Aclacinomycin A Stabilizes Topoisomerase I Covalent Complexes

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ABSTRACT

Aclacinomycin A (aclorubicin) is an anthracycline anticancer agent with demonstrated activity against both leukemias and solid tumors. Previous results suggested that a major activity of aclacinomycin A is the inhibition of topoisomerase II catalytic activity. We have applied a yeast system to test whether aclacinomycin A is a topoisomerase II inhibitor in vivo and to test whether we could identify other important targets of this drug. We have found that overexpression of yeast topoisomerase II confers resistance to aclacinomycin A in yeast, consistent with the hypothesis that this drug is a catalytic inhibitor of topoisomerase II. Interestingly, we have also found that in yeast, aclacinomycin A, like camptothecin, stabilizes topoisomerase I cleavage. We carried out biochemical analysis with purified human topoisomerase I and demonstrated that this drug efficiently stabilizes topoisomerase I covalent complexes, indicating that aclacinomycin A represents a novel class of combined topoisomerase I/II inhibitor.

INTRODUCTION

DNA topoisomerases are the target of a wide range of antitumor agents. Drugs that are active against topoisomerase I (1–3), topoisomerase II (4–7), or both enzymes (8–11) have been identified. The agents that are active against topoisomerase I that are in clinical trials are derivatives of the plant alkaloid camptothecin (reviewed in Refs. 12–15), whereas a range of structurally diverse compounds are active against eukaryotic topoisomerase II. Potent intercalating agents such as doxorubicin, mitoxantrone, and amsacrine (16, 17), as well as nonintercalating agents such as epipodophyllotoxins and fluoroquinolones, are active against eukaryotic topoisomerase II (18, 19).

Antitopoisomerase drugs in clinical use exert their cytotoxic effects by stabilizing an intermediate of the topoisomerase enzyme reaction. A large body of evidence has indicated that the intermediate acts as a type of DNA damage (2, 16, 20, 21). Cells are killed by antitopoisomerase agents because the enzyme is converted into a “poison” rather than by the lack of an essential enzyme activity (16, 22, 23). Inhibitors that stabilize covalent complexes in either topoisomerase I or II have been termed class I inhibitors and have also commonly been referred to as topoisomerase poisons. By contrast, class II drugs act as classical enzyme inhibitors of topoisomerases and have been termed catalytic inhibitors (24). Examples of this class of drug include agents that are competitive inhibitors of ATP binding to topoisomerase II, such as camptothecin and novobiocin (25, 26). These agents are most potent against prokaryotic type II topoisomerases. Recently, several novel class II inhibitors of eukaryotic topoisomerase II have been described. These inhibitors have two different mechanisms for inhibiting topoisomerase II (27). Drugs such as bisdioxopiperazines (i.e., ICRF-187 and ICRF-193) do not interact with DNA, but interact directly with topoisomerase II (28, 29). They are not competitive inhibitors of ATP binding; indeed, they require ATP to interact with topoisomerase II. Experiments by Wang and colleagues (29) indicate that bisdioxopiperazines lock topoisomerase II in a “closed clamp” conformation, a conformation in which topoisomerase II cannot carry out strand passage, hydrolyze ATP, or turn over to start a new reaction cycle. It has also been suggested that merbarone, a barbituric acid derivative, acts as a catalytic inhibitor of topoisomerase II that does not interact with DNA, but it is unknown whether this agent inhibits topoisomerase II like bisdioxopiperazines (30, 31). A second group of catalytic inhibitors bind to DNA strongly and appear to block topoisomerase II binding to DNA. This class of agents includes the anthracycline aclacinomycin A (32, 33).

We have previously described yeast strains for assessing the mechanism of action of antitopoisomerase agents and have applied the system to both class I and II topoisomerase II inhibitors (23, 34). We wanted to demonstrate whether aclacinomycin A is a class II topoisomerase II inhibitor in vivo and to test whether we could identify other important targets of this drug. In agreement with the results of Jensen and colleagues (32, 35), we have found that the action of aclacinomycin A in yeast is consistent with the drug acting as a catalytic inhibitor of topoisomerase II. Interestingly, we have also found that in yeast, aclacinomycin A kills cells through stabilization of topoisomerase I cleavage. We also carried out biochemical analysis with purified human topoisomerase I and demonstrated that this drug efficiently stabilizes topoisomerase I covalent complexes, indicating that aclacinomycin A represents a novel class of combined topoisomerase I/II inhibitor.

MATERIALS AND METHODS

**Yeast Strains.** The yeast strains and their genotypes are listed in Table 1. JN271L was constructed using standard genetic techniques. The isel mutation was derived from strain FL599. Disruption of the yeast TOP1 gene was by one-step gene disruption, as described previously (36). Strains overexpressing topoisomerase II were obtained by transformation with pDEDTOP2 (20). Expression of topoisomerase I from a plasmid was accomplished using the plasmid YEPPlac181TOP1, which carried a 3.8-kb HindIII/HindIII fragment carrying the complete yeast TOP1 gene in the HindIII site of YEPPlac181.

**Drug Sensitivity Determination.** Drug sensitivity in yeast cells was carried out as described previously (20, 37). Briefly, a logarithmically growing culture of yeast cells was diluted to 2 × 10⁶ cells/ml, and drug or DMSO was added. Aliquots were removed, diluted, and plated to YPDA agar. For experiments examining cell survival with cells carrying YEPPlac181TOP1, cells were plated to yeast synthetic medium lacking leucine. Survival is expressed relative to the number of viable colonies at the time of drug addition. Drug sensitivity determinations were carried out at least three times for each strain, and representative results are shown.

**Chemicals.** Oligonucleotides were purchased from The Midland Certified Reagent Co. (Midland, TX). α-32P-labeled cordycepin 5’-triphosphate was purchased from DuPont New England Nuclear (Boston, MA). Polyacrylamide was purchased from Bio-Rad, Inc. (Richmond, CA). Human recombinant topoisomerase I was purified from baculovirus as described previously (38). Camptothecin was provided by Drs. M. Wani and M. Wall (Research Triangle Institute, Research Triangle Park, NC). Aclacinomycin A (NSC 208734) and derivatives were obtained either from the National Cancer Institute Drug Anticancer screen or were provided by Dr. Peter Jensen (Righshospitalet, Copenhagen, Denmark). Ten mg aliquots of each drug were stored at −20°C and thawed and diluted to an appropriate concentration in DMSO just before use.

**Oligonucleotide Labeling and Annealing.** The scissile (upper) strand of the oligonucleotide was labeled with α-32P-labeled cordycepin using terminal transferase and then annealed to the complementary strand. The concentration of the labeled oligonucleotide was determined by liquid scintillation counting. (CANCER RESEARCH 57, 4564-4569, October 15, 1997)
The present work is the source of these strains and genotypes.

### Table 1  Yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>JN271L</td>
<td>ura3-52 leu2Δ his7 ile1</td>
</tr>
<tr>
<td>JN271Ltop1</td>
<td>As JN271L but top1::URA3</td>
</tr>
<tr>
<td>JN271IPDEDTOP2</td>
<td>As JN271L but carrying pDEDTOP2</td>
</tr>
</tbody>
</table>

* The present work is the source of these strains and genotypes.

deoxyribonucleotidyl transferase (Stratagene, La Jolla, CA) as described previously (39). The reaction mixture was subsequently centrifuged through a G25 Sephadex column to remove unincorporated cordycepin. The labeled scissile strand was then annealed to an equimolar amount of unlabeled noncissile (lower) strand by heating the mixture at 95°C for 5 min and cooling down overnight.

**Topoisomerase I Reactions.** DNA substrate (approximately 50 fmol/reaction) was incubated with 1 μl of purified top1 protein (50 units) of purified recombinant topoisomerase I (40) for 15 min at 25°C with or without drug in 10 μl of standard reaction buffer [10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, and 15 μg/ml BSA]. Reactions were stopped by adding SDS (final concentration, 0.5%).

**Gel Electrophoresis and Analysis of Cleavage Products.** Samples were prepared for gel electrophoresis by adding 3.3 volumes of Maxam Gilbert loading buffer (80% formamide, 0.001 M EDTA, 10 mM NaOH, 1 mg/ml xylene cyanol, and 1 mg/ml bromphenol blue) to the reaction mixtures before loading. Electrophoresis was accomplished using 16% denaturing polyacrylamide gels containing 7 M urea and were run at 40 V/cm at 50°C for 2-3 h. Gels were dried on 3MM Whatman paper sheets, and imaging and quantitation were performed using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

### RESULTS

Our first experiments were designed to test whether aclacinomycin A acts as a catalytic inhibitor of topoisomerase II in vivo. We examined the effect of topoisomerase II overexpression on sensitivity to aclacinomycin A, predicting that overexpression of topoisomerase II should lead to resistance to aclacinomycin A. A representative experiment, shown in Fig. 1, demonstrates that JN271L cells were sensitive to aclacinomycin A, with 10 μg/ml drug reducing cell survival and 100 μg/ml drug reducing cell survival by a factor of 10². The same strain carrying pDEDTOP2 was considerably less sensitive to aclacinomycin A, with cell survival reduced only about 20-fold at a drug concentration of 100 μg/ml. These results are similar to what we reported previously with the class II topoisomerase II inhibitor ICRF-193 (41), and they support the hypothesis that aclacinomycin A kills cells partly by acting as a catalytic inhibitor of topoisomerase II.

We observed previously that yeast cells that lack topoisomerase I were hypersensitive to inhibitors of topoisomerase II (41, 42). We observed hypersensitivity to both class I and II topoisomerase II inhibitors. We therefore examined the sensitivity of yeast cells carrying a top1 deletion to aclacinomycin A. The results are shown in Fig. 2. Cells lacking top1 were able to grow in 10 μg/ml drug, and cell killing was attenuated substantially at 100 μg/ml, compared to the isogenic strain carrying wild-type topoisomerase I. These results showed that a lack of topoisomerase I increased cell survival compared to cells with wild-type topoisomerase I, a pattern suggestive of a topoisomerase I poison such as camptothecin.

To test further whether aclacinomycin A might be acting as a topoisomerase I poison, we transformed JN271tal cells with either YEPlac181 (43) or YEPlac181 carrying yeast topoisomerase I expressed from its own promoter (YEPlac181TOP1). If topoisomerase I is a negative survival factor following exposure to aclacinomycin A, then cells carrying YEPlac181TOP1 should be more sensitive to the drug than cells carrying YEPlac181. To confine the experiment to cells carrying plasmid, viability was determined by plating cells to synthetic medium without leucine. The results of this experiment are shown in Fig. 3. There was a minimal inhibition of growth of cells carrying the YEPlac181 when exposed to 10 μg/ml aclacinomycin A and a slight reduction in viable cells at 50 μg/ml aclacinomycin A. By contrast, cells carrying YEPlac181TOP1 have reduced viability in 10 μg/ml aclacinomycin A and a 10⁴-fold reduction in viability after 24 h of exposure to 50 μg/ml aclacinomycin A.

The results above demonstrated that topoisomerase I was very deleterious to cells exposed to aclacinomycin A and strongly suggested that this drug may act as a topoisomerase I poison. To verify the effect of aclacinomycin A, we directly examined cleavage stabilized by purified human recombinant topoisomerase I using an oligonucleotide as a specific DNA substrate for topoisomerase I cleavage. The oligonucleotide included the hexadecameric sequence identified by Westergaard and colleagues as a potent site for topoisomerase I cleavage. The oligonucleotide included the hexadecameric sequence identified by Westergaard and colleagues as a potent site for topoisomerase I cleavage (44, 45). The hexadecameric site was modified by including a guanine 3’ to the topoisomerase recognition sequence to enhance the effect of camptothecin (46). The oligonucleotide was end labeled using ³²P-labeled cordycepin at the 3’-terminus of the (upper) strand as described in “Materials and Methods.” Cleavage at the unique
topoisomerase I site gave rise to a 19-mer product (Fig. 4A, arrowhead). Fig. 4B shows the results obtained using this assay with either aclacinomycin A and with camptothecin as a positive control. For both camptothecin and aclacinomycin A, a higher yield of the cleaved 19-mer product was observed in the presence of the drug (Lanes 3–6) compared to the samples incubated with topoisomerase I in the absence of the drug. For both camptothecin and aclacinomycin A, there was slightly less cleaved product at 100 μM (Lanes 6) than at the next lower concentration, 10 μM (Lanes 5).

We next examined the effect of two other aclacinomycin A derivatives, marcellomycin and rudolfomycin, on cleavage by topoisomerase I. The structure of these compounds along with aclacinomycin A are shown in Fig. 5. The compounds are similar to aclacinomycin A except for the terminal sugar moiety. Fig. 4C shows the results obtained with the three compounds using the specific topoisomerase I cleavage assay. A quantitation of the results for the three aclacinomycin A derivatives, along with camptothecin, are shown in Fig. 4D. A dose-dependent increase in the cleaved 19-mer product was observed for all of the agents except marcellomycin. Rudolfomycin was the most potent anthracycline for producing the cleaved 19-mer, aclacinomycin A was less potent, and marcellomycin was the least potent of the three derivatives. The potency for rudolfomycin was similar to camptothecin up to a concentration of about 10 μM. Taken together, our results show that aclacinomycin A and some of its derivatives are active as topoisomerase I poisons.

DISCUSSION

We present two lines of evidence that aclacinomycin A targets topoisomerase I. First, yeast strains lacking topoisomerase I were more resistant to the cytotoxic action of aclacinomycin A than isogenic strains with wild-type topoisomerase I. This is similar to the results obtained with the topoisomerase I targeting drugs of the camptothecin family (47, 48) and is in marked contrast to the results obtained with agents specific for topoisomerase II (36, 41). Furthermore, we demonstrated by a direct cleavage assay that incubation of topoisomerase I and DNA in the presence of aclacinomycin A leads to enhanced levels of DNA cleavage. Taken together, our results demonstrate that topoisomerase I is a target for aclacinomycin A.

Our results showed that aclacinomycin A also acts on topoisomer-
ACLACINOMYCIN A INHIBITS top1

Fig. 4. DNA cleavage by topoisomerase in the presence of aclacinomycin A. A, a modified \textit{Teratokema} oligonucleotide was used as a specific DNA substrate for topoisomerase I cleavage. Labeling was performed with \textsuperscript{32}P-labeled cordycepin (\textsuperscript{32}P) at the 3'-terminus of the scissile (upper) strand as described in "Materials and Methods." Cleavage at the unique top I site gives rise to a 19-mer product (arrowhead). B, effects of aclacinomycin A on topI-mediated DNA cleavage (camptothecin was used as a control). Reactions were performed as described in "Materials and Methods." The first set of reactions was performed with camptothecin as a positive control; the second set of reactions included aclacinomycin A. Lanes 1, DNA; Lanes 2, topoisomerase I protein. Other lanes are in the presence of topoisomerase I protein and drug with the following concentrations: Lanes 3, 0.1 \textmu M; Lanes 4, 1 \textmu M; Lanes 5, 10 \textmu M; and Lanes 6, 100 \textmu M. C, effects of two new aclacinomycin A derivatives on topoisomerase I-mediated DNA cleavage. Reactions were performed as in B. Lanes 1, DNA; other lanes are in the presence of the following concentrations of drugs: Lanes 2, 0.1 \textmu M; Lanes 3, 1 \textmu M; and Lanes 4, 10 \textmu M. D, quantitation of drug-stabilized cleavage. The intensities of the cleaved bands as determined by the phosphorimager from C along with the results for camptothecin from B are shown.

Jensen and colleagues (32, 33) have demonstrated previously that aclacinomycin A antagonized cell killing by antitopoisomerase II agents. Our results are consistent with their finding that this drug acts as a class II topoisomerase II inhibitor. Agents that inhibit topoisomerase II would be expected to antagonize the cytotoxicity of topoisomerase II poisons such as etoposide and amarscine. In a separate report, Jensen and colleagues (50) also found that, although aclacinomycin A antagonized cell killing by camptothecin, the drug could produce single strand breaks in the presence of purified topoisomerase I and the absence of camptothecin (50). Our results agree with their suggestion that aclacinomycin A is active against topoisomerase I. Our experiments in yeast have enabled us to show that topoisomerase I is an important determinant of cell killing by this drug.

Although the mechanistic details of topoisomerase I poisoning remain to be elucidated, significant information on the structure of various anthracyclines bound to DNA has been obtained. This structural information may give important clues to the mechanism of topoisomerase I poisoning. The detailed X-ray structure of aclacinomycin A bound to DNA has not yet been determined, but its binding to DNA has been studied by DNase I footprinting, alkylation protection, and solution nuclear magnetic resonance spectroscopy. The DNase I footprint showed an increased number of bp protected with the number of saccharide groups present, consistent with DNA binding of the saccharide side chains (51). Chemical probes suggested that the aclacinomycin A protects adenines in the minor groove from alkylation, suggesting that the trisaccharide lies in the minor groove. Nuclear magnetic resonance spectroscopic studies with two different aclacinomycin derivatives also place the trisaccharide in the minor groove of DNA (52). This structure is reminiscent of the determined structure of a morpholino derivative of doxorubicin bound to a DNA hexamer. The anthracycline chromophore intercalation distorts the DNA helix similarly to those observed in other DNA-anthracycline complexes, and the morpholino group lies in the DNA minor groove (53). Significantly, morpholinodoxorubicin has also been shown to act as a topoisomerase I poison (17). These results suggest that interactions in the minor groove of DNA are important for topoisomerase I poisoning, in agreement with the hypothesis of Liu and colleagues (54).

The three aclacinomycin derivatives examined differ mainly in the terminal saccharide and have markedly different potencies for poisoning topoisomerase I. The three compounds have differences in the aglycone chromophore. The differences in the chromophore may lead to different DNA-binding affinities. In addition, the terminal saccharide may affect the precise interaction of the drug with the minor groove of DNA. An alternate possibility is that the different terminal saccharides may differ in their ability to interact with topoisomerase I. If this latter possibility is correct, these compounds may shed insight on how topoisomerase I can be poisoned by anticancer agents. We are presently examining defined series of aclacinomycins to distinguish between these possibilities.

Our results are also of potential relevance to the clinical use of 3 J. L. N., unpublished data.
ACKNOWLEDGMENTS

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REFERENCES


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