Molecular Target of the Antileishmanial Action of Sinefungin

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Sinefungin, a natural nucleoside isolated from cultures of *Streptomyces incarnatus* and *S. griseolus*, is structurally related to *S*-adenosylhomocysteine and *S*-adenosylmethionine. Sinefungin has been shown to inhibit the development of various fungi and viruses, but its major attraction to date resides in its potent antiparasitic activity. This compound has been reported to display antiparasitic activity against malarial, trypanosomal, and leishmanial species. Very little is known about the antiparasitic mode of action of sinefungin. We found that *S*-adenosylmethionine was capable of reversing the inhibitory growth effects of sinefungin in *Leishmania mexicana* and that dATP was capable of reversing inhibitory effects of the drug on DNA polymerase activity when pyrophosphate release was measured. However, when incorporation of [³H]dTTP was used to measure DNA polymerase activity, no inhibition could be observed. Inhibition of DNA polymerase activity by sinefungin occurred only during the initial stages of purification of this enzyme, and inhibition by aphidicolin, a known DNA polymerase inhibitor, paralleled the inhibition by sinefungin. Neither sinefungin nor aphidicolin inhibited partially purified DNA polymerase. *S*-Adenosylmethionine synthetase was partially purified, and sinefungin, at levels active in vitro, had no significant effect. Sinefungin was significantly suppressive against both *L. donovani* and *L. braziliensis panamensis* infections in hamsters when compared with meglumine antimonate (Glucantime).

Sinefungin, a natural nucleoside isolated from cultures of *Streptomyces incarnatus* and *S. griseolus*, is structurally related to *S*-adenosylhomocysteine and *S*-adenosylmethionine (SAM) (10, 12; Fig. 1). Sinefungin has been shown to inhibit the development of various fungi (11, 20) and viruses (22, 23, 28), but its major attraction to date resides in its potent antiparasitic activity (2, 7, 9, 19, 20, 21, 27). This compound displays antiparasitic activity against malarial parasites (27) and *Trypanosoma* (7) and *Leishmania* species (2, 3, 19, 21). Recently, it was reported that sinefungin inhibits protein methylases in *Leishmania* sp. (20), but its major site of inhibition has been reported to involve DNA synthesis (3). Thymidine uptake into the cells was not affected. Uridine incorporation was inhibited to a much lesser extent than thymidine, while leucine incorporation was unaffected. It was shown that nuclear and kinetoplast DNA synthesis was inhibited without inhibition of nucleoside phosphorylation but accompanied by an increase in nucleoside triphosphate levels (3).

In an attempt to elucidate the molecular mode of action of sinefungin in leishmanial parasites, we determined which compounds could reverse its inhibitory action. Because sinefungin is an analog of SAM, we studied its action on SAM synthetase. In recent years, SAM has been found to affect the methylation and properties of such macromolecules as nucleic acids, proteins, phospholipids, and carbohydrates (5, 6, 16).

In an attempt to correlate the antileishmanial activity of this molecule with inhibition of DNA synthesis, we studied the effect of this compound on DNA polymerase.

We also compared the activity of sinefungin in leishmania-infected hamsters with that of meglumine antimonate (Glucantime), a drug used to treat leishmaniasis.

MATERIALS AND METHODS

Sinefungin was generously provided by Malka Robert-Géro, Institut de Chimie des Substances Naturelles, Centre

National de Recherche Scientifique, Gif-sur-Yvette, France, and Eli Lilly & Co., Indianapolis, Ind. Aphidicolin was supplied by A. H. Todd of Imperial Chemical Industries, Cheshire, England. All other chemicals were of the highest purity and were obtained from Sigma Chemical Co., St. Louis, Mo., except for soybean trypsin inhibitor, aprotinin, and leupeptin, which were crude grade. [³H]dTTP (45 Ci mmol⁻¹) and [¹⁴C]-adenosylmethionine (40 Ci mmol⁻¹) were purchased from Amersham Corp., Arlington Heights, Ill. Heparin-Sepharose CL-6B, cellulose phosphate, and denatured DNA cellulose were obtained from Pharmacia Fine Chemicals, Piscataway, N.J.

Cell culture conditions for enzyme isolations. Promastigotes of Walter Reed strain 227 were used in these experiments. This strain has been previously identified as *Leishmania mexicana amazonensis* (J. Decker-Jackson and P. Jackson, personal communication) and was obtained from the Leishmania Section of the Walter Reed Army Institute of Research. Promastigotes were grown in brain heart infusion medium containing 37 g of brain heart infusion medium (Difco Laboratories, Detroit, Mich.) liter of water⁻¹, 10% heat inactivated serum, and 26 µg of hemin ml⁻¹. Cells were grown at 26°C in 2,000-ml wide Fernbach flasks containing 250 ml of brain heart infusion medium. Cells were harvested after 4 days during the exponential growth phase. The cell density was 4 × 10⁷ to 6 × 10⁷ cells ml⁻¹.

Inhibition and reversal studies. Promastigotes of *L. mexicana* were grown in the defined medium of Steiger and Steiger (26) supplemented with 5% heat-inactivated calf serum. When specific purines were being tested for reversal of the inhibitory action of sinefungin, the purine source (adenosine) was omitted and the test purine was added to the medium. The leishmanial cells were initially grown in brain heart infusion medium, and when in log phase (2 × 10⁶ cells per ml), these cells were used as an inoculum (0.5 ml) and aseptically transferred to 4.5 ml of Steiger and Steiger medium in test tubes (14.5 by 1.5 cm). The compound to be

FIG. 1. Sinefungin and related compounds.

tested was added, and the cells were incubated in a slanted position at 26°C. Optical density readings at 660 nm were taken every 24 h for a total of 96 h. Growth experiments were done in duplicate.

Purification of leishmanial DNA polymerase. Cells (1.5 liters) were harvested in 250-ml centrifuge bottles and centrifuged at $15,000 \times g$ for 10 min. The cells were washed twice in buffer containing 50 mM Tris hydrochloride (pH 7.5). The cell pellet (usually 4 to 6 g [wet weight]) was suspended in lysis buffer (8 to 12 ml) containing 10 mM Tris hydrochloride (pH 7.5), 20% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 1.0 M HCl, and 0.3% Triton X-100. This cell suspension was diluted 1:100 with the following stock solution of protease inhibitors: soybean trypsin inhibitor (4.8 mg ml⁻¹), aprotinin (4.8 mg ml⁻¹), leupeptin (2 mg ml⁻¹). Isolated cells were homogenized at 4°C with a Teflon homogenizer or sonicated five times for 10 s each time with 2-min cooling intervals. This was carried out with a Braun-Sonic 2000 ultrasonic disruptor at 4°C. The suspension was centrifuged at $18,000 \times g$ for 1 h at 4°C. This supernatant fluid was subjected to protamine sulfate treatment, dialysis, and column chromatography. DNA was removed by adding sufficient 2% protamine sulfate to the crude enzyme to result in a 1:10 dilution. The precipitate from the protamine sulfate step was removed by centrifugation at $18,000 \times g$ for 15 min at 4°C. The supernatant was dialyzed against 2 liters of standard buffer, which consisted of 20 mM Tris hydrochloride (pH 7.5), 1 mM dithioerythritol, 1 mM EDTA, and 20% glycerol. The precipitate that formed after dialysis was removed by centrifugation at $18,000 \times g$ for 20 min at 4°C.

Chromatography. The supernatant was applied to a heparin-Sepharose column (8.5 by 1.3 cm) equilibrated with 0.1 M KCl in standard buffer. The column was washed with

20 mM Tris hydrochloride (pH 7.5) buffer until the A_{280} was less than 0.1. The DNA polymerase was then eluted with 0.5 M KCl in standard buffer. Active fractions were pooled, and protease inhibitors were added as described above. The pooled fractions were dialyzed overnight against 2 liters of standard buffer containing 0.1 M KCl. After dialysis, more protease inhibitors were added as described above, and the pooled fractions were applied to a cellulose-phosphate column (7.0 by 1.3 cm) equilibrated with 0.1 M KCl in standard buffer. The column was washed with the same buffer until the A_{280} was less than 0.1. The DNA polymerase was then eluted with 0.35 M KCl in standard buffer. The active fractions were pooled, and protease inhibitors were added. The fractions were dialyzed against 2 liters of standard buffer overnight. The pooled dialyzed fractions were applied to a denatured-DNA-cellulose column (13.0 by 1.0 cm) equilibrated with standard buffer. The column was washed with 15 ml of standard buffer, and the enzyme was eluted by using 15-ml step gradients (0.1 M KCl, 0.25 M KCl) in the same buffer.

Protein assays. Protein concentrations were determined by either the dye-binding method (Bio-Rad Laboratories, Richmond, Calif.) or a modified method. The modified method was performed in 96-well microplates by adding 80 μ l of Bio-Rad dye and 20 μ l of a column fraction. The plate was then read in a Dynatech 600 microplate reader at 575 nm.

DNA polymerase radioactive assay. Enzymes were assayed for activated DNA-dependent activity in a reaction mixture containing 10 mM Tris hydrochloride (pH 7.5); 41.6 μ M (each) dATP, dCTP, and dGTP; [methyl-³H]dTTP at 200 to 300 cpm pmol⁻¹; 5 mM MgCl₂; 100 μ g of bovine serum albumin ml⁻¹; and 50 μ g of activated calf thymus DNA ml⁻¹. Assays were for 30 min at 30°C. Compounds tested for inhibition were preincubated for 15 min at 30°C with the enzyme to be tested. Assays were routinely carried out in a final volume of 50 μ l. Reactions were terminated by pipetting 10 μ l of a solution containing 2.5% sodium dodecyl sulfate and 0.15 M sodium PP_i (17). The reaction mixture was then pipetted onto DEAE-cellulose disks (Whatman DE81), followed by immersion in 0.5 M Na₂HPO₄. The filters were washed five times for 5 min each time in this buffer, twice in distilled H₂O, twice in 95% ethanol, and once in ether and air dried. The disks were counted in Fisher Scint Verse II in a Beckman scintillation counter.

One unit of activity is defined as the incorporation of 1 pmol of dTTP into DNA in 30 min under standard assay conditions.

DNA polymerase spectrophotometric assay. The DNA polymerase spectrophotometric assay is described in Sigma technical bulletin 7275, issued in August 1983. This bulletin describes the spectrophotometric determination of PP_i and the assay of DNA polymerase.

Isolation and assay of SAM synthetase. Using the method of Hoffman and Kunz (13), we optimized our enzyme assay for *L. mexicana* 227 promastigotes. Methionine adenosyltransferase activity was measured at 35°C in 100 μ l of a standard assay mixture containing 150 mM KCl, 20 mM MgSO₄, 5 mM dithiothreitol, 50 mM Tris (pH 7.5), 5 mM ATP, and 10 μ M L-[¹⁴C]methionine. The cationic [¹⁴C]adenosylmethionine formed was isolated by spotting 80- μ l portions of reaction mixtures on 2.3-cm-diameter disks of Whatman P81 cellulose phosphate cation-exchange paper, removing unreacted methionine by collecting the disks, and washing them three times in a beaker of cold 0.1 M ammonium formate (pH 3.0), once with 95% ethanol, and once with ether. [¹⁴C]-adenosylmethionine was quantified by liquid

scintillation counting of dried disks under 5 ml of Fisher Scint Verse II.

SAM synthetase was isolated by suspending 8 g of pelleted *L. mexicana* 227 cells in buffer containing 50 mM Tris (pH 7.5), 10 mM MgSO_4 , 1 mM EDTA, and 1 mM dithiothreitol. The cells were sonicated three times for 15 s each time, and the cell suspension was centrifuged at 4°C for 90 min at $40,000 \times g$ in an SW55 Ti rotor. The cell extract (5.3 ml) was applied to a DEAE-cellulose column, and the above buffer was passed through the column until the A_{280} was less than 0.1. The enzyme was then eluted with a linear gradient of KCl (0 to 0.3 M) in a volume of 80 ml.

Mutagenicity testing. The Ames salmonella test for detecting carcinogens and mutagens was performed as previously described (1), in the presence and absence of mammalian liver S9 fraction. The strains used included TA1535, TA1538, TA98, and TA100. Sinefungin, at levels from 2 to 2,000 $\mu\text{g}/\text{ml}$, was used in the test system, and results were compared with those of a known mutagen, aflatoxin (0 to 0.3 $\mu\text{g}/\text{ml}$). Tests were done in triplicate.

Animals. Male golden hamsters (*Mesocricetus auratus*) weighing approximately 60 g were obtained from Harlan Sprague-Dawley (Madison, Wis.), housed in plastic hamster cages on double-screened stainless steel racks, and given Rodent Blox (Wayne, Eatonton, Ga.) and tap water ad libitum. The hamsters were maintained in a climate-controlled room at a temperature of 22°C in the presence of 12 h of light and 12 h of darkness each day.

Testing procedure for *L. donovani*. Each hamster was infected with amastigotes of *L. donovani* WR378 obtained from heavily infected donor hamster spleens. Splenic suspensions were prepared by grinding the spleens in sterile saline in a Ten Broeck tissue grinder and diluting them with saline so that 0.2 ml contained approximately 10×10^6 amastigotes. Each experimental hamster was infected by intracardiac injection of 0.2 ml of the amastigote suspension.

Before initiation of treatment, initial body weights of hamsters were recorded to serve as a basis to determine weight changes during treatment and to determine the quantity of drug to be used. Treatment was initiated on day 3 postinfection and continued through day 6. Control groups received either vehicle only or one of three dosage levels of the reference compound, meglumine antimonate, twice daily intramuscularly. Sinefungin was administered at three dosage levels intramuscularly twice daily. Hamsters were observed during this period for such clinical signs of toxicity as death, nervous disorders, roughened hair coat, and sluggish activity.

One day after completion of treatment (day 7), hamsters were weighed and killed with CO_2 , livers were removed and weighed, and liver impressions were made for enumeration of amastigotes. Subsequently, the total numbers of parasites per liver (25), percent weight change, and percent amastigote suppression were calculated for all experimental groups by using an IBM-XT microcomputer.

Testing procedure for *L. braziliensis panamensis*. In preparation for infection with *L. braziliensis panamensis* WR539 and weekly during the experiment, the hair was clipped on the dorsal tail head, and a commercial depilatory agent (Nair; Carter-Wallace, Inc., New York, N.Y.) was applied to the area to remove the remaining hair. Each hamster was inoculated intradermally with 15×10^6 promastigotes near the base of the tail with a 0.25-ml glass syringe equipped with a 30-gauge, 1.27-cm-long needle. Promastigotes were grown in Schneider drosophila medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine

FIG. 2. Growth inhibition of *L. mexicana* by sinefungin. Cells were grown in the defined medium of Steiger and Steiger (26) without adenosine and supplemented with 5% heat-inactivated fetal bovine serum. Growth was monitored by turbidity measured at 660 nm at 72 h.

serum (GIBCO), harvested by centrifugation, and diluted with Schneider drosophila medium without fetal bovine serum so that each 0.05 ml contained 15×10^6 parasites.

Treatment was begun on day 19 postinfection and continued through day 22, being administered twice daily intramuscularly at the desired dosage levels. Hamsters were weighed before initiation of treatment for determination of dosage volume and to serve as a basis for the determination of weight change for indication of drug toxicity. Groups of hamsters receiving vehicle only or one of three dosage levels of meglumine antimonate were included as controls. Final weights were recorded for each group of animals 1 day following completion of treatment (day 23).

One week following completion of treatment (day 29), lesions were measured with a quantitated template which determined the diameter of each lesion in square millimeters.

Mean lesion size, percent weight change, and percent suppression were calculated for all groups with an IBM-XT microcomputer.

RESULTS

Antileishmanial effect of sinefungin compared with that of aphidicolin. The antileishmanial activity of sinefungin has been reported for a number of species but not for *L. mexicana* (23). Compared with previous reports, *L. mexicana* appears to be the most sensitive strain tested. Figure 2 shows the sensitivity of this strain to sinefungin in the medium of Steiger and Steiger. In our growth experiments, the average concentration of sinefungin that produced 50% inhibition was 3 nM. We compared this activity with that of a known DNA polymerase inhibitor, aphidicolin. At 2 μM , aphidicolin produced 50% growth inhibition in *L. mexicana* (Fig. 3).

Since SAM and S-adenosylhomocysteine are structurally related to sinefungin, these compounds were tested both for reversal of inhibition by sinefungin and for their growth effects on *L. mexicana*. It was found that S-adenosylhomocysteine (0 to 100 μM) had no effect on the growth of the parasite and could not reverse growth inhibition by sinefungin. SAM (50% effective dose [ED_{50}], 30 μM) stimulated growth of the parasite when added alone to the medium

FIG. 3. Growth inhibition of *L. mexicana* by aphidicolin. Cells were grown in 5 ml of the defined medium of Steiger and Steiger (26) without adenosine and supplemented with 5% heat-inactivated serum. Growth was monitored by turbidity measured at 660 nm at 72 h.

and completely reversed the inhibitory action of sinefungin (at 5 nM). SAM is one of nature's most versatile molecules, serving as a methyl donor and precursor in the biosynthesis of polyamines which influence nucleic acid biosynthesis. Therefore, we tested the following compounds for reversal of growth inhibition by sinefungin: putrescine, spermine, spermidine, methionine, folic acid, thymidine, and adenosine. These compounds were chosen because SAM interacts with these compounds or their products as a methyl donor or they are needed for synthesis of SAM. None of these compounds were able to reverse growth inhibition by sinefungin in *L. mexicana*.

Effect of sinefungin on SAM synthetase. The results of our reversal experiments suggested that this enzyme was a possible target for the inhibitory action of sinefungin, since SAM, the product of this reaction, could reverse inhibition but methionine, the precursor, could not. This enzyme catalyzes the following reaction: L-methionine + ATP → SAM + PP_i + P_i (19). This enzyme also appeared to be a logical target, since its inhibition would inhibit DNA synthesis. This is because SAM, the product, is necessary for methylation of DNA.

The isolation procedures described eliminated 99% of the starting protein and resulted in a 430-fold increase in activity of the enzyme (data not shown). Assay for DNA polymerase by the procedures described showed that polymerase activity coeluted with SAM synthetase. Further chromatography of SAM synthetase (data not shown) on phenyl-Sepharose, CL-6B Sepharose, and Sephadex G-150, resulted in an 80% reduction in activity of the enzyme, and DNA polymerase activity no longer eluted with this enzyme. In these enzyme preparations, sinefungin was slightly (14%) inhibitory at concentrations of up to 1 mM, depending on temperature, ATP concentration, and addition of the product of the reaction, SAM.

Effect of sinefungin on DNA polymerase activity by spectrophotometric assay. Using a coupled assay system as described by Sigma technical bulletin 7275, we first determined that sinefungin, at the levels being used, had no effect on the coupling assay system. Since sinefungin most closely resembles dATP, compared with the other deoxynucleoside tri-

phosphates, we varied the dATP concentration in the presence of 0.5 μM sinefungin. With this assay system, dATP had a K_m of 55.5 μM (Fig. 4A), and sinefungin inhibited the enzyme with a K_i of 15 nM (Fig. 4B). SAM and S-adenosylhomocysteine (up to 40 μM), which are structurally related to sinefungin, showed no inhibition. Complete reversal of inhibition by sinefungin was obtained by various dATP concentrations (Fig. 5). To ensure that we had indeed observed DNA polymerase activity, we tested a number of known DNA polymerase inhibitors in this assay system. *N*-Ethylmaleimide, KCl, ethidium bromide, berenil, and aphidicolin were found to be inhibitory. Aphidicolin at 20 μM inhibited the enzyme activity by 50%, which correlates reasonably well with its *in vitro* antileishmanial activity (Fig. 3). Since it is well documented that *N*-ethylmaleimide and aphidicolin are specific DNA polymerase inhibitors and since we were able to inhibit activity completely with higher concentrations of these compounds, it was assumed that it was DNA polymerase that had been assayed. We were able to detect DNA polymerase activity by using this assay until

FIG. 4. (A) Lineweaver-Burk plot obtained by using the spectrophotometric assay detecting PP_i release as a measurement of DNA polymerase activity. Symbols: ■, assay in the presence of 0.5 μM sinefungin; ●, assay with no inhibitor present. The substrate varied was dATP. All other deoxynucleoside triphosphates were present at 44 μM. The K_m for dATP was 55.5 μM. (B) Dixon plot of increasing concentrations of sinefungin in the presence of S₁ (44.4 μM dATP) and S₂ (22.2 μM dATP). All other deoxynucleoside triphosphates were present at 44.4 μM. The K_i for sinefungin was 15 nM. Abbreviations: I, sinefungin inhibitor; V, velocity; S, substrate dATP.

the enzyme was purified further with denatured DNA-cellulose (Table 1). After this point in purification, we could not detect any inhibition by either sinefungin or aphidicolin at concentrations of up to 200 μ M. Fearing that perhaps the assay system was at fault, we decided to use a radioactive assay system used by most investigators in the field.

Effect of sinefungin and aphidicolin on *L. mexicana* DNA polymerase by radioactive assay. Table 1 shows the purification scheme obtained for the isolation of DNA polymerase with the activity quantitated by use of [3 H]TTP. Much to our surprise, at all steps we could not detect any inhibition of activity by sinefungin or aphidicolin. Neither could we detect inhibition by sinefungin by using [3 H]dATP as the isotope in the assay. Other investigators using this assay technique have reported similar results with aphidicolin as a tool to detect DNA polymerase α in trypanosomes (24) and in the related protozoan *Crithidia fasciculata* (14). These findings have produced a great amount of interest because aphidicolin was demonstrated to be a specific direct inhibitor of animal DNA polymerase but is without effect on polymerase β or γ (15). With aphidicolin as a tool, it has been shown that DNA polymerase α is clearly the principal polymerase required for DNA replication in all of the animals and plants studied (15).

Effect of sinefungin in the salmonella/mammalian mutagenicity test. Sinefungin drastically inhibits DNA synthesis (3). We found that growth inhibition could be reversed with SAM only when it was added to the medium at the same time as sinefungin. If SAM was added hours after sinefungin, complete reversal did not occur. These results indicated that sinefungin may interfere with the methylation of DNA, a function which has been implicated in regulating gene expression (4-6). To determine whether sinefungin was a possible mutagen, the Ames test was performed with the strains described in Materials and Methods in the presence or absence of mammalian S-9 liver fraction. The latter is required for the activation of several known mutagens and carcinogens (1). Aflatoxin produced 600 to 2,000 revertants. In the presence of sinefungin, the number of revertants was lower than the number of spontaneous revertants (data not shown).

Antileishmanial effect of sinefungin in hamsters. Sinefungin significantly suppressed growth of both *L. donovani* (Table 2) and *L. braziliensis panamensis* (Table 3) in hamsters. A comparison of the suppressive activity of sinefungin with that of meglumine antimonate against *L. donovani* indicates that sinefungin was approximately four times more effective than the reference compound. The ED₅₀ of sinefungin was approximately 4 mg/kg of body weight as compared with 16 mg of antimony per kg of body weight for meglumine antimonate. The activity of sinefungin was approximately 30-fold greater against *L. braziliensis panamensis* than was that of meglumine antimonate. The ED₅₀ for sinefungin was

FIG. 5. Reversal of inhibition by sinefungin of DNA polymerase in the presence of increasing concentrations of dATP. All other deoxynucleoside triphosphates were present at 44 μ M. Enzyme activity was assayed spectrophotometrically as described in Materials and Methods.

approximately 22 mg/kg of body weight, while that of meglumine antimonate was 660 mg of antimony per kg of body weight.

Although treatment of hamsters with sinefungin did not result in significant loss of body weight, the hair coats of hamsters receiving this compound appeared slightly roughened, suggesting some toxicity. Additional work would be required to draw valid conclusions regarding the toxicity of sinefungin.

DISCUSSION

In our particular strain of *L. mexicana*, sinefungin has been shown to be one of the most potent antileishmanial compounds tested in our in vitro promastigote test system. With an in vivo hamster test system, sinefungin was shown to be 4 to 5 times more effective against *L. donovani* and 30 times more effective against *L. braziliensis panamensis* than was meglumine antimonate.

In vivo, sinefungin has been reported to cure mice infected with various *Trypanosoma* species, with no toxic side effects (7). Also, no toxic side effects were observed in mice treated with *L. donovani* and *L. tropica*, and the potency of sinefungin relative to that of sodium stibogluconate, a known antileishmanial drug, was reported to be 73 times greater (19). Although sinefungin has been shown to inhibit DNA synthesis in *Leishmania* spp. (3), we showed that it is not mutagenic in the Ames test. Nearly 90% of all carcinogens tested by this method have been found to be mutagenic (1).

The mode of action of sinefungin appears to involve inhibition of DNA replication. DNA replication is enzymatically complex and requires many activities in addition to

TABLE 1. Purification scheme for DNA polymerase

Purification step	Total vol (ml)	Total protein (mg)	U (ml ⁻¹) ^a	Total activity (U)	Sp act (U mg ⁻¹)	Purification (fold)
Lysed cells	40.8	1,448.4	6.70	273.18	0.189	
Crude extract ^b	38.5	1,068.38	7.7	296.45	0.277	1.47
Protamine sulfate supernatant	37.5	441.0	15.29	573.36	1.3	6.88
Heparin-Sepharose	14.5	41.62	119.77	1,736.67	41.73	220.79
Cellulose phosphate	10.2	4.38	26.58	271.14	61.82	327
DNA-cellulose	3.1	0.108	29.08	90.15	834.72	4,416.51

^a One unit of activity is defined as the incorporation of 1 pmol of dTMP into DNA in 30 min under the assay conditions described in Materials and Methods.

^b From centrifugation at 15,000 $\times$ g for 1 h.

TABLE 2. Comparison of the suppressive activities of meglumine antimonate and sinefungin against *L. donovani* in golden hamsters

Compound and total dosage (mg/kg) ^a	No. of animals	No. of deaths	% Wt decrease	Mean no. of parasites/liver (10 ⁶)	% Suppression ^b
Vehicle control	6	0	10	525	
Meglumine antimonate					
416	6	0	12	1	100
52	6	0	10	131	75
13	6	0	7	278	47
Sinefungin					
104	6	0	10	31	94
52	6	0	10	77	85
6.5	6	0	6	206	61

^a Dosage levels of meglumine antimonate were based on percent antimony. Those of sinefungin were based on the total weight of the compound.

^b Sinefungin was approximately four times more potent than meglumine antimonate at the ED₅₀.

DNA polymerase. A difficulty in analyzing the role of eucaryotic DNA polymerases is caused by the lack of in vitro systems containing all of the components needed for replication. Biochemical characterization of eucaryotic DNA replication is still in its infancy.

The precursor for DNA synthesis is a nucleoside triphosphate, which loses two phosphate groups in the reaction. It appears, from the results presented here, that sinefungin and aphidicolin interfere with a pyrophosphatase activity associated with DNA polymerase at certain stages of purification. This pyrophosphatase activity was inhibited in the spectrophotometric assay by both aphidicolin and sinefungin. It is interesting that, when this activity was lost during purification, a dramatic increase in the specific activity occurred in the [³H]TMP incorporation assay (Table 1; 13-fold increase between the cellulose phosphate and DNA-cellulose steps). Because of the complexity of the reaction catalyzed by DNA polymerase, it is impossible to estimate the exact relevance of our findings. However, the results

TABLE 3. Comparison of the suppressive activity of meglumine antimonate and sinefungin on *L. braziliensis panamensis* in golden hamsters

Compound and total dosage (mg/kg) ^a	No. of animals	No. of deaths	% Wt decrease	Mean lesion area (mm ²)	% Suppression ^b
Vehicle control	6	0	6.8	121	
Meglumine antimonate					
4,128	6	0	3.8	17	86
2,064	6	0	2.1	26	79
832	6	0	2.2	44	63
Sinefungin					
208	6	0	10.6	42	66
104	6	0	9.9	26	79
26	6	0	7.0	50	59

^a Dosage levels of meglumine antimonate were based on percent antimony. Those of sinefungin were based on the total weight of the compound.

^b Sinefungin was approximately 30 times more potent than meglumine antimonate at the ED₅₀.

presented here and in previously published reports on the nature of DNA polymerase α in parasitic protozoa are compatible with the following interpretation. Solari et al. (24) and Holmes et al. (14) reported the isolation of a DNA polymerase α in parasitic protozoa which was resistant to inhibition by aphidicolin, yet the growth of the parasites were strongly inhibited by aphidicolin. Solari et al. reported that DNA synthesis in vivo was inhibited by aphidicolin. These investigators found that the isolated DNA polymerase α -like enzyme failed to cross-react with mammalian DNA polymerase α from different species (14, 24). These investigators have suggested that the DNA polymerases α of these parasites are immunologically distinct from the host enzyme and that the structural differences between the parasite and host enzymes could be exploited for the development of agents to combat parasitic diseases. Our results suggest that, in the case of *L. mexicana*, an aphidicolin-sensitive activity is associated with DNA polymerase activity and that this activity is lost early in purification. The enzymatic activity associated with aphidicolin inhibition fails to be detected in the commonly used radioactive assays for DNA polymerase α activity. Sinefungin also appears to inhibit this pyrophosphatase activity. The reported in vitro study showing that sinefungin drastically inhibits DNA synthesis (3) suggests that this enzyme is crucial for in vivo DNA replication. It is interesting that SAM synthetase has tripolyphosphatase activity, and in our early purification steps, this enzyme coeluted with DNA polymerase. The observation, that only SAM could reverse the in vivo growth inhibition of sinefungin, suggests that SAM synthetase is somehow involved in regulating DNA synthesis via methylation of the DNA. Since neither aphidicolin nor sinefungin inhibited partially purified SAM synthetase, the possibility exists that these compounds inhibit DNA replication only when SAM synthetase and DNA polymerase are together. That dATP was able to reverse inhibition by sinefungin in the in vitro DNA polymerase spectrophotometric assay suggests that this compound, which is sterically similar to sinefungin, prevented the inhibitor from binding at the appropriate site. Future studies will concentrate on the purification and characterization of this sinefungin- and aphidicolin-sensitive pyrophosphatase activity, and studies will be conducted on the effect of this activity on DNA replication.

The data presented substantiate the report that DNA replication is inhibited by sinefungin in leishmanial parasites (3), and the site of action appears to be unique in these lower eucaryotes.

Since the DNA polymerase of the *Trypanosomatidae* has been shown to be biochemically and immunologically distinct from its mammalian counterpart (8, 14, 23), it is a rational target for chemotherapy. Sinefungin has been shown to be antiparasitic at levels not toxic to mammalian cells, and its major mode of action appears to be to interfere with DNA replication. Sinefungin appears to have potential as an antiparasitic drug, and further elucidation of its mode of action will provide a framework for the synthesis of an ever more selective drug.

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