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# Differential Effect of Sinefungin and Its Analogs on the Multiplication of Three *Leishmania* Species

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**Sinefungin and seven analogs were evaluated in vitro for activity against promastigote multiplication of *Leishmania donovani*, *L. tropica*, and *L. enrietti*. Of these compounds, sinefungin, the cyclic derivative, and A9145C were leishmanicidal at concentrations ranging from 0.13 to 2.6 μM. Sinefungin was the most active of these three compounds against *L. donovani*, and A9145C was most active against *L. enrietti*. None of the remaining derivatives exhibited significant activity against any one of the three species at the highest dose tested. All agents were assayed for activity against protein methylases I and III. The results of these tests showed that there is no relationship between the inhibition of growth and inhibition of protein methylases I and III.**

Sinefungin (compound 1), its cyclic derivative (compound 2), and a dehydro analog, A9145C (compound 3) (Fig. 1), are natural nucleosides produced by *Streptomyces griseolus* (7) and *Streptomyces incarnatus* (Rhône-Poulenc patent no. 7611141, April 1976). Sinefungin exhibited antifungal (6) and antiparasitic activity in vitro and in vivo (1–3, 10, 16). Its activity as a transmethylase inhibitor in various avian and mammalian cells was also established (4, 13, 15, 17). In a preliminary study, Bachrach et al. (1) have shown that 3 μM sinefungin completely inhibits the growth of promastigotes of some species of *Leishmania*.

The aims of this work were to determine whether the effect of sinefungin was irreversible and to establish a relationship among growth inhibition, chemical structure of these nucleosides, and their activity on leishmanial protein methylases.

## MATERIALS AND METHODS

**Nucleosides.** Compounds 1 and 3 were generously provided by R. S. Gordee, Lilly Research Laboratories, Indianapolis, Ind. Compound 2, 9-[6-amino-5,6,7,8,9-pentadeoxy-8-methyl-β-D-ribomonafuranosyl]-adenine (compound 6), and 9-[6-cyano-5,6,7,8,9-pentadeoxy-8-methyl-β-D-ribo6-ene-nonafuranosyl-1,4-9-adenine (compound 7) (Fig. 1) were synthesized in our laboratory (5; P. Blanchard, N. Dodic, J.-L. Fourrey, M. Géze, F. Lawrence, H. Malina, P. Paolantonacci, M. Vedel, C. Tempête, M. Robert-Géro, and E. Lederer, *in The Biochemistry of S-adenosylmethionine as a Basis for Drug Design*, in press). *S*-adenosylhomocysteine (SAH) and *S*-adenosylmethionine (SAM) were purchased from Sigma Chemical Co., St. Louis, Mo., and difluoromethyl ornithine (DFMO) was purchased from Merrell Co., Strasbourg, France. The compounds were dissolved in phosphate-buffered saline at 100 to 1,000 times the concentrations used for the experiments and sterilized by filtration (0.22-μm-pore-size filter, Millex; Millipore S.A., Molsheim, France). Stock solutions were stored at −20°C. The purity of the molecules was controlled by thin-layer chromatography.

**Cells.** The promastigotes of *Leishmania donovani* LRC L52, *L. tropica* LRC L32, and *L. enrietti* LRC L327 originated from the strain collection of the World Health Organization International Reference Center for Leishmaniases and had been maintained at the Department of Medical Protozoology, the Hebrew University-Hadassah Medical School Jerusalem, Israel. They were forwarded by L. Schnur, of the above address.

**Growth of organisms.** Promastigotes of the three species were grown in a medium composed of 45% Dulbecco modified Eagle medium, 45% RPMI 1640 medium containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.4]), and 10% heat-inactivated fetal calf serum. Streptomycin at 5 μg/ml, penicillin at 5 U/ml, and kanamycin at 5 μg/ml were also added.

**Antileishmanial effect of sinefungin and analogs.** Promastigotes ($2.5 \times 10^6$) were inoculated into 5 ml of the above medium. Cultures were performed in 25-ml Nunclon flasks at 26°C. One day after seeding, the promastigotes were counted in each flask with a hemacytometer, and the compound to be tested was added at the desired concentration. Each test was made in duplicate, and the number of promastigotes was counted from two dilutions, generally 1/10 and 1/20, once a day. Untreated cultures were run in parallel. On day 3 of treatment, the cells were centrifuged and washed in 5 ml of phosphate-buffered saline, and the promastigotes were suspended in a new medium. As all the molecules used were water soluble, this procedure removed all measurable test compounds. The number of promastigotes was counted again 3 and 14 days later. Irreversible growth arrest and decrease in cell number was attributed to a leishmanicidal effect.

**Preparation of cell extracts.** About $10^9$ mid-log-phase promastigotes were centrifuged at 4,500 × *g* for 10 min at 10°C. Pellets were washed twice in phosphate-buffered saline and lysed by four cycles of freezing and thawing. Cell lysis was monitored with the aid of the microscope. Lysed cells were centrifuged at 1,200 × *g* for 30 min at 4°C, and the supernatants were stored for 24 to 36 h at −20°C.

**Protein methylases.** *S*-Adenosylmethionine:protein–arginine c methyltransferase (EC 2.1.1.23; protein methylase I [PMI] and *S*-adenosylmethionine:protein–lysine methyltransferase (EC 2.1.1.25; protein methylase III [PMIII]), which catalyse the posttranslational methylations of arginine and lysine residues of proteins, respectively, were measured by a published procedure (12). Kinetic constants were obtained from Lineweaver-Burk plots (8). Protein concentra-

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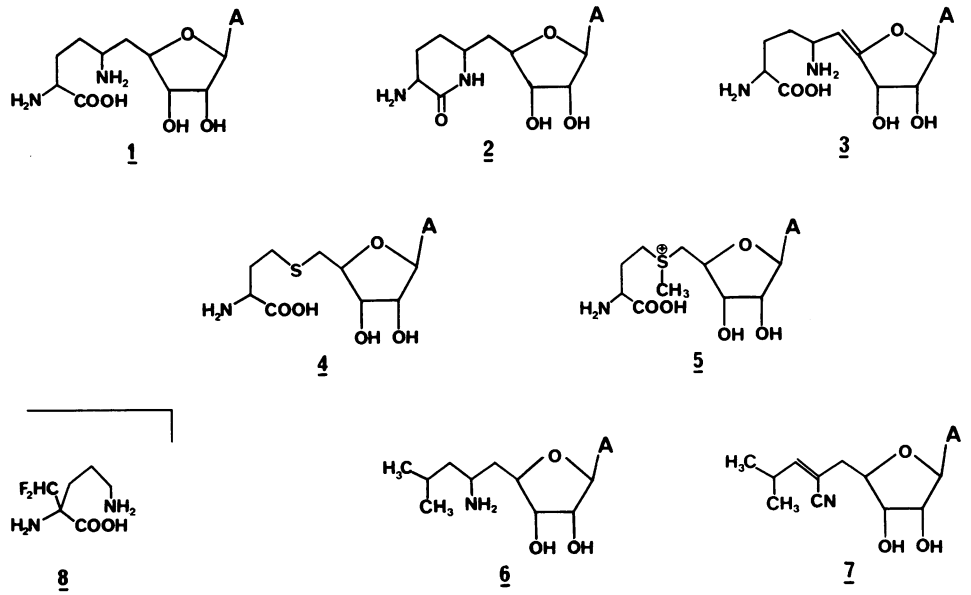

FIG. 1. Structures of compounds tested. A, Adenine residue. The common names of these molecules are as follows: compound 1, sinefungin; compound 2, cyclic derivative of sinefungin; compound 3, A9145C; compound 4, SAH; compound 5, SAM; compound 6, 9-[6-amino-6,7,8,9-pentadeoxy-8-methyl-β-D-ribononafuranosyl]-adenine; compound 7, 9-[6-cyano-5,6,7,8,9-pentadeoxy-8-methyl-β-D-ribo-6-ene-nonafuranosyl]-adenine; and compound 8, 2-difluoromethyl-2,5-diamino-pentanoic acid, or DFMO.

tion was determined by the method of Lowry et al. (9) with crystalline bovine serum albumin as the standard.

## RESULTS

The three *Leishmania* species studied were *L. donovani*, the etiologic agent of visceral leishmaniasis, or kala azar; *L. tropica*, which causes cutaneous lesions in humans; and *L. enrietti*, the agent of leishmaniasis in guinea pigs. Sinefungin was inhibitory in vitro for the promastigotes of all species but its effect varied greatly from one species to another, *L. donovani* being the most susceptible and *L. enrietti* being the least susceptible (Table 1). The growth arrest took less than one generation to become fully established. When observed under the light microscope, the sinefungin-treated promastigotes were initially elongated and they became immobile and spherical. After a 3-day exposure, promastigotes were transferred to drug-free medium for up to 14 days to distinguish between reversible and irreversible growth arrest. Under these conditions, the concentration of sinefungin required for irreversible growth arrest was 2.6 μM for *L. enrietti*, 0.26 μM for *L. tropica*, and 0.13 μM for *L. donovani* (Table 1). After treatment with lower concentrations, growth was restored when the cells were transferred to drug-free medium.

Because of the marked differences in the susceptibility of the three *Leishmania* species to the inhibitory effect of sinefungin, we investigated the action of various structurally related compounds on the multiplication of promastigotes. Changes in the side chain of sinefungin as in the analogs 6 and 7 (Fig. 1) result in the complete loss of the inhibitory

TABLE 1. Effect of various concentrations of sinefungin on the multiplication of three *Leishmania* species

| Organism | Concn of sinefungin (μM) | No. of promastigotes ($10^5$) (±15%) on day of incubation | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 6[a] | 14[a] |
| *L. donovani* | 0 | 12.0 | 38.0 | 170 | 300 | 700 | 500 |
| | 0.026 | 12.0 | 20.0 | 22.0 | 23.0 | 50.0 | 80.0 |
| | 0.130 | 12.0 | 10.6 | 10.8 | 11.0 | 10.8 | 11.0 |
| | 0.260 | 12.0 | 10.4 | 10.8 | 11.0 | 6.0 | 4.3 |
| | 2.600 | 12.0 | 10.2 | 10.2 | 10.0 | 4.0 | 4.2 |
| *L. tropica* | 0 | 8.0 | 22.0 | 50.0 | 150 | 300 | 500 |
| | 0.026 | 8.0 | 19.0 | 29.0 | 35.0 | 60.0 | 290 |
| | 0.130 | 8.0 | 12.0 | 18.0 | 18.0 | 40.0 | 150 |
| | 0.260 | 8.0 | 10.0 | 9.0 | 9.2 | 11.0 | 13.0 |
| | 2.600 | 8.0 | 7.8 | 6.3 | 5.0 | 4.5 | 3.6 |
| *L. enrietti* | 0 | 8.0 | 60.0 | 150 | 170 | 300 | 500 |
| | 0.026 | 8.0 | 16.0 | 32.0 | 62.0 | 100 | 500 |
| | 0.130 | 8.0 | 13.5 | 21.0 | 37.0 | 80.0 | 450 |
| | 0.260 | 8.0 | 10.6 | 10.6 | 10.5 | 40.0 | 100 |
| | 2.600 | 8.0 | 6.2 | 4.2 | 4.3 | 4.4 | 3.5 |

[a] Drug-free period.

TABLE 2. Minimal leishmanicidal concentration (μM) of sinefungin and related compounds[a]

| Organism | Minimal leishmanicidal concn (μM) of compound[b]: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| *L. donovani* | 0.13 | 0.26 | >2.6 | >10 | >100 | >26 | >26 | >4 |
| *L. tropica* | 0.26 | 2.6 | >2.6 | >100 | >100 | >26 | >26 | >4 |
| *L. enrietti* | 2.6 | 2.6 | 0.13 | >100 | >100 | >26 | >26 | >4 |

[a] Mean values of three to five independent experiments with each analog are shown.
[b] Compounds are as shown in Fig. 1: 1, sinefungin; 2, cyclic derivative of sinefungin; 3, A9145C; 4, SAH; 5, SAM; 6 and 7, synthetic analogs of sinefungin; and 8, DFMO.

effect for all three species (Table 2). Identical results were obtained with SAH (compound 4) and SAM (compound 5), in which the CH—$NH_2$ group of sinefungin is replaced by S and $S^+$—$CH_3$, respectively. No inhibition was observed when adenosine and ornithine, the components of sinefungin, were added to the culture. DFMO (compound 8), a side chain analog and a known inhibitor of ornithine decarboxylase, had no effect on promastigote growth.

A9145C (compound 3), which differs from sinefungin only by the presence of a double bond at the 5′ end, is much less effective towards *L. donovani* and *L. tropica* but irreversibly inhibits growth of *L. enrietti* promastigotes at 0.13 μM. The cyclic derivative of sinefungin (compound 2) was the only analog tested that was leishmanicidal for the three species. The activity of this agent against the three species varied: growth of *L. donovani* was inhibited irreversibly by 0.26 μM, but a 10-fold higher concentration was necessary to inhibit *L. tropica* and *L. enrietti*. These results suggest that either the molecular target of sinefungin is different in the three species or, if it is the same, it behaves differently.

Vedel et al. have shown previously that sinefungin and A9145C are very potent inhibitors of SAM-mediated transmethylations (17). Thus, in an attempt to explain the differences in susceptibility among the three species to the inhibitory effect of sinefungin and the analogs, we studied two protein methylases, PMI and PMIII, of these parasites. As the existence of these enzymes was not yet described in promastigotes, procedures published for avian and mammalian protein methylases were used to measure the activities. Both methylases were detected in the three species (Table 3). The in vitro activity of both enzymes is comparable in *L. donovani* and *L. enrietti*, whereas those of *L. tropica* are weaker.

The response of PMI and PMIII towards the various analogs varied with the species. All compounds that inhibited protein methylation in vitro were competitive with respect to SAM. Only SAH and sinefungin were inhibitors of all species, but with one exception, there was no correlation between the ability of these molecules to inhibit protein methylases and growth of promastigotes. The exception was the PMIII of *L. donovani*, with a $K_m$ of SAM/$K_i$ of sinefungin ratio of 66. Thus, in the case of this parasite, PMIII may be one of the molecular targets of sinefungin.

## DISCUSSION

In some respects the antileishmanial effects of sinefungin, its cyclic derivative, and A9145C resemble those reported for allopurinol riboside and formycin B. It has been shown that the inhibitory effect of allopurinol riboside varies considerably among species of *Leishmania* promastigotes: *L. brasiliensis* being the most susceptible, *L. donovani* being intermediate, and *L. mexicana* being relatively resistant (11). One of the differences between the effects of allopurinol riboside and those of sinefungin is that the reduction of the growth rate by the former requires 18 to 21 h to become fully established, and the compound seems to be only leishmanistatic (11).

Insignificant variations in the susceptibility of *Leishmania* species towards formycin B were reported, but as in the case of sinefungin no lag periods were observed before onset of growth inhibition (14). Formycin B was leishmanicidal only at a concentration of 10 μM, whereas the leishmanicidal concentrations of sinefungin for *L. donovani* and *L. tropica* were in the submicromolar range.

A preliminary study of the relationship between growth inhibition and chemical structure showed that changes in the side chain of sinefungin, such as replacement of the CH—$NH_2$ group by S or $S^+$—$CH_3$ in SAH or SAM, respectively, lead to the loss of the inhibitory activity. The presence of the terminal amino and carboxyl groups also seems to be important since the synthetic analogs lacking these functions are practically inactive. Cyclization of the side chain preserves but lowers activity against *L. tropica* and *L. donovani*. Interestingly, the introduction of a double bond at the 5′ end abolishes activity against *L. donovani* and *L. tropica* but enhances activity against *L. enrietti* 20-fold. These results suggest that sinefungin and its cyclic analog may share a common molecular target in *L. donovani* and *L. tropica*, with sinefungin having greater affinity. A9145C is seemingly not recognized by the same target in these species. The superior activity of this analog against *L. enrietti* may be ascribed to differences in its molecular target or its sensitivity of receptors in the different species. Studies on additional structural analogs are needed for clarification of this problem.

TABLE 3. Apparent kinetic constants of PMI and PMIII in *L. donovani*, *L. tropica*, and *L. enrietti* promastigotes[a]

| Promastigote | Kinetic constant of | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PMI | | | | | | | | PMIII | | | | | | | |
| | $V_m$[b] | $K_m$ of SAM (μM)[c] | $K_i$ (μM) of compound | | | | | | $V_m$[b] | $K_m$ of SAM (μM)[c] | $K_i$ (μM) of compound | | | | | |
| | | | 1 | 2 | 3 | 4 | 6 | 7 | | | 1 | 2 | 3 | 4 | 6 | 7 |
| *L. donovani* | 1,120 | 24 | 145 | 253 | ND[d] | 3 | 52 | ND | 1,560 | 120 | 1.8 | ND | ND | 4.8 | ND | 980 |
| *L. tropica* | 310 | 14 | 57 | ND | ND | 19 | ND | ND | 350 | 37 | 47 | | | 3.0 | | |
| *L. enrietti* | 1,050 | 60 | 120 | 1,370 | 500 | 2 | | | 1,050 | 50 | 73 | ND | ND | 4.9 | | |

[a] Protein methylase activities were measured as described by Paik et al. (12).
[b] $V_m$, Picomoles of $CH_3$ incorporated per milligram of protein at 27°C for 1 h.
[c] SAM concentration varied from 1 to 80 μM.
[d] ND, Inhibition not detectable when the drug was tested at a concentration of 200 μM.

According to our results, leishmanial protein methylases respond differently to sinefungin and analogs than do such enzymes in other organisms (15). With the exception of PMIII of *L. donovani*, this nucleoside is not a good in vitro inhibitor of protein methylases of promastigotes. The findings that SAH is a potent inhibitor for these enzymes but has no effect on parasite multiplication can probably be explained by its intracellular degradation by SAH hydrolase. A clear relationship, between the antileishmanial activity of this nucleoside and its inhibition of a protein methylase in these parasites could not be established. Thus, the exact mechanism of inhibition by sinefungin remains unresolved.

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