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INHIBITION OF LEISHMANIAL DNA SYNTHESIS BY SINEFUNGIN

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Abstract—RNA, DNA and protein biosynthesis were studied in *Leishmania donovani* and *L. tropica* promastigotes cultured with or without sinefungin. Thymidine incorporation was significantly impaired by this compound. Neither the uptake of thymidine nor its phosphorylation were inhibited. Furthermore the ratios of deoxyribonucleotide to the corresponding ribonucleotide were not significantly affected by sinefungin. Analysis of the DNA indicates that the inhibition of thymidine incorporation affects mostly nuclear DNA, kDNA being less affected by this drug. No such effect on thymidine incorporation was observed in macrophages, the host cells of these parasites.

Sinefungin **1** (Fig. 1), a natural nucleoside isolated from cultures of *Streptomyces incarnatus* and *S. griseolus* is an antifungal and antiparasitic agent *in vitro* and *in vivo* [1–10].

This nucleotide is structurally-related to *S*-adenosylmethionine **4** (AdoMet) and to *S*-adenosylhomocysteine **5** (AdoHcy), respectively substrate and inhibitor of methyltransferases, and was shown to inhibit these enzymes in various cells [11–14]. However, contrarily to what was expected, sinefungin is a poor inhibitor of leishmanial protein methylases I and III *in vitro* and *in vivo* [10, 15]. As the growth inhibition provoked by sinefungin may be related to other cellular events we studied the effect of this nucleoside on macromolecular synthesis in promastigotes in *Leishmania*. Our results showed that DNA synthesis was drastically inhibited in promastigotes grown in the presence of sinefungin and that this inhibition was not the consequence of either thymidine uptake or its phosphorylation. The inhibition of DNA synthesis is specific for the parasite, since no such inhibition could be detected in host cells (macrophages).

MATERIALS AND METHODS

Growth of organisms. Leishmania tropica (strain LRC L32), *L. enrietti* (strain LRC L327) and *L. donovani* (strain LRC L52) originated from the strain collection of the World Health Organisation's International Reference Center for leishmaniasis (WHO–LRC) and were kindly provided by Dr. L. F. Schnur (Hebrew University, Hadassah Medical School, Jerusalem, Israël).

Mouse peritoneal macrophages, cell line P388D (obtained by chemical carcinogenesis) were kindly provided by Dr A. Adam (Institute of Biochemistry, Faculté des Sciences d'Orsay, France).

The promastigotes and the macrophages were grown in a semi-defined medium as described previously [10]. The temperature of the cultures was 26° for the *Leishmania* promastigotes and 37° for the macrophages.

Nucleosides. Sinefungin **1** and A9145 C **3** (Fig. 1) were kindly provided by Dr. R. Nagarajan (Lilly Research Laboratories, Indianapolis, IN). The cyclic derivative of sinefungin **2** (Fig. 1) was synthesized in our laboratory by Dr. M. Gèze [16]. AdoHcy, AdoMet, adenine, thymine, uracyl, guanine and their respective nucleosides and nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO). Radioactive products were from Amersham (U.K.) [^{14}C-U]adenosine, 500 mCi/mmol, [^{14}C-U]guanosine 450 mCi/mmol, [5-^{3}H]uridine 30 Ci/mmol, [methyl-^{3}H]thymidine 52 Ci/mmol and L-[4,5-^{3}H]leucine 138 Ci/mmol.

Effect of sinefungin on macromolecular synthesis. Promastigotes (5.10^6 cell/ml) were cultured with and without sinefungin [10] for various lengths of time, and then the appropriate radioactive precursor was added for 1 hr to the medium: L[4.5-^{3}H]leucine, 5 μCi/ml or [5-^{3}H]uridine, 2.5 μCi/ml or [methyl-^{3}H]thymidine, 2.5 μCi/ml. After 1 hr labelling, the cells were centrifuged and rinsed twice in cold phosphate buffered saline (PBS). The uptake into the soluble pool and the incorporation into nucleic acids and proteins, were obtained from the cold-TCA-soluble, hot-TCA-soluble and insoluble materials respectively [17]. Protein concentration was determined by the Lowry procedure [18] using bovine serum albumin as the standard.

Effect of sinefungin on nucleosides phosphorylation. Promastigotes were cultured and labelled as described for macromolecular synthesis. The rinsed cells were extracted 2 min with 0.5 M cold perchloric acid. After neutralization and centrifugation the extract was chromatographed along with unlabelled markers on cellulose thin layer plates (Eastman-Kodak 6064) using tert-amylalcohol–formic acid–water (60–40–20 vol/vol) as solvent [19].

Deoxyribunucleotide content was determined on the same extract which had been treated with periodate and methylamine [20].

DNA analysis. DNA was extracted as described by Blin and Stafford [21].

Equilibrium sedimentation in CsCl gradients was

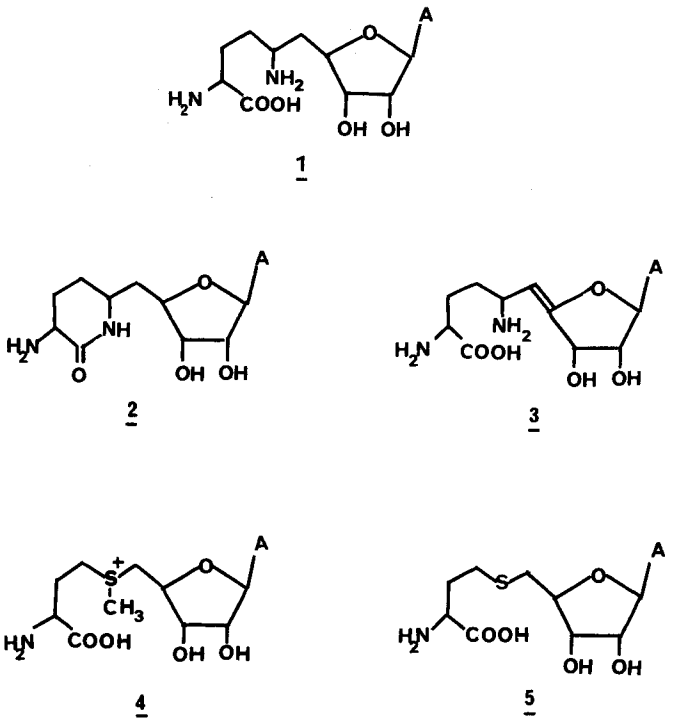

Fig. 1. Structure of **1** Sinefungin; **2** Sinefungin lactam; **3** A9145C; **4** *S*-adenosylmethionine (AdoMet); **5** *S*-adenosyl-homocysteine (AdoHcy). A = adenosine.

carried out as described by Simpson and Berliner [22].

RESULTS

Effects of sinefungin on macromolecular biosynthesis

RNA, DNA and protein biosynthesis were studied in the absence and in the presence of sinefungin concentrations which inhibit promastigote multiplication (0.026 μM and 0.26 μM) [10]. As shown in Table 1 thymidine incorporation was significantly impaired in *L. donovani* promastigotes. After 6 hr i the presence of 0.026 μM and 0.26 μM sinefungin th incorporation of thymidine into DNA was inhibite by 70 and 91% respectively, despite an increase in th amount of radioactivity in the thymine-containin components of the acid-soluble pool. In compariso to these results, uridine incorporation and uptak were both moderately decreased; however, th uptake of uridine into the TCA-soluble fraction wa inhibited to a lesser degree than the incorporatio into TCA-insoluble material suggesting a slight effe

Table 1. Macromolecular biosynthesis in *L. donovani* promastigotes cultured with or without sinefungin

Labelled precursor	Sinefungin concentration (μM)	TCA-soluble fraction	TCA-insoluble fraction
Leucine	0.	24 640 (100%)	59 680 (100%)
	0.026	123 ± 10%	97 ± 5 %
	0.26	117 ± 8%	81 ± 6%
Uridine	0.	1 234 280 (100%)	492 300 (100%)
	0.026	79 ± 9%	64 ± 3%
	0.26	59 ± 13%	39 ± 2%
Thymidine	0.	963 060 (100%)	511 830 (100%)
	0.026	102 ± 3%	30 ± 4%
	0.26	125 ± 5%	9 ± 3%

Leishmania donovani promastigotes (5.10^6 cell/ml) were cultured with or without sinefungin; after 6 hr the cultures were labelled for 1 hr with radioactive precursors: 2.5 μCi/ml for leucine uridine or thymidine. Cells were then centrifuged, rinsed twice in phosphate buffered saline (PBS) and treated with 5% cold TCA* as described in [17]. The results of three independent experiments are expressed as the percentage of incorporation with respect to the control. The 100% values are given in cpm/mg protein.

* Trichloroacetic acid.

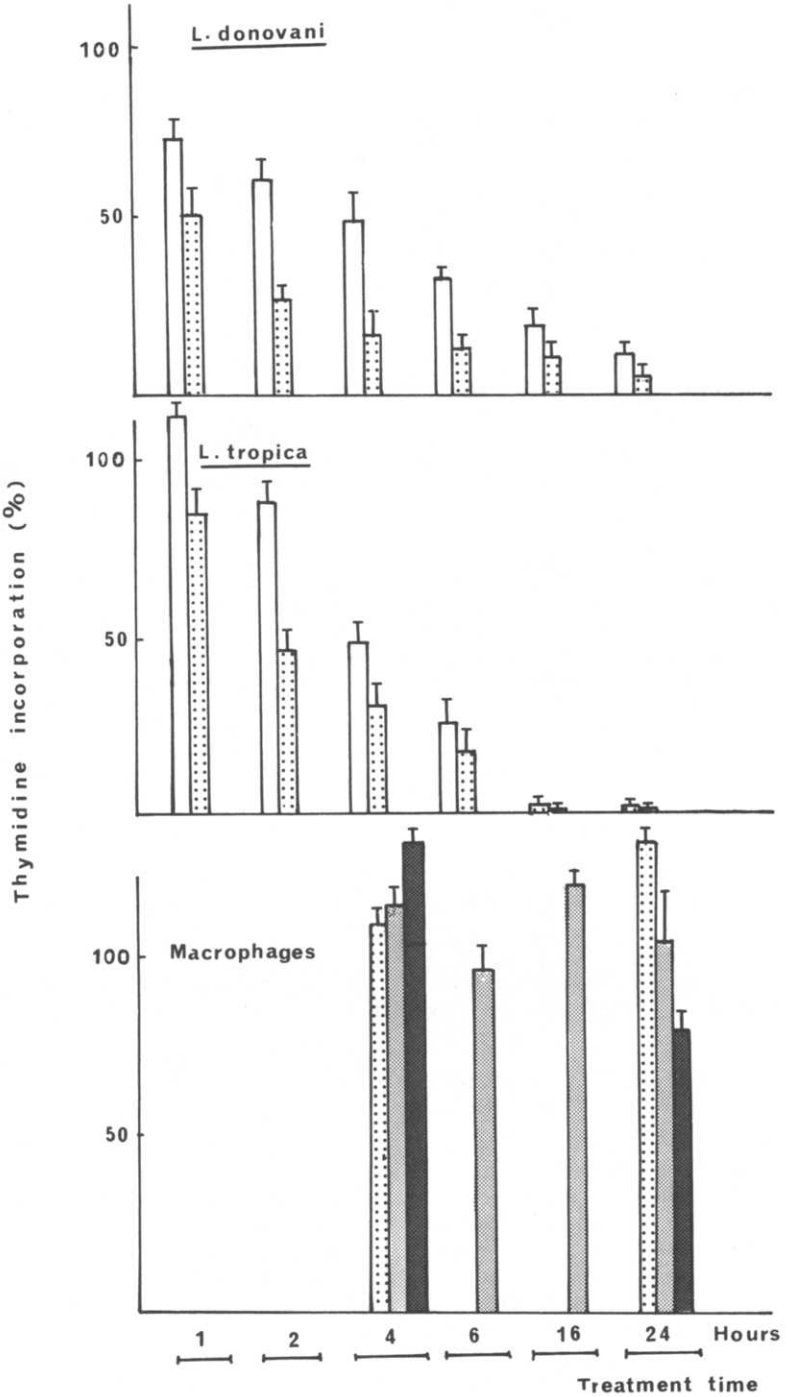

Fig. 2. Species specificity of sinefungin on thymidine incorporation. Cells were grown as described in Materials and Methods, in the presence or in the absence of various sinefungin concentrations for various lengths of time and then labelled with [methyl-^{3}H]-thymidine for 1 hr. Cells were washed twice with cold PBS and treated with ice-cold 5% trichloroacetic acid. The radioactivity in the TCA-insoluble fractions was measured. Results are expressed as the percentage of specific radioactivity found in treated cells with respect to control cells and are mean values of 4–6 independent experiments with each cell type. ☐ 0.026 μM; ■ 0.26 μM; ▨ 2.6 μM; ■ 26 μM sinefungin.

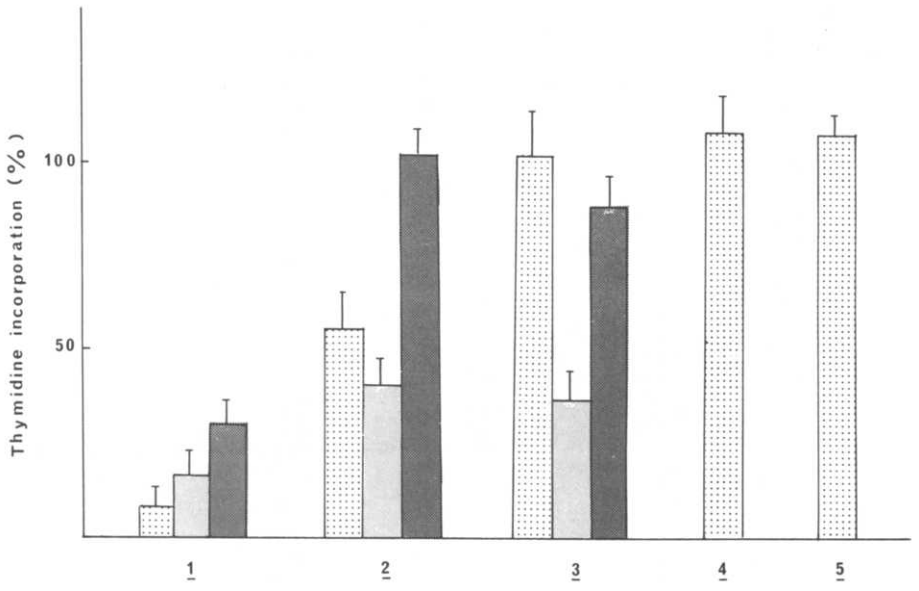

Fig. 3. Comparative effect of sinefungin and analogues on thymidine incorporation in different species of *Leishmania*. Cells were grown as described in Materials and Methods in presence or absence of various sinefungin-related compounds for 6 hr, and then labelled with [methyl-^{3}H]-thymidine for 1 hr. Cells were then washed twice with cold PBS and treated with ice-cold 5% trichloroacetic acid [17]. The radioactivity in TCA-insoluble fraction was measured. Results are expressed as the percentage of specific radioactivity found in treated-cells with respect to control cells. ▒ *L. donovani*; ▒ *L. tropica*; ■ *L. enrietti*. Compounds: **1** Sinefungin; **2** Sinefungin lactam; **3** A9145C; **4** *S*-adenosylmethionine (AdoMet); **5** *S*-adenosyl-homocysteine (AdoHcy). The concentrations used were: **1** and **3**: 0.26 μM; **2**: 0.52 μM (DL forms); **4**: 12 μM; **5** : 2.6 μM. 100% expressed as cpm/mg of protein corresponded to: 5 274 600, 870 950 and 5 173 600 for *L. donovani, L. tropica* and *L. enrietti* respectively. Results are mean values of three to five independent experiments with each species.

on RNA synthesis. Leucine incorporation into protein was weakly inhibited by the drug under these conditions.

Characteristics of the inhibition of DNA synthesis by sinefungin

As shown in Fig. 2 the inhibition of thymidine incorporation by sinefungin was time and concentration dependent in both *Leishmania* species tested. This inhibition was more pronounced in *L. donovani* than in *L. tropica* at both concentrations up to two hours.

The inhibition of the DNA synthesis by low doses of sinefungin seemed specific for the parasites since in macrophages (host cells) no such effect could be observed: even treatments with 10 to 100 times higher concentrations and incubation times up to 24 hr did not alter thymidine incorporation into the macrophages. (Fig. 2).

Because of the differences in the susceptibility of the growth of *Leishmania* species to sinefungin [10] we investigated the action of structurally-related compounds on DNA synthesis.

The lactam **2** was active on *L. donovani* and *L. tropica* and the dehydro derivative A9145C **3** inhibited the DNA synthesis in *L. tropica*; however, these compounds were less powerful than sinefungin; AdoMet **5** and AdoHcy **5** were ineffective on thymidine incorporation in *L. donovani* (Fig. 3).

Analysis of DNA synthesized in control and treated cells

The DNA synthesized in the presence or absence of sinefungin has been analyzed on a CsCl gradient (Fig. 4).

The buoyant densities of nuclear DNA (nDNA) and kinetoplastic DNA (kDNA) were not significantly affected by sinefungin treatment: the values were estimated at 1.712 ± 0.06 and 1.708 ± 0.05 for nDNA and 1.675 ± 0.012 and 1.683 ± 0.013 for kDNA from control and treated cells respectively.

After 6 hr treatment with 0.26 μM sinefungin, the specific radioactivities of nDNA and kDNA obtained from three independent experiments carried out in quadruplicate represented respectively 23 ± 4% and 57 ± 18% that of control cells.

Phosphorylation of nucleosides in cells cultured with or without sinefungin

A prerequisite for cellular DNA synthesis is the supply of deoxyribonucleoside triphosphate (dXTP). *De novo* synthesis of desoxyribonucleotides occurs exclusively by the reduction of the corresponding ribonucleotides, catalysed by the allosterically-

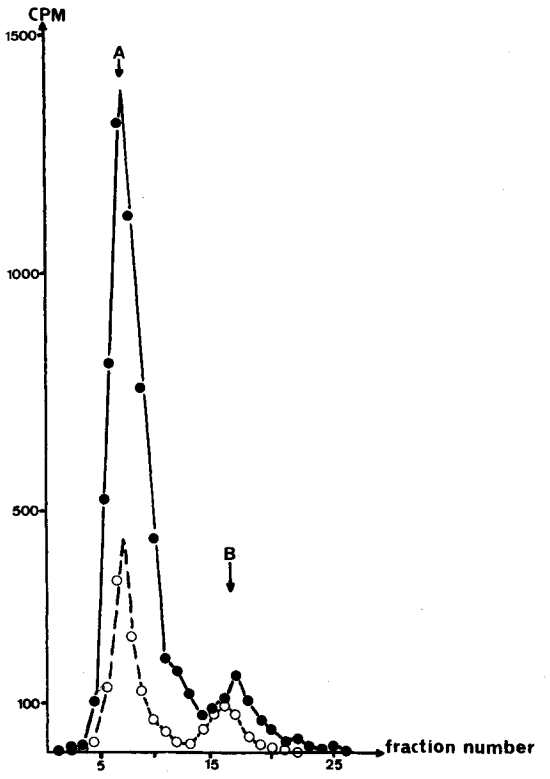

Fig. 4. CsCl gradient analysis of DNA from *L. donovani* promastigotes cultured with and without sinefungin. ○ = treated by sinefungin, ● = untreated; A = nuclear DNA (nDNA), B = kinetoplastic DNA (kDNA). Promastigotes were grown in the presence or in the absence of 0.26 μM sinefungin for 6 hr and then labelled with [methyl-^{3}H]-thymidine for 1 hr. DNA was extracted as described by Blin and Stafford [21] and analysed on a CsCl gradient as described by Simpson and Berliner [22].

controlled enzyme ribonucleotide reductase [23]. An alternative source of DNA precursor is the salvage of exogenous deoxyribonucleosides which serve as substrates for nucleoside kinases. In order to know if the inhibition of thymidine incorporation into DNA results from an inhibition of thymidine phosphorylation or from a decreased supply of deoxyribonucleoside-5′-phosphates, we measured the phosphorylation of ribo- and deoxyribonucleosides in cells cultured with or without sinefungin. Figure 5 shows that in control cells labelled with thymidine 15.3 ± 2.7% of the counts in the soluble pool were in TTP. Treatment of *L. donovani* promastigotes with sinefungin up to 15 hr did not cause any depletion of TTP but rather an accumulation: the percentage of TTP radioactivity was increased (up to 47% of the counts of the soluble pool are in TTP after 6 hr treatment with 2.6 μM sinefungin) as well as the radioactivity of the soluble pool.

Similar results were obtained with cells labelled with adenosine or guanosine, although the intensity of the response to sinefungin treatment was weaker and shorter than for thymidine: thus in control cells labelled with adenosine 14.2 ± 2.8% of the radioactivity of the soluble pool are found in ATP. In treated cells the percentage of ATP radioactivity was increased (it represented 29.1% of the radioactivity of the soluble pool after 4 hr treatment with 2.6 μM sinefungin) and ATP radioactivity was accumulated in treated cells for as long as 15 hr. When cells were labelled with guanosine 11.5 ± 2.6% of the radioactivity of the soluble pool were in GTP. Sinefungin treatment causes an increase of the radioactivity of the soluble pool and an increase in the percentage of counts in GTP (up to 16.4% after 2 hr of contact with 2.6 μM sinefungin).

The uptake of uridine was drastically reduced for short periods of treatment and moderately reduced for longer periods of exposure to sinefungin. The percentage of UTP radioactivity in the soluble pool of control cells was more variable and lower than that measured for TTP, ATP and GTP (8.3 ± 4.4%). Upon sinefungin treatment this value was increased (up to 15.8% after 4 hr at 2.6 μM) however, the UTP did not accumulate.

Similar results have been obtained for *L. tropica* promastigotes (results not shown). The quantification of the deoxyribonucleotides (after periodate treatment) reveals an increase in deoxyribonucleoside-5′-triphosphate pools of the treated promastigotes. The percentage of deoxyribonucleoside-5′-phosphate found in control cells has been evaluated to 1.8 ± 0.3 and 2.8 ± 1.0% of the radioactivity of the soluble pool for adenosine and guanosine derivatives respectively. In sinefungin-treated cells these values were increased in a similar way to those of ribonucleoside-5′-phosphate (Fig. 6).

Thus the supply of ribo- and deoxyribo-nucleoside-5′-triphosphate within the cell was not lowered upon sinefungin treatment.

DISCUSSION

Sinefungin shows antileishmanial activity towards promastigotes [3, 10]. Our data indicate that this molecule inhibits drastically the incorporation of thymidine into promastigotes DNA. This inhibition is time- and concentration-dependent. The decline in the labelling of DNA does not result from an inhibition of transport of thymidine into the cell since the amount of label found in the acid-soluble pool increased. We observed differences in susceptibility of the different species of *Leishmania* towards inhibitory effects of the sinefungin and its structural analogues. In all species, sinefungin was the most active of the three compounds tested, *L. donovani* being more susceptible than *L. tropica* and *L. enrietti*. *L. donovani* and *L. tropica* were similarly sensitive to the cyclic derivative of sinefungin. A9145C was less effective towards *L. donovani* and *L. enrietti*. These differences in susceptibility of DNA synthesis towards sinefungin and related molecules are roughly similar to those observed for the growth of the promastigotes.

The DNA synthesis in macrophages was not affected by sinefungin. This was not due to a lack of uptake of the drug since sinefungin is able to penetrate infected macrophages and to inhibit amastigotes multiplication [3, 8]. Similarly, the synthesis

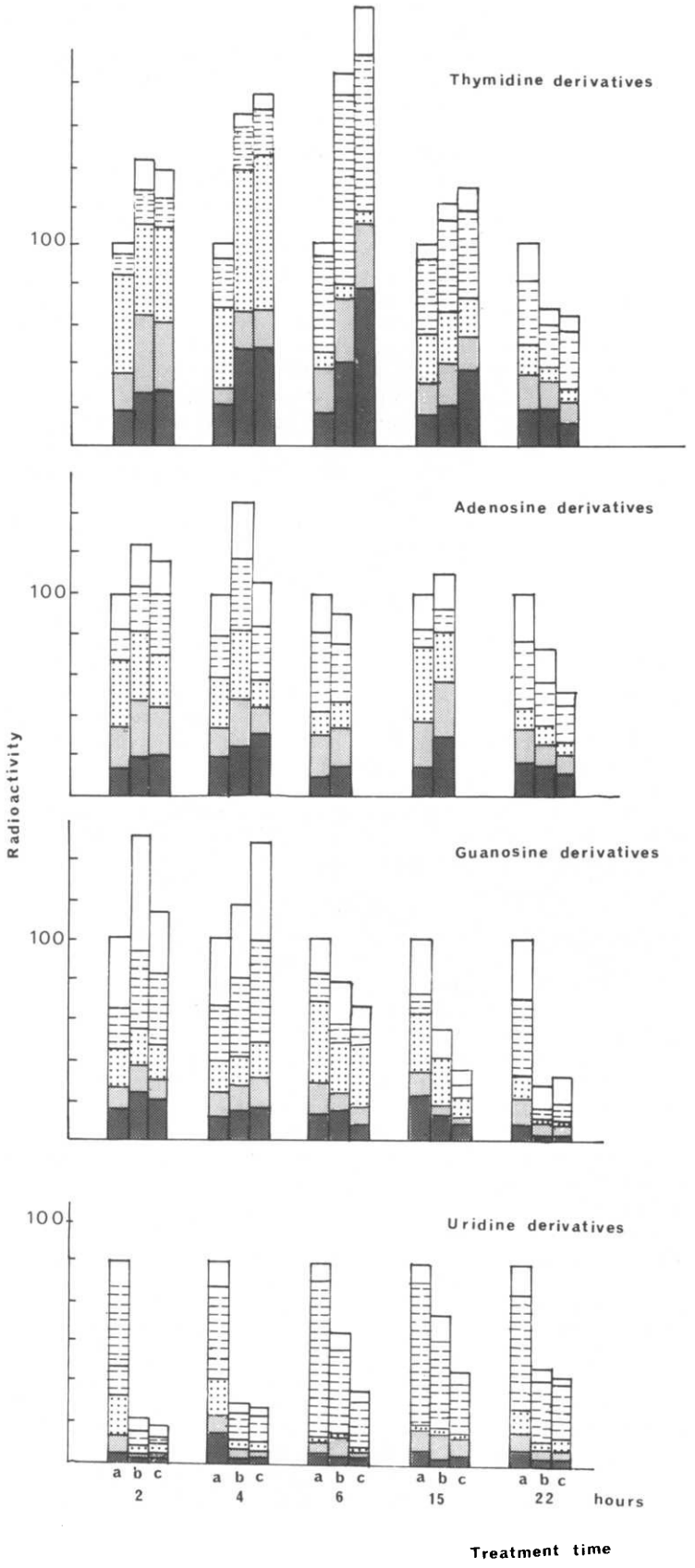

Fig. 5. Nucleoside phosphorylation in promastigotes of *L. donovani* cultured with or without sinefungin. *Leishmania* promastigotes (5.10^6 cell/ml) were cultured with or without sinefungin (0.26 or 2.6 μM) for various lengths of time, and then labelled for 1 hr with 5 μCi/ml of nucleoside. Cells were then centrifuged, rinsed twice with PBS and extracted 2 min with cold 0.5 M perchloric acid. The perchloric extract was neutralized with KOH on ice, using phenol red as internal indicator. After centrifugation to remove the perchlorate salts, 20 μl were chromatographed along with unlabelled carriers, on thin layer cellulose plates, using tert-amylalcohol:formic acid:water (3:2:1 vol/vol) as solvent. The radioactivity accompanying the carriers was determined by liquid scintillation counting. Results are expressed as the percentage of radioactivity found in nucleotide mono-, di- and triphosphate, with respect to the total radioactivity found in each lane. The specific radioactivity of the soluble pools of control cells for each length of treatment is taken as 100. Results are mean value of three independent experiments. ■ XTP; ▨ XDP; ▩ XMP; ▤ nucleoside + base; □ other; (a) control cells; (b) 0.26 μM sinefungin; (c) 2.6 μM sinefungin.

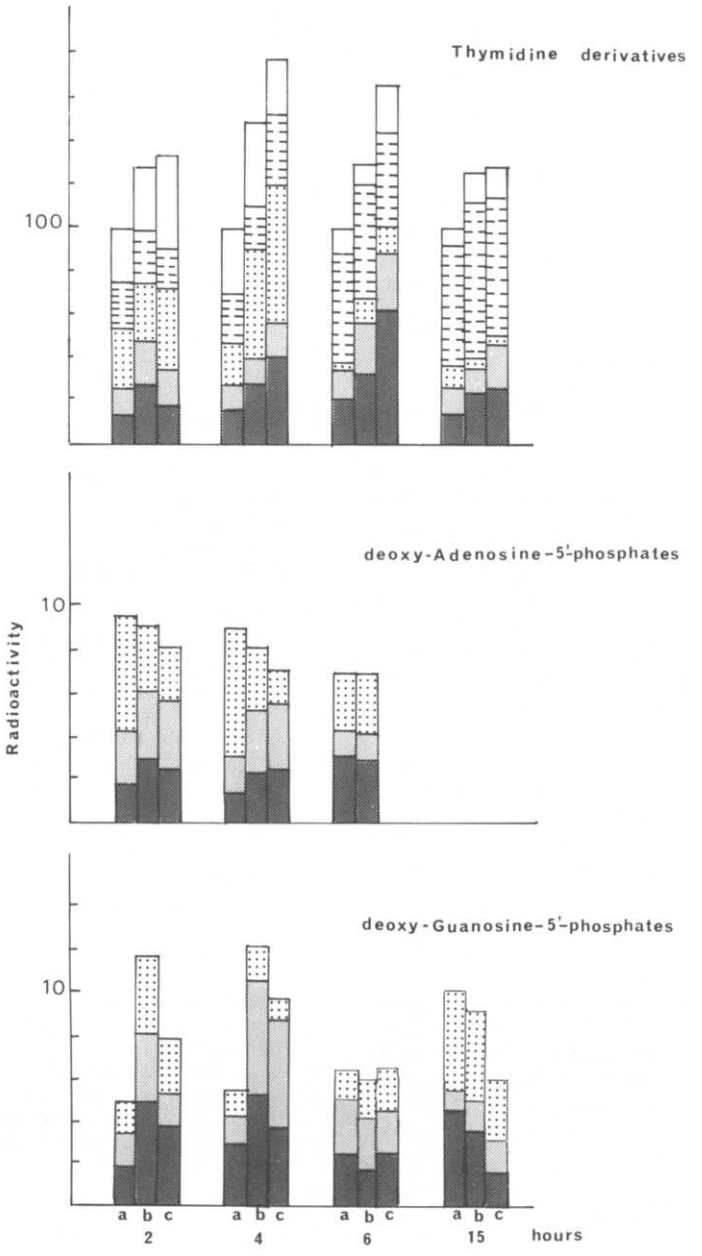

Fig. 6. Deoxyribonucleoside phosphorylation in promastigotes of *L. donovani* cultured with or without sinefungin. Promastigotes (5.10^6 cell/ml) were cultured with or without sinefungin (0.26 and 2.6 μM) for various lengths of time and then labelled for 1 hr with 5 μCi/ml of nucleoside. Cells were then centrifuged, rinsed twice with PBS and extracted 2 min with cold 0.5 M perchloric acid. The percholoric extract was neutralized with KOH on ice. After centrifugation an aliquot was treated with periodate and methylamine [20]. After 30 min at 37° the reaction was stopped by a rapid chilling and addition of rhamnose. Then 20 μl were chromatographed along with unlabelled carriers, on thin-layer cellulose plates using tert-amylalcohol : formic acid : water (3 : 2 : 1 vol/vol) as solvent. The radioactivity accompanying the carriers was determined by liquid scintillation counting. Results are expressed as the percentage of radioactivity found in nucleotide mono-, di- and triphosphate with respect of total radioactivity found in each lane. Results are mean values of three independent experiments. ■ dXTP; ▨ dXDP; ▩ dXMP; ▤ nucleoside + base; (a) control cells; (b) 0.26 μM sinefungin; (c) 2.6 μM sinefungin.

of DNA in Rous Sarcoma Virus-infected chick embryo fibroblasts was not affected by sinefungin treatment while the transformation of these cells was greatly reduced by this drug [12].

Buoyant density analysis of the DNA of *L. donovani* has shown that incorporation of thymidine was less inhibited in kDNA than in nDNA. This could reflect either a lower penetration of sinefungin into

the kinetoplast or a different sensitivity of the target. The values of buoyant densities for kDNA and nDNA of *L. donovani* were similar in control and treated cells.

Attempts to explain the mechanism of action of sinefungin have shown that this molecule does not inhibit the phosphorylation of thymidine or other nucleosides in cell culture. Furthermore, the ratio deoxyribonucleoside to ribonucleotide was not significantly affected by the drug. These results indicate that the nucleoside kinases and the ribonucleotide reductases activities are not modified by sinefungin.

The activities of the purine phosphoribosyltransferases from *Leishmania mexicana* (which convert adenine, guanine, hypoxanthine and xanthine to their monophosphates) are not inhibited by sinefungin up to 1 mM [24].

It has been reported that sinefungin caused actively-dividing cells of *Saccharomyces cerevisiae* to arrest within one cell cycle as unbudded cells. These cells were blocked in the cell cycle initiation step "start" [25]. The active concentration range was of the same order of magnitude in both cases: 0.7 μM for yeast [25] and 0.26 μM for *Leishmania* [10]. As in yeast, there is a modification of the *Leishmania* morphology upon treatment: the promastigotes become rounded. Sinefungin-treated cells in yeast and parasite show both:

(a) the reversibility of growth inhibition when cells, treated with low concentrations of sinefungin, are transferred into drug-free medium;

(b) the effect of the drug is prevented by *S*-adenosyl methionine. However, methionine prevents growth inhibition in yeast but not in *Leishmania*.

The precise mechanism of inhibition by sinefungin of growth and thymidine incorporation into DNA of *Leishmania* promastigotes is not yet completely elucidated. The fact that this compound has no effect on protein methylases I and III but can be antagonised by AdoMet suggests that the cellular target in the promastigotes might be specific methylase, probably related to DNA replication.

In this respect it is interesting to consider the results of Noguchi *et al.* [26] describing an enzymatic complex named "replitase". This complex contains DNA polymerase and DNA methylase activities and is fully associated with newly-synthesized DNA. Whether such a "replitase"—which can be a potential target for sinefungin—exists in *Leishmania* is now under investigation in our laboratory.

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