **29**, 10934–10939.
27. Yanagida, M. & Wang, J. C. (1987) in *Nucleic Acids and Molecular Biology*, eds. Eckstein, F. & Lilley, D. M. J. (Springer, Berlin), Vol. 1, pp. 196–209.
28. Creighton, A. M. (1970) in *Progress in Antimicrobial Anticancer Chemotherapy*, ed. Umezawa, H. (University Park Press, Baltimore), Vol. 1, p. 167.
29. Creighton, A. M. (1979) in *Advances in Medical Oncology, Research and Education*, ed. Fox, M. (Pergamon, Elmsford, NY), Vol. 5, p. 83.
30. Sharpe, H. B. A., Field, E. O. & Hellmann, K. (1970) *Nature (London)* **226**, 524–526.
31. Traganos, F., Darzynkiewicz, Z. & Melamed, M. R. (1981) *Cancer Res.* **41**, 4566–4576.
32. Ishida, R., Tanabe, K., Narita, T., Sato, M., Yui, R., Utsumi, K. R. & Andoh, T. (1993) *Molecular Biology of DNA Topoisomerases and Its Application to Chemotherapy*, Proceedings of the International Symposium on DNA Topoisomerases in Chemotherapy, Nagoya, Japan, 1991, eds. Andoh, T., Ikeda, H., and Oguro, M. (CRC, Boca Raton, FL), pp. 207–214.
33. Clarke, D. J., Johnson, R. T. & Downes, C. S. (1993) *J. Cell Sci.* **105**, 563–569.