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Identification of the Aminocatechol A-3253 as an In Vitro Poison of DNA Topoisomerase I from *Candida albicans*

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The aminocatechol A-3253 is active against several pathogenic fungi, including *Candida albicans*, *Cryptococcus albidus*, and *Aspergillus niger*. A-3253 interferes with both the in vitro biosynthesis of (1,3)-β-glucan and the activity of topoisomerases I isolated from *Candida* spp. It is likely that one or more of the enzymes involved in glucan biosynthesis rather than topoisomerase I is the primary intracellular target of A-3253, since a strain of *Saccharomyces cerevisiae* lacking topoisomerase I is as susceptible to A-3253 as cells containing wild-type levels of topoisomerase I. However, the interaction of A-3253 with topoisomerase I in vitro is of interest since the *Candida* topoisomerase is more susceptible to A-3253 than is the topoisomerase I isolated from human HeLa cells. A-3253 is both a reversible inhibitor of topoisomerase I catalysis and a reversible poison of topoisomerase I, and in both reactions the fungal topoisomerase I is more susceptible than the human topoisomerase I to A-3253. In contrast, an earlier study found that the human topoisomerase I is more susceptible than the fungal topoisomerase to camptothecin (J. M. Fostel, D. A. Montgomery, and L. L. Shen, Antimicrob. Agents Chemother. 36:2131–2138, 1992). Taken together with the response to camptothecin, the greater susceptibility of the *Candida* topoisomerase I to A-3253 suggests that there are structural differences between the human and fungal type I topoisomerases which can likely be exploited to allow for the development of antifungal agents which act against the fungal topoisomerase and which have minimal activity against the human enzyme.

There is an increasing need for novel antifungal agents to treat the growing population of patients at risk of systemic fungal infections. The most robust antifungal agent would be one whose target is unique to fungi and which has a fungicidal mechanism of action, an important criterion for immunocompromised patients. Of the two clinically available classes of agents with activity against systemic fungal infections, the azoles have fungistatic rather than fungicidal activities, while the polyene amphotericin B is associated with some toxicity to the host (36). Agents that inhibit (1,3)-β-glucan biosynthesis, as does A-3253, have been reported previously (1, 24, 26, 32, 35), and some are in development as potential new antifungal agents. However, it is possible that these compounds may perturb cell membranes (15, 16, 27, 34). Thus, additional targets of antifungal agents are needed to allow for the discovery of novel and specific antifungal agents.

The type II topoisomerase is likely to be a fungicidal target (8, 21, 29), as is the type I topoisomerase, in light of the observation that camptothecin, a poison of type I topoisomerase, has a fungicidal mechanism in *Saccharomyces cerevisiae* (7, 20). It is therefore of interest to evaluate topoisomerases as potential targets for new antifungal agents.

Topoisomerases modulate the topological structure of DNA in the cell and play important roles in DNA replication, transcription, and recombination, as well as chromosome segregation (for reviews, see references 5, 31, 37, and 38). Eukaryotic cells possess both type I and type II topoisomerases, which are distinguished on the basis of their interactions with DNA. Type I topoisomerase (topoisomerase I) changes the linking number of DNA in steps of one, while the type II topoisomerase (topoisomerase II) changes the linking number of DNA in steps of two (for reviews, see references 4, 14, 22, 23, and 25). Furthermore, topoisomerases are the targets of several antineoplastic agents, such as camptothecin and etoposide, which act by stabilizing a complex between the topoisomerase and DNA. The stabilization of this complex in a growing cell triggers a process that leads to cell death; hence, these agents have been termed topoisomerase poisons (for a review, see reference 17).

The eukaryotic type I topoisomerases are ubiquitous and have regions of protein structure homology predicted by the sequences of the genes encoding these enzymes (19). To be effective, an antifungal agent must show specificity for the fungal cell over the host tissue, which would be enhanced by good selectivity of the agent for the fungal topoisomerase I over the human enzyme. Consequently, to establish topoisomerase I as a target of an antifungal agent, there must be features that distinguish the fungal and human enzymes and that allow the fungal enzyme to be targeted by concentrations of the topoisomerase poison that will have a minimal effect on the host enzyme.

To begin to address this, the type I topoisomerase from *Candida albicans* was isolated and characterized (9). That study revealed that stabilization by camptothecin of the fungal topoisomerase I cleavage complex in vitro requires a concentration 10-fold greater than the minimum concentration of camptothecin needed to enhance nicking by the human topoisomerase I. This observation, taken together with the observations of the responses of fungal and human enzymes to A-3253 reported here, suggest that there are structural differences between the two enzymes which may allow for the identification of new antifungal agents which target the fungal topoisomerase I and not the human enzyme.

MATERIALS AND METHODS

The aminocatechol A-3253 (Fig. 1) was synthesized at Abbott Laboratories by Mary A. Zeng, dissolved in dimethyl sulfoxide (DMSO) at 4 mg/ml, and used fresh. Camptothecin was purchased from Sigma Chemical Co. (St. Louis, Mo.), dissolved in DMSO at 4 mg/ml, and stored at −20°C. *C. albicans* ATCC 10321 was used to prepare topoisomerases I and II. *C. albicans* CCH 442 was used for

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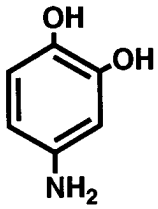

FIG. 1. Chemical structure of A-3253.

(1,3)-β-glucan synthesis, chitin synthesis, and membrane integrity determinations. Frozen HeLa cells were obtained from GIBCO/BRL (Grand Island, N.Y.) and were used to prepare topoisomerase I. *S. cerevisiae* JN394 (*ISE2 rad52*::*LEU2 ura3-52 trp1-289 ade1 his7 leu2*) was provided by James C. Wang (Harvard University, Cambridge, Mass.). *S. cerevisiae* JN394t1 (*ISE2 rad52*::*TRP1 top1*::*LEU2 ura3-52 trp1 ade1 his7 leu2*) was provided by John Nitiss (Children's Hospital, Los Angeles, Calif.).

Topoisomerase I preparation. The *Candida* topoisomerase I was isolated essentially as described previously (9) by batch adsorption to hydroxylapatite resin (Bio-Rad Laboratories, Hercules, Calif.) after disruption of the cells in a glass bead beater (BioSpec, Bartlesville, Okla.) and then step elution. After adsorption to phosphocellulose resin (Whatman, Fairfield, N.J.), topoisomerase I was eluted with 10% glycerol–0.9 M NaCl–15 mM KPO_4 (pH 7.2)–0.1 mM EDTA and was adsorbed to a second hydroxylapatite column to aid concentration.

The protein concentrations in the preparations were determined by the method of Bradford (2) with reagents from Bio-Rad Laboratories, and bovine serum albumin (BSA) was used as a standard. The concentration of the *Candida* topoisomerase I preparation was at 0.64 mg/ml, while that of the HeLa topoisomerase I preparation was at 0.24 mg/ml. The *Candida* topoisomerase had an activity of 600 relaxation units per μl, and thus a specific activity of 930,000 U/mg. The respective values for the HeLa topoisomerase were 500 relaxation units per μl and 2,000,000 U/mg. Earlier work suggested that the specific activity of the purified *Candida* topoisomerase I is equivalent to that of the purified human topoisomerase I (9); therefore, the catalytic activities of the two enzyme preparations were used to normalize both DNA relaxation and DNA nicking reactions for comparison.

Topoisomerase I reactions. Catalytic activity was determined as described previously (9), with one unit defined as the activity required to relax 50% of the DNA in a 20-μl reaction mixture containing 0.2 μg of pBR322 DNA, 25 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, and 0.5 mM EDTA with 50 μg of BSA per ml in 15 min at 30°C. Nicking reactions contained 500 catalytic units (0.53 μg of *Candida* topoisomerase I; 0.24 μg of human topoisomerase I), except as noted, and were performed in 20-μl reaction mixtures containing 0.1 μg of supercoiled pBR322 DNA, 25 mM Tris-HCl (pH 8.0), 50 mM KCl, and 0.5 mM EDTA with 50 μg of BSA per ml at 30°C for 5 min. Reactions were terminated by the addition of sodium dodecyl sulfate (SDS) to 0.5% and then proteinase K digestion and agarose electrophoresis in TBE (90 mM Tris, 90 mM borate, 2.5 mM EDTA).

For analysis of the nicked and linear products, ethidium bromide was included at 0.5 μg/ml in the gel and running buffers. Ethidium bromide overwinds the covalently closed (form I) topoisomers, but nicked (form II) species remain relaxed. Thus, both the supercoiled starting material and the covalently closed relaxed products of catalysis have similar electrophoretic mobilities, while the nicked DNA product of the stabilized cleavage complex has a slower mobility. Quantification of the reaction products was by analysis of photographic negatives with a GS300 densitometer with the GS370 software package (Hoeffer, San Francisco, Calif.). For each reaction mixture, the total amount of each DNA species was determined, and the nicked DNA was expressed as a fraction of the total DNA in the reaction mixture. Replicate tracings of each photograph were reproducible to within ±1%, and replicates of each experiment were reproducible to within ±5%. Data from a representative experiment are given in Fig. 2 through 7.

Topoisomerase II reactions. The catalytic activity of topoisomerase II was determined with knotted P4 DNA as a substrate (18). The reaction mixtures were 20 μl in volume and contained 0.1 μg of knotted P4 DNA, 25 mM Tris-HCl (pH 8.0), 80 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, and 1 mM ATP with 30 μg of BSA per ml. Reactions were run for 15 min at 30°C, stopped with SDS to 0.5%, and analyzed by agarose gel electrophoresis as described previously (29). One catalytic unit was defined as the activity required to unknot 50% of the DNA. The cleavage activity of topoisomerase II was determined in the same buffer; cleavage reactions contained 200 catalytic units of topoisomerase II (0.4 μg) and 0.1 μg of supercoiled ColE1 DNA.

(1,3)-β-Glucan synthesis. Measurements of the inhibition of (1,3)-β-glucan synthesis in microsomes isolated from *C. albicans* were performed as described by Frost et al. (10). The reaction volume was 100 μl, and the reaction mixture contained 100 μg of protein. The extent of UDP–[^{14}C]glucose incorporated in 30 min was determined by measuring the radioactive material retained on glass fiber filters after precipitation with 10% trichloroacetic acid.

TABLE 1. Activity of A-3253 against several fungal strains

Organism tested	MIC (μg/ml)	
	A-3253	Amphotericin B
Candida albicans ATCC 10231	3.12	1.56
Candida albicans ATCC 38247	>100	25
Candida albicans ATCC 62376	12.5	1.56
Candida albicans CCH 442	12.5	1.56
Candida albicans 579a	25	1.56
Candida kefyr ATCC 28838	12.5	1.56
Candida tropicalis NRRL Y-112	6.25	1.56
Aspergillus niger ATCC 16404	50	1.56
Cryptococcus albidus ATCC 34140	12.5	3.12
Saccharomyces cerevisiae GS1-36	12.5	0.78
Torulopsis glabrata ATCC 15545	0.78	1.56

Chitin synthesis. The inhibition of chitin synthesis in *Candida* microsomes was measured as described by Cabib et al. (3). The reaction volume was 50 μl, and the reaction mixture contained 100 μg of protein. The extent of [^{14}C]UDP–*N*-acetylglucosamine incorporated in 30 min was determined by measuring the radioactive material retained on glass fiber filters after precipitation with 5% trichloroacetic acid.

Membrane integrity assay. The effect of A-3253 on membrane integrity was determined by measuring the loss of [^{14}C]isobutyric acid from *C. albicans* cells during a 1-h incubation with A-3253 at 100 μg/ml as described previously (11).

Determination of growth effects. The MIC determinations for *Candida* species and other fungi (see Table 1) were performed by a broth microtiter modification of the method of Shadomy and Pfaller (28). The MIC determination for *S. cerevisiae* JN394 and JN394t1 (see Table 3) were performed in SC synthetic complete medium (30). A total of 200 ml of cells suspended at 5×10^4 cells per ml was added to microtiter plates containing threefold serial dilutions of the test compounds dissolved in 20 μl of 10% DMSO. Growth determinations were performed by visual inspection of the plates after the cells in wells to which no compound was added had reached confluence.

RESULTS

Biological activity of A-3253. The aminocatechol A-3253 (Fig. 1) was identified in a discovery program for antifungal agents. A-3253 has activity against a number of fungi, with MICs for wild-type strains ranging from 0.78 to 50 μg/ml (Table 1). Interestingly, A-3253 was not active against the polyene-resistant strain ATCC 38247 of *C. albicans*. A-3253 also had weak or no activity against a variety of gram-negative and gram-positive bacteria (data not shown). A-3253 had in vitro activity both as a topoisomerase I poison (at 25 μg/ml and above) and as an inhibitor of glucan biosynthesis (with a 50% inhibitory concentration of 9 μg/ml). A-3253 did not strongly interfere with the other biological activities tested (Table 2).

Since A-3253 has activity against two different fungal enzymes in vitro, it was of interest to determine which one might be the primary target in the cell. The importance of topoisomerase I as a biological target for A-3253 was assessed by measuring the effect of A-3253 on the growth of *S. cerevisiae* JN394t1, which lacks a functional topoisomerase I (Table 3). In agreement with the results obtained by others (20), JN394t1 is resistant to camptothecin, while the parent strain, JN394 (with wild-type topoisomerase I), is susceptible to camptothecin. In contrast, both JN394 and JN394t1 are equally susceptible to A-3253. This suggests that another cellular enzyme is more susceptible to A-3253 than topoisomerase I in the cell. It is possible that one or more of the enzymes involved in the synthesis of (1,3)-β-glucan may be the primary cellular target of A-3253.

Differential in vitro susceptibilities of human and fungal topoisomerases I to A-3253. During the initial characterization, we observed that A-3253 appeared to have potency for *Can-*

TABLE 2. In vitro activity of A-3253

Activity tested	Effect of A-3253
Topoisomerase I nicking	Stimulation by ≥25 μg/ml
Topoisomerase I catalysis (DNA relaxation)	30-fold inhibition at 1 μg/ml; >90-fold inhibition at 10 μg/ml
Topoisomerase II cleavage	No effect at 200 μg/ml
Topoisomerase II catalysis (DNA unknotting)	IC_{50}[a], 150 μg/ml
(1,3)-β-Glucan biosynthesis	IC_{50}, 9 μg/ml
Chitin biosynthesis	No effect at 100 μg/ml
Cell membrane integrity	No effect at 100 μg/ml

[a] IC_{50}, 50% inhibitory concentration.

dida topoisomerase I different from that for the human enzyme. For this reason we characterized the activity of A-3253 as a topoisomerase poison in vitro. The hallmark of a topoisomerase poison is the ability to stabilize the cleavage complex formed between topoisomerase I and DNA. When this complex is trapped with a protein denaturant such as SDS, the denatured topoisomerase is found to be covalently attached to the 3′ end of a new nick in the DNA backbone. The presence of a topoisomerase poison increases the production of nicked DNA by topoisomerase I.

This effect is illustrated in Fig. 2. Each reaction mixture in lanes 2 to 7 of Fig. 2A and B contained 500 catalytic units of topoisomerase I. Figure 2A shows reactions with *Candida* topoisomerase I; Fig. 2B shows reactions with the human topoisomerase I isolated from HeLa cells. As the concentration of A-3253 is increased (lanes 4 to 7 of Fig. 2A) an increasing proportion of the DNA in reaction mixtures containing the *Candida* topoisomerase I was recovered as the nicked form (form II). Minimal stimulation of nicking was seen when A-3253 was added to reaction mixtures containing the human topoisomerase I (Fig. 2B, lanes 4 to 7). Both *Candida* and human topoisomerases responded to camptothecin as expected (Fig. 2A and B, lanes 3).

The amount of each DNA species was quantified by densitometry, and the amount of nicked DNA, expressed as a percentage of total DNA in the reaction mixture, is plotted in Fig. 2C. In that experiment, nicking by the *Candida* topoisomerase I was stimulated by concentrations of A-3253 of 25 μg/ml and above. Almost 75% of the DNA was nicked by topoisomerase in the presence of A-3253 at 200 μg/ml. This is comparable to the effect of camptothecin at 100 μg/ml, which usually leads to 85% of the DNA nicked by this amount of *Candida* topoisomerase I. In contrast, A-3253 did not stimulate nicking by the human topoisomerase I unless it was present at a concentration of 200 μg/ml in the reaction mixture, when only 30% of the DNA was nicked (Fig. 2C). The use of camptothecin resulted in 90% of the DNA being nicked by this amount of human topoisomerase I.

Stimulation of topoisomerase nicking by A-3253 is reversible and is not masked by exogenous protein. The topoisomerase-DNA cleavage complex dissociates in the presence of salt in excess of 0.5 M or when the temperature is raised, and the addition of camptothecin to the cleavage complex does not alter this reversibility (13). Figure 3 demonstrates that the stimulation of *Candida* topoisomerase I nicking by A-3253 is also reversible. In the experiment whose results are given in Fig. 3, the reactions in lanes 2 to 5 were stopped by the addition of SDS; this was followed by the addition of 0.5 M NaCl. For the reactions in lanes 6 to 9 the salt was added prior to the addition of SDS.

The proportion of nicked form II DNA in reaction mixtures containing A-3253 at 50 μg/ml (lanes 4 and 8) and 200 μg/ml (lanes 5 and 9) is substantially lower when the reaction is terminated by the addition of salt (lanes 8 and 9) than when the reaction is stopped by the addition of SDS (lanes 4 and 5). This behavior is also observed for camptothecin (lanes 3 and 7), in agreement with previous observations (13). Heating of the reaction mixture to 65°C for 10 min also reversed nicking

TABLE 3. Effect of disruption of the *TOP1* gene on activity of A-3253

Organism tested	Relevant phenotype[a]	MIC (μg/ml)[b]		
		A-3253	CPT	AmB
JN394	*ISE2 rad52*::*LEU2*	20.5	1.56–3.12	1.56–3.12
JN394t1	*top1*::*LEU2* *ISE2 rad52*::*TRP1*	20.5	>100	1.56–3.12

[a] *ISE2* and disruption of *RAD52* confer increased susceptibility to topoisomerase poisons (20). TOP1 encodes topoisomerase I (12, 33).
[b] CPT, camptothecin; AmB, amphotericin B.

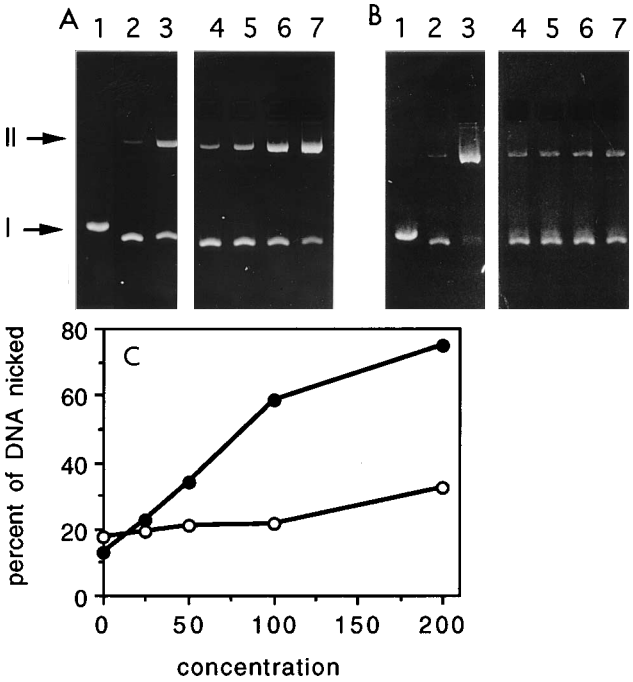

FIG. 2. Effect of A-3253 on nicking by topoisomerase I from *C. albicans* (A) and human cells (B). (A and B) Lanes 1, DNA substrate; lanes 2 to 7, 500 U of topoisomerase and no added agent (lanes 2), camptothecin at 100 μg/ml (lanes 3), and A-3253 at 25, 50, 100, and 200 μg/ml (lanes 4 to 7, respectively). The arrow labeled II indicates form II (nicked) DNA; the arrow labeled I indicates form I (covalently closed) DNA. (C) Topoisomerase nicking in the presence of A-3253. Filled circles, *Candida* topoisomerase I; open circles, human topoisomerase I.

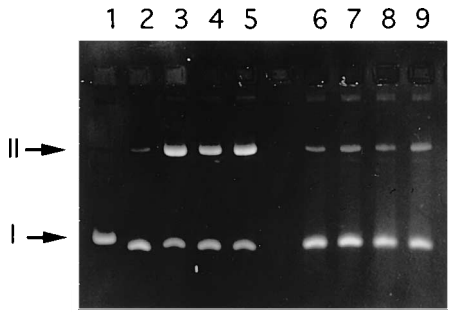

FIG. 3. A-3253 stimulation of topoisomerase cleavage is reversible. Lane 1, DNA substrate; lanes 2 to 5, reactions terminated with SDS prior to the addition of 0.5 M NaCl; lanes 6 to 9, reactions terminated with 0.5 M NaCl prior to the addition of SDS. Lanes 2 and 6, topoisomerase alone; lanes 3 and 7, topoisomerase with camptothecin at 100 μg/ml; lanes 4 and 8, topoisomerase with A-3253 at 50 μg/ml; lanes 5 and 9, A = 3253 at 200 μg/ml. The arrow labeled II indicates form II DNA; the arrow labeled I indicates form I DNA.

in the presence of A-3253 (data not shown). The interaction of A-3253 with *Candida* topoisomerase I is therefore similar to that of camptothecin in that both agents are able to enhance the nicking of DNA by *Candida* topoisomerase I by a reversible mechanism.

The low reactivity of the human topoisomerase I preparation compared with that of the *Candida* topoisomerase I preparation could arise from interference by a contaminant in the human topoisomerase I preparation. To address this, the activities of the *Candida* and human topoisomerase preparations were tested alone and after being mixed together. The topoisomerases were more active when they were mixed than when either one was tested alone (data not shown), suggesting that the weaker response of the human enzyme is not due to a *trans*-acting inhibitor in the preparation. The presence of BSA at 200 μg/ml in the reaction mixtures did not greatly alter the efficiency of nicking by either *Candida* or human topoisomerase I (data not shown).

A-3253 interferes with relaxation of DNA by topoisomerase I. The effect of A-3253 on topoisomerase I catalysis was determined by measuring the relaxation activity of serial dilutions of human and *Candida* topoisomerase I in the presence and absence of A-3253 (Fig. 4). Topoisomerase activity is measured by the conversion of the supercoiled substrate to relaxed topoisomers. Dilutions of each enzyme corresponding to 30, 10, 3, and 1 U per reaction mixture were made and tested for their activities (Fig. 4A, lanes 1 to 4 and 5 to 8 for the *Candida* and human topoisomerases, respectively). The corresponding lanes of Fig. 4B show parallel reactions performed in the presence of A-3253 at 1 μg/ml.

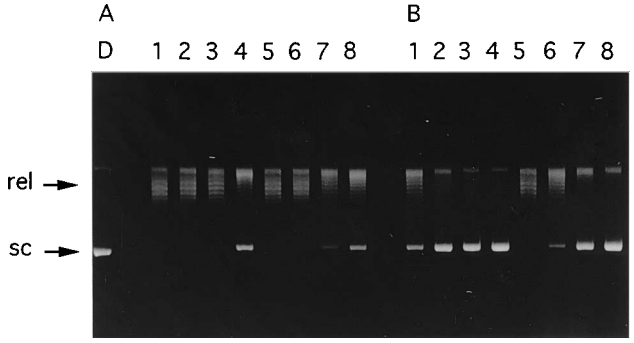

FIG. 4. Effect of A-3253 on catalysis by *Candida* topoisomerase I. (A) Reaction mixtures contained no A-3253; (B) reaction mixtures contained A-3253 at 1 μg/ml. Lane D, unreacted DNA substrate. (A and B) Lanes 1 to 4, *Candida* topoisomerase I at 30, 10, 3, and 1 unit, respectively; lanes 5 to 8, human topoisomerase I at 30, 10, 3, and 1 unit, respectively. rel, relaxed topoisomers; sc, supercoiled DNA.

In the presence of A-3253, 30 U of *Candida* topoisomerase I has activity equivalent to that of 1 catalytic unit (compare Fig. 4B, lane 1, which has 30 catalytic Units plus A-3253, with Fig. 4A, lane 4, which has 1 catalytic Unit and no A-3253). This indicates that the *Candida* enzyme is inhibited approximately 30-fold by A-3253 at 1 μg/ml. The human topoisomerase I is inhibited approximately threefold by A-3253 at this concentration. Thus, A-3253 at 1 μg/ml inhibits the *Candida* topoisomerase to a greater extent than it inhibits the human topoisomerase I. A-3253 inhibits both enzymes by over 95% at concentrations greater than 10 μg/ml (data not shown).

Inhibition of relaxation by A-3253 is reversible. It is possible that the inhibition of topoisomerase I catalytic activity by A-3253 occurs via an irreversible modification of the enzyme. To address this, the *Candida* topoisomerase was preincubated with A-3253 and was then diluted in buffer without A-3253, and the activity was measured (Fig. 5B). For comparison, an aliquot of the enzyme was preincubated without A-3253 and was then diluted and assayed in parallel (Fig. 5A). Another aliquot of the *Candida* topoisomerase was preincubated with A-3253 and was then diluted and assayed in the continued presence of A-3253 at 133 μg/ml (Fig. 5C).

In those reactions, topoisomerase was initially incubated at a concentration of 500 U/μl with (or without) A-3253 at 133 μg/ml and was then diluted 5-fold (lanes 1 and 2), 50-fold (lanes 3 and 4), or 500-fold (lanes 5 and 6), and the activity of 1 μl was measured in a 20-μl reaction mixture. Thus, the final concentration of A-3253 in the relaxation reactions is 1.33 μg/ml or less, and the amount of the initial topoisomerase in the reactions corresponded to 100 (lanes 1 and 2), 10 (lanes 3 and 4) or 1 (lanes 5 and 6) catalytic unit. Comparison of the activity in Fig. 5A (without A-3253 exposure) with that in Fig. 5B (after exposure to A-3253) reveals that any inhibitory effect from incubation with A-3253 is reversible, since the corresponding lanes of Fig. 5A and B have the same activities.

The presence of DNA alters the response of *Candida* topoisomerase I to A-3253. The observation that A-3253 reversibly inhibits catalysis by *Candida* topoisomerase I raised the question of whether this agent also has a negative effect on the formation of the cleavage complex by the *Candida* topoisomerase I. This was addressed by preincubating *Candida* topoisomerase I with A-3253 for 10 min in a reaction buffer and then adding DNA and allowing the reaction to continue for another 5 min. Under these conditions, no stimulation of nicking by A-3253 was seen (Fig. 6).

This result could arise from inactivation of the topoisomerase or of the A-3253 present during the preincubation period. This question was addressed in two ways. First, following exposure of topoisomerase to A-3253, camptothecin was added along with the DNA. The results of this reaction (Fig. 6) demonstrate that exposure to A-3253 interferes with the response of the topoisomerase to camptothecin. There was also no increase in the production of nicked DNA when fresh A-3253 was added to the reaction after the 10-min preincubation; the proportions of nicked DNA were only 12 and 10% in reaction mixtures containing topoisomerase exposed to A-3253 at 25 and 100 μg/ml, respectively. This suggests that the lack of response following preincubation is due to an effect on the topoisomerase rather than to the loss of active A-3253.

In another experiment, DNA was included in the preincubation step with the enzyme and A-3253. As before, this ex-

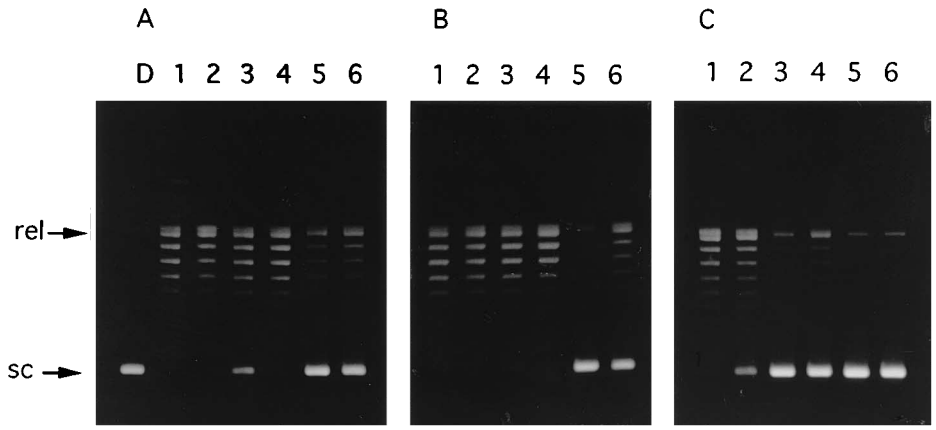

FIG. 5. A-3253 inhibition of catalysis is reversible. *Candida* topoisomerase I (500 U/μl) was incubated for 10 min in the absence (A) or presence of A-3253 at 133 μg/ml (B and C) and was then diluted 5-fold (lanes 1 and 2), 50-fold (lanes 3 and 4), or 500-fold (lanes 5 and 6), and the activity in 1 μl was measured in a 20-μl reaction mixture. The dilution buffer contained no A-3253 (A and B) or A-3253 at 133 μg/ml (C). Lane D, unreacted substrate DNA. The reactions in lanes 1, 3, and 5 were run for 5 min; the reactions in lanes 2, 4, and 6 were run for 15 min. rel, relaxed topoisomers; sc, supercoiled DNA.

periment was performed in parallel, with camptothecin added to one set of reactions after preincubation. The presence of DNA in the preincubation mixture appears to protect the response of the topoisomerase to camptothecin (Fig. 6). The response of the topoisomerase to camptothecin under these conditions is comparable to the response of the topoisomerase without exposure to A-3253. DNA also partially protects the response of the topoisomerase to A-3253 (Fig. 6).

The results in Fig. 6 suggest that the DNA in the preincubation mixture can protect the topoisomerase from the loss of activity during incubation with A-3253. It is possible that the protective effect could arise from binding of A-3253 to the DNA. To address this, different reaction mixtures in which the DNA concentration was altered were prepared. The concentration of topoisomerase was altered in parallel, to keep the ratio of topoisomerase to DNA constant. The standard reaction mixture contained 500 U (0.5 μg) of *Candida* topoisomerase I and 0.1 μg of ColE1 DNA and is indicated by the reaction labeled 1 in Fig. 7. Reactions were performed with half (250 U of topoisomerase and 0.05 μg of ColE1), twice (1,000 U and 0.2 μg of ColE1), and three times (1,500 U and 0.3 μg of ColE1) this amount. These reaction mixtures contained either A-3253 or camptothecin at 100 μg/ml or no topoisomerase poison.

The results in Fig. 7 indicate that the proportion of DNA nicked by topoisomerase in the presence of A-3253 was not decreased as the concentrations of DNA and topoisomerase were increased. In each of the four reaction mixtures approximately 30% of the DNA was nicked after incubation with topoisomerase without added poison. Even though the absolute amount of nicked DNA produced in each reaction increased as the total amount of DNA in the reaction mixture was increased, the percentage of nicked DNA in each reaction mixture remained the same since the ratio of topoisomerase to DNA was kept constant. In the presence of A-3253 approximately 70% of the DNA in the different reaction mixtures was nicked, while on average 90% of the DNA was nicked in

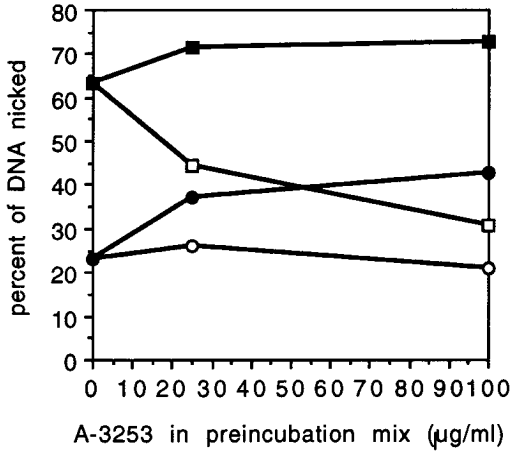

FIG. 6. Effect of exposure of *Candida* topoisomerase to A-3253 prior to reaction. Open circles, a total of 500 U of *Candida* topoisomerase was incubated for 10 min in 20 μl of reaction buffer with increasing concentrations of A-3253 and then 0.1 μg of ColE1 DNA was added, the mixture was incubated for 5 min, and then the reaction was stopped with SDS. Open squares, as for the open circles, but camptothecin was added to 100 μg/ml with the DNA. Filled circles, as for the open circles, but DNA was added at the start of the reaction, not after 10 min of incubation. Filled squares, as for the filled circles, but camptothecin was added after 10 min of incubation.

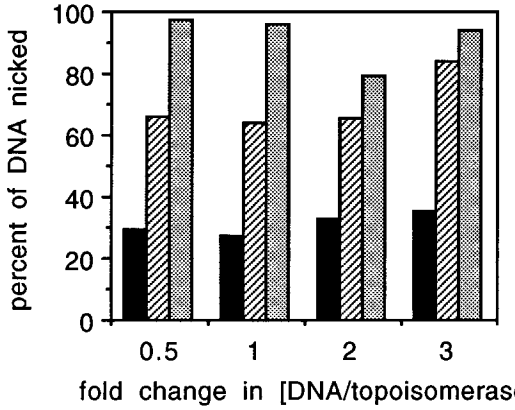

FIG. 7. Effect of DNA concentration on response to A-3253. Reaction mixtures of 20 μl contained A-3253 at 100 μg/ml (striped bars), camptothecin at 100 μg/ml (stippled bars), or no agent (solid bars). The reaction labeled 1 contained the standard 500 U of *Candida* topoisomerase and 0.1 μg of ColE1 DNA. The reaction labeled 0.5 contained half this (250 U and 0.05 μg of DNA). Reactions 2 and 3 contained two and three times this (1,000 U and 0.2 μg of DNA, and 1,500 U and 0.3 μg of DNA, respectively).

reaction mixtures containing camptothecin. If A-3253 were binding to DNA in the reaction mixture, it would be expected that increasing the concentration of DNA in the reaction mixture while holding the concentration of A-3253 constant would lead to a decrease in the stimulation of nicking by A-3253. No marked change in the proportion of DNA nicked in the presence of A-3253 was observed over this range of DNA concentrations. Thus, altering the level of DNA in the reaction mixture over this concentration range did not appear to interfere with the response of the topoisomerase to A-3253.

DISCUSSION

The most useful antifungal agent is one whose target is unique to fungi and which has a fungicidal mode of action. Agents that meet these two criteria are expected to be well tolerated by the host and also to eradicate systemic fungal infections in immunocompromised patients. Camptothecin, a poison of topoisomerase I, has a fungicidal mode of action (7, 20), suggesting that poisons of topoisomerase I could make up a novel class of fungicidal agents. For topoisomerase I to be established as a target of antifungal agents, there must be sufficient differences between the fungal and human enzymes to allow agents with selectivity for the fungal topoisomerase to be obtained. The response of the *Candida* and human topoisomerases to A-3253 reported here demonstrates that agents with more activity against the fungal enzyme than against the human topoisomerase can be found.

Although the major intracellular target of A-3253 has not been identified, this agent inhibits the growth of several species of pathogenic fungi, with MICs ranging from 0.8 to 50 μg/ml. A-3253 inhibits one or more of the enzymes involved in the in vitro biosynthesis of (1,3)-β-glucan, and it is possible that inhibition of this pathway in the cell leads to the inhibition of fungal growth. A-3253 also stabilizes the topoisomerase I cleavage complex in vitro; however, yeast cells lacking topoisomerase I retain their susceptibilities to A-3253, suggesting that topoisomerase I is not the most susceptible target for A-3253 in the cell. Nevertheless, the observation that *Candida* and human type I topoisomerases exhibit different in vitro susceptibilities to A-3253 is of interest since a study of the interaction between A-3253 and the topoisomerases may allow structural differences between the *Candida* and human topoisomerases I to be characterized.

The observation that the topoisomerase-DNA cleavage complex is reversible indicates that the topoisomerase in the cleavage complex remains active in the presence of A-3253 and DNA. When salt is added, the topoisomerase dissociates from the cleavage complex and reseals any nicks in the DNA to regenerate intact, covalently closed DNA topoisomers. This would not be predicted if A-3253 were increasing the production of nicked DNA by chemically modifying the topoisomerase in such a way as to render it irreversibly inactive. It is therefore likely that A-3253 is binding reversibly to the topoisomerase, the DNA, or the cleavage complex in such a way as to interfere with catalysis and to favor the formation of the cleavage complex.

When the *Candida* topoisomerase I is incubated with A-3253 prior to the addition of DNA, no stimulation of nicking is observed when DNA is added, in contrast to the stimulation of topoisomerase-dependent DNA nicking by A-3253 if there is no preincubation step. Preincubation with A-3253 also reduces the stimulation of nicking by camptothecin, suggesting that A-3253 may interfere with the ability of the enzyme to respond to both A-3253 and camptothecin.

The presence of DNA during exposure to A-3253 appears to protect the topoisomerase from the inhibitory effects of A-3253. DNA could produce this protective effect by binding A-3253, and thus limiting the concentration available to act against the topoisomerase, or by binding the topoisomerase and thereby affording protection from A-3253. The former explanation is not likely because the concentration of DNA in the reaction mixture was varied over a sixfold range without greatly affecting the efficiency of stimulation of DNA nicking by either camptothecin or A-3253. Further experiments are needed to elucidate the basis for this protective effect.

The nature of the interactions between A-3253 and topoisomerase in the presence and absence of DNA remains to be characterized. Regardless of the details of the interaction between A-3253 and topoisomerase, however, the effects of this compound on enzyme activity suggest that A-3253 interacts with a site or sites on the topoisomerase that can affect catalysis and the formation of the cleavage complex. In addition, the differential responses of the fungal and human topoisomerases to A-3253 suggest that there are structural differences between these two enzymes at the site(s) of interaction with A-3253. Thus, A-3253 is a topoisomerase poison that can be used as a probe to allow the further characterization of differences between *Candida* and human topoisomerases I.

After the manuscript was submitted, we learned of a study describing the selective inhibition of topoisomerases isolated from *Pneumocystis carinii* compared with that of topoisomerases from mammalian cells (6). This observation further supports the hypothesis that the fungal topoisomerases have sufficient structural differences from mammalian topoisomerases to allow for the differential targeting of the fungal enzymes by chemical agents.

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