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Characterization of DNA Topoisomerase I from *Candida albicans* as a Target for Drug Discovery

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***Candida albicans* is an opportunistic pathogen responsible for life-threatening infections in persons with impaired immune systems. Topoisomerase I is a potential target for novel antifungal agents; however, in order for this enzyme to be a therapeutically useful target, it needs to be demonstrated that the fungal and human topoisomerases differ sufficiently as to allow the fungal topoisomerase to be selectively targeted. To address this question, we isolated the topoisomerase I from *C. albicans* and compared its biochemical properties with those of the mammalian enzyme. Similar to other eukaryotic type I topoisomerases, the *C. albicans* type I topoisomerase has an apparent molecular mass of 102 kDa and covalently links to the 3′ end of DNA, as shown after the reaction is interrupted by sodium dodecyl sulfate. Topoisomerase poisons such as camptothecin act by stabilizing the cleavage complex formed by the topoisomerase I and DNA. We observed that the *C. albicans* and mammalian type I topoisomerases differ in that the *C. albicans* cleavage complex is approximately 10-fold less sensitive to camptothecin than the mammalian cleavage complex is. In addition, we found that the antifungal agent eupolauridine can stabilize the cleavage complex formed by both the *C. albicans* and human topoisomerases and that the response of the *C. albicans* topoisomerase I to this drug is greater than that of the human enzyme. Thus, the topoisomerase I from *C. albicans* is sufficiently distinct from the human enzyme as to allow differential chemical targeting and will therefore make a good target for antifungal drug discovery.**

Topoisomerases are enzymes which modulate the topological structure of DNA (for recent reviews, see references 7, 20, and 30). Topoisomerases are able to relax supercoiled DNA and unlink catenated DNA circles, among other functions. These enzymes perform these reactions by making a transient break in the DNA backbone, passing another segment of DNA through this break, and then resealing the break. Type I topoisomerases break only one DNA strand, while type II topoisomerases can introduce a double-strand break into DNA. In the eukaryotic cell, type I topoisomerases are associated with elongating transcription and replication forks, while type II topoisomerases are required for segregation of daughter chromosomes after cell division (1, 12, 13, 18, 34, 37).

Topoisomerases form a complex with DNA in which the transiently broken ends of the DNA are constrained. When a protein denaturant is added, a DNA break is revealed, and the denatured topoisomerase is found to be covalently attached to one end of the break (5, 11, 16, 33, 35). This complex between topoisomerase and DNA has been termed the cleavage complex (26; reviewed in reference 23).

Certain drugs stabilize the cleavage complex, such as the anticancer drug camptothecin, which targets the eukaryotic topoisomerase I (19). A structure-activity relationship for camptothecin derivatives has been established, and a correlation is seen between the cytotoxicity of the compound in replicating cells and its ability to stabilize the cleavage complex formed by purified topoisomerase I with DNA (17, 22). Type II topoisomerases are the targets of the antibacterial quinolone drugs, which act on the prokaryotic topoisomerase II (16, 35), and anticancer drugs such as adriamycin and etoposide, which target the eukaryotic topoisomerase II (10, 31, 36). For all these classes of compounds, it is the stabilization of the topoisomerase cleavage complex in the cell that can trigger a process leading to cell death (reviewed in references 9, 23, and 32).

Candida albicans is a normal human commensal organism; however, in people with impaired immune systems, such as patients with cancer undergoing chemotherapy, organ transplant recipients, and patients with AIDS, *C. albicans* can cause secondary infections that are life-threatening. The mortality rate from systemic candidiasis is greater than 70%, and candidiasis contributes to the deaths of 7 to 43% of immunocompromised patients (statistics vary with the site of patient care) (38). At present, the choices for antifungal therapies are limited, and in addition, fungal strains resistant to the current therapies have become an increasing problem in hospitals (28). Thus, there is a need for new antifungal therapeutic agents.

We hypothesized that compounds which stabilize the cleavage complex formed by topoisomerases in the fungal cell would be fungicidal, as is the case for the bactericidal quinolones and cytotoxic poisons of mammalian topoisomerases. In order for a topoisomerase poison to be therapeutically useful as an antifungal agent, it must be specific for the fungal topoisomerase and have minimal effects on the corresponding human enzyme. As an initial step in the process of discovering such an agent, we isolated the topoisomerase I from *C. albicans* and compared the properties of this enzyme with the type I topoisomerases isolated from bovine and human cells. Several properties are shared by the type I topoisomerases from these sources, including apparent molecular mass and reaction requirements.

We found differences in the efficiencies of cleavage complex formation by the fungal and mammalian type I topoisomerases and, in addition, that the cleavage complex formed by the mammalian topoisomerase I is stabilized by lower concentrations of camptothecin than the fungal cleavage complex is. This result demonstrates that there are biochemical differences between the fungal and human type

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I topoisomerases that may allow chemical targeting of one with respect to the other.

Eupolauridine is a natural product isolated from the bark of species of the genus *Eupomatia* (3) which has been demonstrated to have anticandidal activity (21). During these studies, we found that eupolauridine also stabilizes the cleavage complex formed by both the fungal and mammalian type I topoisomerases, a property of this compound that was hitherto unknown. The fungal topoisomerase shows a greater response to eupolauridine than the human topoisomerase I, demonstrating that agents which show some specificity for the fungal topoisomerase can be identified.

MATERIALS AND METHODS

Materials. The supercoiled ColE1 DNA used as a substrate was a gift of John Baranowski (Abbott Laboratories). Camptothecin was purchased from the Sigma Chemical Co., and eupolauridine was synthesized by Larry Klein and Clinton Yeung (Abbott Laboratories). Drugs were dissolved in dimethyl sulfoxide at a concentration of 4 mg/ml and stored at 4°C. Proteinase K, leupeptin, and pepstatin were from Boehringer Mannheim. Bacteriophage T4 polynucleotide kinase and bovine serum albumin (BSA) were purchased from Bethesda Research Laboratories.

Enzyme activity. Topoisomerase I catalytic activity was measured by ATP-independent DNA relaxing activity. The standard buffer used was made up of 25 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, and 50 μg of BSA per ml. Reactions were in 20 μl and contained 0.2 μg of supercoiled ColE1 DNA per reaction. Reaction mixtures were incubated for 15 min at 37°C and stopped by the addition of sodium dodecyl sulfate (SDS) to 0.5%; this was followed by digestion with proteinase K in loading buffer (0.67% SDS, 67 mM EDTA, 26.7% sucrose, 0.67% bromphenol blue). One unit of relaxation activity was taken as the activity required to relax 50% of the supercoiled ColE1 DNA under these conditions. DNA products were analyzed by electrophoresis for 16 h at 20 mA in 0.8% agarose gels in TBE buffer (90 mM Tris, 90 mM borate, 2.5 mM EDTA).

The formation of the cleavage complex was measured by incubating the topoisomerase I with DNA in TINA buffer (25 mM Tris-HCl [pH 7.4], 50 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM EDTA, and 50 μg of BSA per ml) for 5 min at 37°C and then stopping the reaction by the addition of SDS to 0.5%. Reaction mixtures were digested with proteinase K and were subjected to electrophoresis as described above, with the exception that ethidium bromide was included in the electrophoresis buffer at a concentration of 0.4 μg/ml. The extent of DNA nicking was measured by densitometry of photographic negatives of the ethidium bromide-stained DNA by using an LKB laser densitometer (Pharmacia). The total amount of DNA was determined for each reaction mixture, and the nicked DNA was expressed as a percentage of the total DNA.

Isolation of *C. albicans* topoisomerase I. Wild-type *C. albicans* ATCC 10321 cells were grown in YPD broth (1% Bacto yeast extract; 2% Bacto Peptone; 2% glucose) at 30°C until the optical density of the culture at 600 nm reached 8, at which point the culture was in the mid- to late-log phase. The cells were quickly chilled on ice, harvested by centrifugation at 4,600 × *g* for 20 min at 4°C in a Sorvall H-6000A rotor, washed with buffer T (50 mM Tris-HCl [pH 7.6], 20 mM KCl, 1 mM EDTA, and 10% glycerol with 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium bisulfite, 1 μg of pepstatin per ml, 1 μg leupeptin per ml, and 0.1 mM dithiothreitol; the last five components were added just prior to use), frozen in dry ice-ethyl alcohol, and stored at −80°C.

Cells from 12 liters of culture were lysed mechanically by using a glass bead beater with 0.5-mm-diameter glass beads (BioSpec) in buffer T. The lysate was brought to 0.5 M NaCl with 5 M NaCl, stirred slowly for 30 min at 4°C, and then centrifuged at 30,000 × *g* for 40 min in a Sorvall SS-34 rotor. The supernatant was then decanted to give fraction I, the cell lysate. This material was mixed with 24 g of hydroxylapatite resin (Bio-Rad), and the mixture was stirred slowly for 40 min at 4°C. The resin suspension was centrifuged at 4,000 × *g* for 5 min in a Sorvall SS-34 rotor, the supernatant was discarded, and the resin was resuspended in 250 ml of buffer P plus 0.2 M KPO_4 (pH 7.5) (buffer P is 15 mM KPO_4 [pH 7.5], 0.1 mM EDTA, and 10% glycerol with 1 mM phenylmethylsulfonyl fluoride, 1 μg of pepstatin per ml, 1 μg of leupeptin per ml, and 0.1 mM dithiothreitol; the last four components were added just prior to use). After a 10-min incubation, the resin was recovered by centrifugation as described above and was washed again with buffer P–0.2 M KPO_4 (pH 7.5). The washed resin was resuspended in 25 ml of buffer P–0.7 M KPO_4 (pH 7.5) to elute the topoisomerases and was incubated with gentle agitation for 10 min at 4°C. The resin was removed by centrifugation, and the elution step was repeated two more times. The eluates were pooled to give fraction II, the hydroxylapatite eluate.

Fraction II was mixed with 50 ml of phosphocellulose P11 resin (Whatman) in the presence of a volume of buffer P equal to 10 times the volume of fraction II. Prior to use, the phosphocellulose resin was hydrated as recommended by the manufacturer, incubated with 1 M KPO_4 (pH 7.5), and then equilibrated with buffer P–50 mM NaCl. After 60 min at 4°C, the phosphocellulose resin was recovered by centrifugation at 4,000 × *g* for 15 min in a Sorvall GSA rotor, gently resuspended with buffer P–50 mM NaCl, and packed into a column to allow elution of the topoisomerases. The column was washed with buffer P–0.35 M NaCl, and then topoisomerase II was eluted with buffer P–0.56 M NaCl. Buffer P–0.9 M NaCl was then used to elute the type I topoisomerase. The fractions containing topoisomerase I activity were pooled to give fraction III, the phosphocellulose eluate.

For each ml of fraction III, 0.2 g of $(NH_4)_2SO_4$ was slowly added, and this was loaded onto a 10-ml butyl-Sepharose (Bethesda Research Laboratories) column that had been equilibrated with buffer P–0.9 M NaCl–0.2 g of $(NH_4)_2SO_4$ per ml. The column was washed with this buffer and was then developed with a gradient of high to low salt concentrations to elute the bound topoisomerase. The starting buffer was buffer P–0.9 M NaCl–0.2 g of $(NH_4)_2SO_4$ per ml, and the final buffer was buffer P–0.1 g of $(NH_4)_2SO_4$ per ml. Fractions containing peak topoisomerase activity were pooled to give fraction IV. Fraction IV was concentrated by using Centricon 30 columns (Amicon) and was stored in 50% glycerol–25 mM Tris (pH 8.0)–50 mM KCl–0.1 mM EDTA–10 mM 2-mercaptoethanol at −20°C.

During purification, topoisomerase I activity was monitored by the DNA relaxation assay described above. The protein concentration was determined by the Coomassie blue dye binding method of Bradford (4). Topoisomerase II activity was measured by the unknotting assay described by Liu et al. (24) by using knotted DNA from bacteriophage P4 as a substrate.

Sources of mammalian type I topoisomerases. Topoisomerase I from bovine calf thymus was purchased from Bethesda Research Laboratories. Topoisomerase I was isolated from HeLa cells by a procedure suggested by Leroy Liu (Johns

TABLE 1. Purification of topoisomerase I from *C. albicans*

Fraction	Step	Sp act (U/mg)[a]
I	Nuclear lysate	30–100
II	Hydroxylapatite	9,000
III	Phosphocellulose	70,000
IV	Butyl-Sepharose	125,000

[a] Specific activity of the topoisomerase I at different stages of purification. Units of relaxation activity were determined as described in the text. Protein concentration was determined by the Coomassie-blue dye binding method of Bradford (4) by using BSA as a standard. The specific activity of fraction I was difficult to measure because of the presence of nucleic acids and nucleases; thus, a range of values observed for this fraction from several independent lysates is given. No topoisomerase II activity was detected in fraction III or IV.

Hopkins University). Briefly, the cells were disrupted with a Dounce homogenizer, the nuclei were recovered and extracted with 0.35 M NaCl, and the resulting material was chromatographed on a BioRex-70 column (Bio-Rad) developed with a gradient of 0.2 to 1 M NaCl in buffer P. The peak topoisomerase activity was then adsorbed to hydroxylapatite and eluted with a step of 0.8 M KPO_4 (pH 7.5) in buffer P. This material was concentrated and stored as described above.

Label transfer. The modification of the DNA transfer reaction (5) described here was suggested by Tao Hsieh (Duke University). A single-stranded DNA oligomer (5′-CCGAATTCGG-3′; obtained from Bethesda Research Laboratories) was labeled at the 5′ end with bacteriophage T4 polynucleotide kinase. The reaction mixture contained 20 U of kinase, 0.7 μg of DNA, 25% glycerol, 10 mM $MgCl_2$, 5 mM Tris-HCl (pH 9.5), and 1.1 μM [γ-^{32}P]ATP (3,000 Ci/mmol; obtained from Amersham) and was incubated at 37°C for 30 min. The resulting labeled DNA was added directly to the topoisomerase I reaction buffer, at a final dilution of 5 μl of kinase reaction mixture per 60 μl of topoisomerase reaction mixture. The mixture was incubated for 3 min at 37°C and then stopped by the addition of SDS to a final concentration of 1.5%. The topoisomerase in the reaction was precipitated by the addition of trichloroacetic acid to 10%, recovered by centrifugation, washed with acetone, and then resuspended in SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer (125 mM Tris [pH 6.8], 700 mM 2-mercaptoethanol, 20% glycerol, 4% SDS, 0.02% bromphenol blue), boiled, and analyzed by SDS-PAGE by using precast 7.5% acrylamide gels (Integrated Separation Systems). After electrophoresis, the gel was stained with Coomassie dye, dried, and autoradiographed with Kodak XAR film for 16 h.

RESULTS

Isolation of topoisomerase I. Topoisomerase I was extracted from *C. albicans* cells and further purified by chromatography by using hydroxylapatite, phosphocellulose, and butyl-Sepharose resins. The details of this procedure are given in Materials and Methods. At each step, the active topoisomerase I was localized by measuring the DNA relaxation activity in the column fractions, and the most active fractions were pooled.

The specific activity of each step in the purification procedure is given in Table 1. The presence of the endogenous nucleic acids and nucleases in the initial cell lysate interfered with the determination of topoisomerase I activity in fraction I. For this reason, a range of estimates for activity in this fraction is given, reflecting the activity determined in several independent cell lysates. The specific activities observed for fractions II and III were more reproducible, since nucleic acids were removed by the hydroxylapatite step and nucleases were removed by chromatography on phosphocellulose (data not shown). No topoisomerase II activity was detected in the preparation after phosphocellulose chromatography (data not shown).

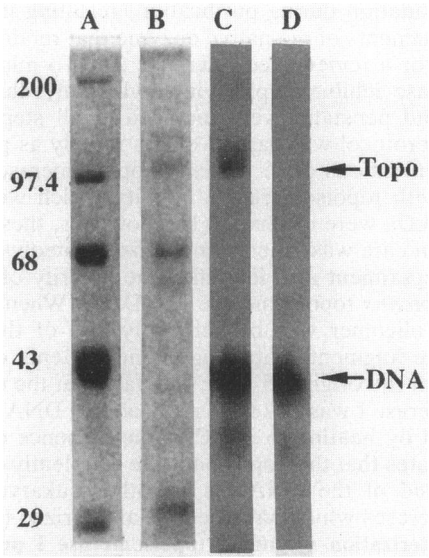

FIG. 1. Label of active topoisomerase I species. Lane A, molecular mass markers (in kilodaltons; from Bethesda Research Laboratories); lane 2, *C. albicans* topoisomerase fraction IV after the label transfer reaction (see text). Both A and B are stained with Coomassie dye. Lane C, the autoradiograph of lane B; lane D, autoradiograph of a lane containing only the DNA substrate. The band labeled Topo was seen only in reactions containing topoisomerase I.

Identification of active species. A label transfer experiment was performed to identify the active species in the topoisomerase I preparation. In this experiment, a single-stranded DNA oligomer was radiolabeled at the 5′ position as described in Materials and Methods, and this substrate was used to react with the topoisomerase I preparation. The topoisomerase I forms a cleavage complex with this substrate, and the addition of SDS allows the recovery of denatured topoisomerase I covalently linked to DNA.

This material was analyzed by SDS-PAGE, and the results are shown in Fig. 1. Lane A contains markers of the indicated molecular masses, and lane D is the autoradiograph of a lane containing radiolabeled DNA without topoisomerase I. The protein composition of the topoisomerase I preparation, detected by Coomassie blue staining of the gel, is shown in lane B, and the autoradiograph of this material is in lane C. The autoradiograph contains a band that exactly comigrated with a protein band seen in lane B which has a mobility consistent with a protein of 102 kDa. The other band in lane C had a mobility similar to that of the DNA band seen in control reactions containing only DNA (lane D).

This experiment demonstrated that the fungal topoisomerase I is recovered as a single species with an apparent molecular mass of 102 kDa. This is very similar to the mammalian topoisomerase I, which has a molecular mass of 100 kDa (25). Type I topoisomerases isolated from other organisms have been found to be very sensitive to proteo-

lytic degradation during purification, resulting in the isolation of fragments of the native enzyme that retain relaxation activity (for a review, see reference 27). To minimize this, the protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, and pepstatin were included in all steps; and the isolation protocol was carried out as rapidly as possible. In initial preparations of *C. albicans* topoisomerase I, protein species with topoisomerase I activity which were smaller than 100 kDa were observed (15); however, these were not seen when care was taken to minimize proteolysis.

This experiment also identified the polarity of linkage of the *C. albicans* topoisomerase I to DNA. When the single-stranded oligomer was broken, only one of the resulting pieces, the fragment containing the new 3′ end, could carry the label at the original 5′ end. The fact that the *C. albicans* topoisomerase I was linked to radiolabeled DNA after being denatured by heating to 100°C in the presence of 2% SDS demonstrates that this topoisomerase covalently links to the new 3′ end of the DNA, as do other eukaryotic type I topoisomerases which have been characterized (6).

Characterization of fungal topoisomerase I activity. The relaxation of supercoiled DNA was used as a measure of topoisomerase I catalytic activity. The topoisomerase requires monovalent salt for activity, and activity was maximized in a reaction mixture containing between 100 and 150 mM monovalent salt, similar to the requirements of the HeLa topoisomerase I as measured in our laboratory (data not shown) and consistent with earlier reports (25). The *C. albicans* topoisomerase I does not require exogenous magnesium for catalytic activity (data not shown), as is characteristic of other eukaryotic type I topoisomerases (8).

The formation of the camptothecin-stabilized cleavage complex by topoisomerase I was measured by incubating the enzyme with supercoiled DNA in reaction buffer containing camptothecin at a concentration of 100 μg/ml. The reaction was stopped with SDS, the topoisomerase was hydrolyzed with proteinase K, and the resulting products were analyzed by agarose gel electrophoresis. As has been observed for the mammalian topoisomerase I, the formation of the cleavage complex by the fungal enzyme is sensitive to ionic strength and is maximal when the monovalent salt concentration is 50 mM (data not shown).

The effect of divalent cations on the formation of the camptothecin-stabilized cleavage complex was also determined, and these results are given in Table 2. The level of cleavage complex formation in a typical experiment was determined by densitometric quantitation of the nicked DNA band as a function of the total DNA in each reaction. Over a concentration range of 1 to 10 mM, the formation of the cleavage complex was maximized by the presence of either $CaCl_2$ or $MgCl_2$. This is very similar to the response of the bovine topoisomerase I, also given in Table 2, and the HeLa topoisomerase I preparation used (data not shown).

Measurement of the formation of the cleavage complex by the fungal topoisomerase I. Since the formation of the cleavage complex is a stoichiometric reaction by the topoisomerase, the inclusion of more topoisomerase I molecules in the reaction mixture leads to a higher yield of nicked DNA molecules. Therefore, in order to compare the efficiency of cleavage complex formation by different enzyme preparations, a similar molar concentration of topoisomerase I must be present in the two reactions. Since the fungal topoisomerase I preparation was only partially purified, we compared the intensity of Coomassie staining of the 102-kDa protein band corresponding to an equivalent number of enzymatic units of the purified fungal and HeLa topoisomerase I preparations used in our laboratory (data not shown). By this measure, we estimated that the fungal and HeLa type I topoisomerases would be expected to have similar specific activities if they were homogeneous, and so reactions containing equal numbers of catalytic units were expected to contain comparable molar concentrations of topoisomerase I.

TABLE 2. Effects of divalent cations on nicking by topoisomerase I[a]

Cation (concn [mM])	% Nicked DNA	
	Bovine topo I	*C. albicans* topo I
$MgCl_2$		
0	29	11
1	36	40
3	38	38
10	30	25
$CaCl_2$		
0	29	11
1	51	28
3	34	31
10	20	18

[a] The extent of nicking after SDS denaturation of the cleavage complex was quantified by densitometry and expressed as a percentage of the total DNA in the reaction mixture. Each reaction mixture contained 75 catalytic units of topoisomerase I (topo I) and 100 μg of camptothecin per ml.

The topoisomerase reaction produces both nicked circles (form II DNA), which result from the disruption of the cleavage complex, and relaxed topoisomers that are the product of the catalytic activity of the topoisomerase. Since under standard conditions for agarose gel electrophoresis these species have indistinguishable electrophoretic mobilities, the intercalator ethidium bromide was included in the electrophoresis buffer. Ethidium bromide does not alter the mobility of the nicked species; however, the presence of ethidium bromide greatly increases the mobility of the relaxed topoisomers, which then migrate in a position different from those of the nicked products. The effect of ethidium bromide on electrophoretic mobility can be seen in Fig. 2. Form II DNA, the nicked species, was the species with the slowest mobility on the gel. The covalently closed species (form I) included both the negatively supercoiled starting material (lane E) and the relaxed topoisomers produced by the topoisomerase in the reactions (other lanes in Fig. 2).

Figure 2 reveals that while camptothecin stimulates nicking by both the mammalian and fungal type I topoisomerases (Fig. 2, lanes A to C and F to G, respectively), the camptothecin-dependent stimulation of nicking is greater in the reaction mixture containing the mammalian topoisomerase I (lanes A to C) than in the reaction mixture containing the fungal topoisomerase I (lanes F to G). In addition, the effect of the topoisomerase I concentration on the yield of nicked DNA is illustrated in Fig. 2. Reaction mixtures containing fewer units of topoisomerase I yield smaller amounts of the nicked product (compare lane C with lanes A and B and lane G with lanes F and E in Fig. 2).

Camptothecin has less of an effect on the fungal cleavage complex than it does on the mammalian cleavage complex. The minimum concentration of camptothecin that stabilizes the cleavage complex was determined for the fungal and HeLa type I topoisomerases, as shown in Fig. 3. Four different topoisomerase I concentrations were used in this experiment: 75, 150, 300, and 1,000 catalytic units per

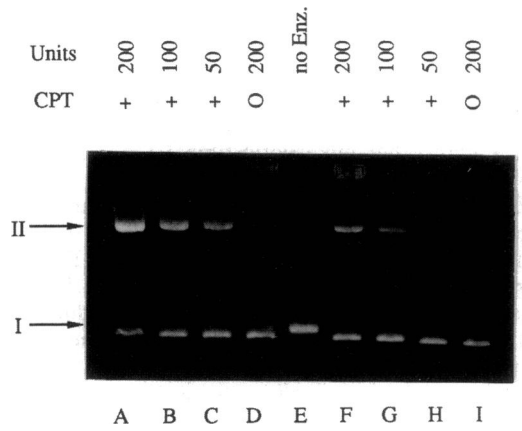

FIG. 2. Effect of topoisomerase I concentration on DNA nicking. Lanes A to D, reaction mixtures contained the bovine calf thymus topoisomerase I; lanes F to I, reaction mixtures contained the *C. albicans* topoisomerase I; lane E, reaction mixtures contained no enzyme. The levels of enzyme, measured by catalytic units, in each reaction mixture are indicated by the numbers above each lane. Camptothecin at a concentration of 400 μg/ml was included in the reaction mixtures in lanes A to C and F to H. Form II DNA is the nicked species; form I DNA includes both supercoiled and relaxed topoisomers. The gel was run in the presence of 0.4 μg of ethidium per ml.

reaction. For reactions containing less than 1,000 catalytic units of topoisomerase I (Fig. 3A to C), concentrations of camptothecin of less than 0.1 μg/ml were ineffective in stabilizing the fungal topoisomerase I cleavage complex, whereas comparable levels of the HeLa topoisomerase I were susceptible to camptothecin concentrations that were at least 10-fold lower than the minimal concentration detected with the fungal topoisomerase I.

Figure 3A to C reveals that a camptothecin concentration of 0.3 μg/ml leads to a twofold increase in nicking by the HeLa topoisomerase I. In order to allow the camptothecin-dependent stimulation of nicking by the fungal topoisomerase I to be measured, the level of topoisomerase I in the reaction was increased to 1,000 catalytic units, a level high enough to allow measurable nicking by the fungal enzyme in the absence of camptothecin (Fig. 3D). Under these conditions, approximately 33% of the DNA molecules were nicked by the fungal topoisomerase I, and approximately 50% of the DNA was nicked by the HeLa topoisomerase I. With this level of *C. albicans* topoisomerase I, a camptothecin concentration of 3 μg/ml caused a twofold increase in nicking (Fig. 3D).

As shown in Fig. 3D, the stimulation by camptothecin of nicking by the fungal topoisomerase I fluctuated for camptothecin concentrations of 0.001 and 0.03 μg/ml and then increased with camptothecin concentrations of 0.1 μg/ml and greater. In reactions containing the HeLa topoisomerase I, camptothecin at 0.003 μg/ml caused a small increase in nicking by the HeLa topoisomerase I, and nicking increased with higher concentrations of camptothecin. At camptothecin concentrations greater than 0.1 μg/ml, double-strand breaks were observed in the DNA after reaction with 1,000 catalytic units of the HeLa topoisomerase I. It is likely that these are due to closely positioned single-strand nicks made by the topoisomerase I. When the DNA is nicked more than once per molecule, the percentage of nicked DNA can no longer be used to estimate the level of nicking in the reaction mixture, so for this reason, the level of nicking in reaction mixtures containing linear DNA was not quantified.

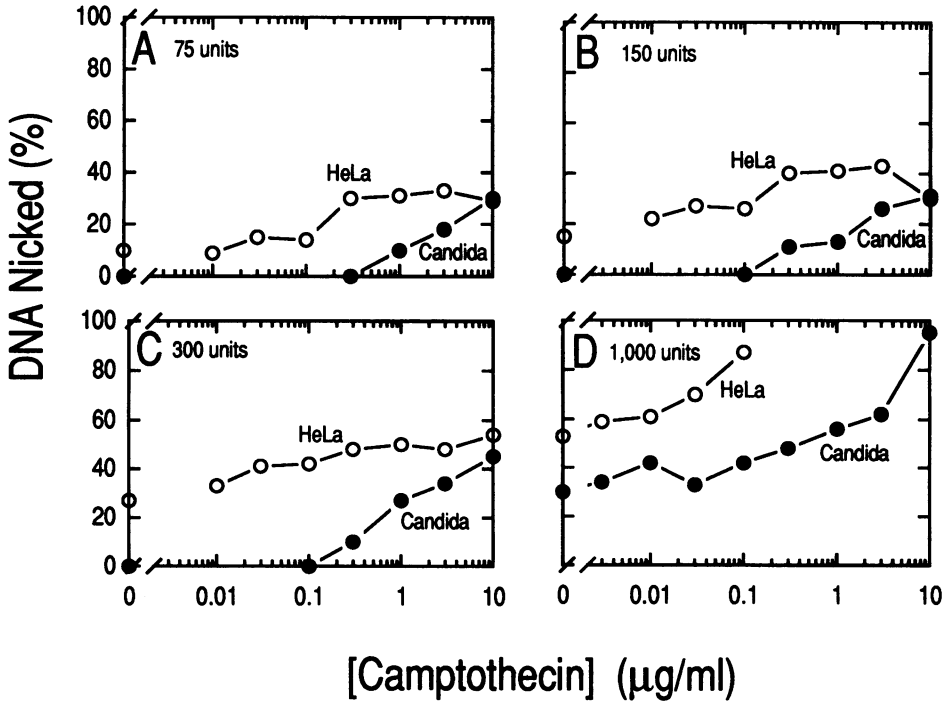

FIG. 3. Response of the fungal and HeLa type I topoisomerases to camptothecin. The extent of each reaction was determined by densitometry. The reaction mixtures in panels A to D contained 75, 150, 300, and 1,000 catalytic units of topoisomerase I, respectively. Open circles indicate reaction mixtures with the HeLa topoisomerase I; filled circles are the *C. albicans* topoisomerase I.

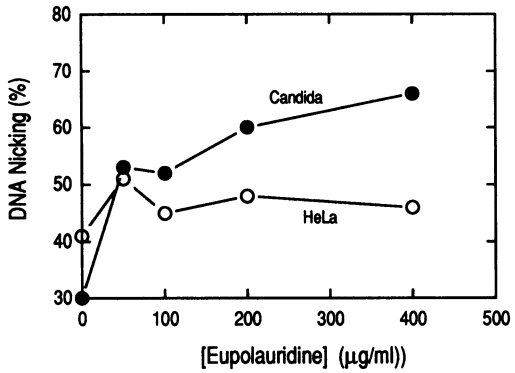

FIG. 4. Stimulation of topoisomerase I-dependent nicking by eupolauridine. Reactions contained 1,000 U of *C. albicans* topoisomerase I (filled circles) or 1,000 U of HeLa topoisomerase I (open circles).

Eupolauridine stimulates nicking by both the fungal and HeLa type I topoisomerases. The effect of eupolauridine on topoisomerase-dependent nicking is shown in Fig. 4. Eupolauridine stimulates nicking by the *C. albicans* topoisomerase I in a dose-dependent manner over a range of concentrations from 50 to 400 μg/ml. At the highest concentration used, eupolauridine stimulates nicking by the fungal topoisomerase I approximately twofold. This stimulation of DNA nicking required the presence of topoisomerase I, since the reaction containing eupolauridine without enzyme did not show an increase in DNA nicking (data not shown).

Eupolauridine also appears to stimulate nicking by the HeLa topoisomerase I, although it has less of an effect on the HeLa enzyme than on the fungal topoisomerase I, as shown in Fig. 4. The response of the HeLa enzyme was not enhanced by the presence of higher concentrations of eupolauridine in the reaction, however.

DISCUSSION

Candidiasis is a medical problem of growing importance, and there is a need for improved therapeutic agents to combat this disease. We selected DNA topoisomerase I as a target for a mechanism-based screen for novel antifungal agents. For this strategy to be effective, *C. albicans* must contain abundant topoisomerase I, and agents which are selective for the fungal topoisomerase I and which are able to reach physiologically relevant concentrations in the fungal cell must be found.

As an initial step in this project, we isolated and characterized the fungal topoisomerase I. The apparent molecular mass of the fungal topoisomerase I is 102 kDa, which is in good agreement with that observed for the HeLa topoisomerase I, which has an apparent molecular mass of 100 kDa as measured by mobility on SDS-polyacrylamide gels (25). The topoisomerase I isolated from *Saccharomyces cerevisiae* has a mobility on SDS-polyacrylamide gels consistent with a protein with a molecular mass of 95 kDa (16), which is slightly less than that of the *C. albicans* topoisomerase I. The differences between the structures of these different eukaryotic type I topoisomerases will be more easily understood once the sequence of the *C. albicans* topoisomerase I is determined.

Like other eukaryotic type I topoisomerases, the *C. albicans* topoisomerase I links to the 3′ end of the nick introduced into DNA when the reaction is interrupted with SDS. The reaction conditions that are optimal for catalysis and formation of the cleavage complex by the *C. albicans* topoisomerase I are very similar to those optimal for the mammalian topoisomerase I. These observations suggest that the *C. albicans* topoisomerase I shares a number of features with other eukaryotic type I topoisomerases that have been characterized previously.

Differences between the *C. albicans* and mammalian type I topoisomerases were observed when the efficiency of nicking by the two enzymes was compared. DNA nicking occurs when the cleavage complex formed between the topoisomerase I and DNA is denatured, leading to a new DNA nick being revealed, with the topoisomerase I covalently linked to the new 3′ end. When equivalent levels of the topoisomerase I from *C. albicans* or mammalian cells were incubated with DNA and the reaction was stopped with SDS, the level of nicking seen in the reaction mixture containing the *C. albicans* topoisomerase I was lower than the nicking seen in the reaction mixture containing the topoisomerase I from HeLa or bovine cells. This may be due to impurities in the fungal topoisomerase I preparation, or it may reflect the possibility that the *C. albicans* topoisomerase I nicks DNA less efficiently or that the *C. albicans* topoisomerase I cleavage complex is less readily denatured than the cleavage complex formed by the mammalian topoisomerase I is.

Camptothecin also has a differential effect on the fungal and mammalian type I topoisomerases. Camptothecin stabilizes the cleavage complex formed by both fungal and mammalian type I topoisomerases; however, the mammalian enzyme responded to approximately 10-fold lower concentrations of this drug than the *C. albicans* topoisomerase I did. This observation demonstrates that the type I topoisomerases from mammalian and fungal cells can be distinguished biochemically.

Studies in *S. cerevisiae* have demonstrated that camptothecin specifically targets the type I topoisomerase in cells (29). When the gene encoding topoisomerase I is disrupted in cells that were initially susceptible to camptothecin, the loss of topoisomerase I activity was correlated with a loss of susceptibility to camptothecin. This effect was reversible: the susceptibility to camptothecin is restored with the reintroduction of a gene encoding topoisomerase I into these cells (2). These studies reveal that camptothecin acts through topoisomerase I and that this effect is fungicidal in this system. Unfortunately, wild-type *S. cerevisiae* cells are resistant to camptothecin because of a permeability barrier, so the *S. cerevisiae* cells used in this study contained mutations to allow cytotoxic levels of camptothecin in the cell (29). For this reason, our observation that camptothecin has no effect on the growth of *C. albicans* (data not shown) is inconclusive, since it is likely that the intercellular drug concentration is too low to be active in *C. albicans* cells, as is the case for *S. cerevisiae*.

The study described above demonstrates that the cytotoxic effect of camptothecin arises from its stabilization of the cleavage complex in the cell rather than its inhibition of topoisomerase activity. By analogy, compounds that stabilize the fungal topoisomerase I cleavage complex are likely to be fungicidal. We found that eupolauridine enhances the recovery of the cleavage complex formed by both the fungal and mammalian type I topoisomerases, and studies by other groups (21) have demonstrated that eupolauridine does indeed inhibit the growth of *C. albicans*. The question of whether topoisomerase I is the only cytotoxic target of eupolauridine can be easily addressed by measuring the

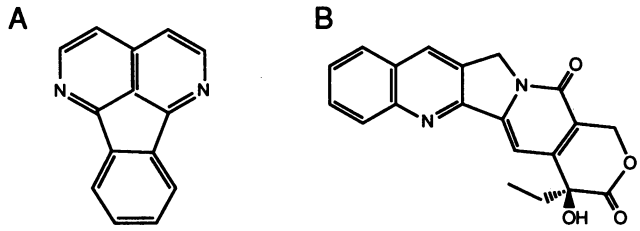

FIG. 5. Chemical structures of eupolauridine (A) and camptothecin (B).

effect of eupolauridine on a strain of *S. cerevisiae* that lacks a functional topoisomerase I gene.

Eupolauridine appears to enhance the recovery of the cleavage complex formed by both the HeLa and fungal type I topoisomerases, a novel activity for this compound. The chemical structure of eupolauridine is shown in Fig. 5A, and the chemical structure of camptothecin is shown in Fig. 5B. The structures of these two compounds appear to be quite different; thus, it is interesting that both can interact with cleavage complexes formed by eukaryotic type I topoisomerases. Unlike camptothecin, eupolauridine appears to bind or intercalate into DNA (data not shown); thus, its future therapeutic usefulness is limited unless this property of the compound can be chemically separated from its effect on the topoisomerase I cleavage complex.

We suggest that topoisomerase I is a good target for the discovery of novel antifungal agents that stabilize the cleavage complex formed by this enzyme and DNA. We isolated and characterized this enzyme and found that it shows similarity in size and reaction requirements to the mammalian topoisomerase I. The fungal topoisomerase I differs from its mammalian counterpart in terms of the efficiency of nicking when the cleavage complex is disrupted by protein denaturants. This observation may reflect the presence of the other components of the topoisomerase I preparation, which is not homogeneous, or this may reflect an underlying biochemical difference between these two enzymes, such as a decrease in the stability or rate of formation of the cleavage complex. This question remains to be addressed.

In addition, we found that both camptothecin, a drug known to target the eukaryotic type I topoisomerase, and eupolauridine, a compound which we observed to stabilize the topoisomerase I cleavage complex, show differential selectivities for the type I topoisomerases isolated from fungal and HeLa cells. The HeLa topoisomerase I cleavage complex is more susceptible to camptothecin, while the fungal topoisomerase I is slightly more susceptible to eupolauridine. This demonstrates that these two type I topoisomerases can be chemically distinguished and that other agents which specifically target the fungal topoisomerase I can likely be found.

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