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THE ANTIFUNGAL ANTIBIOTIC SINEFUNGIN AS A VERY ACTIVE INHIBITOR OF METHYLTRANSFERASES AND OF THE TRANSFORMATION OF CHICK EMBRYO FIBROBLASTS BY ROUS SARCOMA VIRUS

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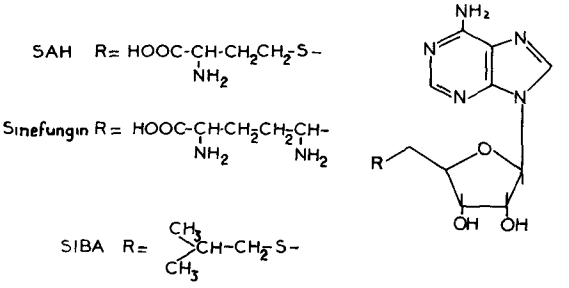
SUMMARY - The antifungal antibiotic Sinefungin is a structural analogue of S-adenosyl-methionine (SAM) and of S-adenosyl-homocysteine (SAH). It is as active as the synthetic SAH analogue S-isobutyl adenosine (SIBA) in inhibiting Rous Sarcoma Virus (RSV) induced transformation of chick embryo fibroblasts (CEF) in cell culture. Sinefungin, like SAH and SIBA is a competitive inhibitor in vitro of tRNA methylases and of protein methylases I and III of CEF. Its ki value for tRNA methylases of normal and transformed cells is respectively 3 to 10 times lower than the ki of SAH. It is thus in vitro the most active tRNA methylase inhibitor described until now. Protein methylase I and III are inhibited to about the same extent by the two molecules whereas SIBA has much higher ki values for the three enzymes in vitro. When methylation was measured in whole cells SAH, SIBA and Sinefungin inhibited [^{14}C]-methyl incorporation to about the same degree in normal cells, but only SIBA and Sinefungin were active in transformed cells.



Abbreviations:
CEF : Chick embryo fibroblast
RSV : Rous sarcoma virus
SAH : S -adenosyl-homocysteine
SAM : S-adenosyl-methionine
SIBA : 5'-deoxy-5'-S-isobutyl-thioadenosine
Tris : Tris-(hydroxy-methyl)aminomethane.

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INTRODUCTION

Sinefungin is an antifungal antibiotic isolated from Streptomyces griseolus (1). Its structure (2)* shows a close chemical relationship to S-adenosyl-methionine (SAM) and to S-adenosyl homocysteine (SAH). It aroused our interest because we had previously shown that several synthetic analogues of SAH, such as 5'-deoxy-5'-S-isobutyl-thioadenosine (SIBA) are inhibitors of cell transformation by oncogenic viruses: Rous sarcoma virus (3) polyoma virus (4) mouse sarcoma virus (5) mouse mammary tumor virus (6) Friend virus (7) Herpes virus (8).

A recent paper by Pugh et al. (9) on the effect of Sinefungin on the methylation of viral mRNA prompts us to report our experiments with Sinefungin which extend the observations of these authors.

MATERIALS AND METHODS

Chemicals : S-adenosyl-L-homocysteine and 5'-deoxy-5'-S-isobutyl-thioadenosine (SIBA) were from Sefochem Fine Chemicals (Israel). Sinefungin was a gift of Dr R.S. Gordee, Lilly Research Laboratories, Indianapolis, USA.

E. coli K12 tRNA was supplied by M. A. Escaut (ICSN, CNRS, Gif sur Yvette, France). All chemicals used were of the highest purity available.

Labelled materials : S-adenosyl-methionine methyl [^{14}C] 50 mCi/mM, methionine methyl [^{14}C] 55 mCi/mM, L-leucine [^{3}H] 30 Ci/mM, uridine [^{3}H] 23 Ci/mM and thymidine [^{3}H] 49 Ci/mM were from the Commissariat à l'Energie Atomique, Saclay (France).

Media and conditions of cultivation : Secondary cultures of CEF were prepared and cultivated as described before in Eagles MEM with 5 % calf serum and antibiotics (3) ; when methionine [^{14}C] methyl incorporation was measured Ham F10 (10) medium was used.

Inhibition of cell transformation : The action of the inhibitors on focus formation was tested as described earlier (3). The virus used was a clonal isolate (SR4) of Schmidt-Ruppin strain RSV, type D. The inhibitors were dissolved in the medium and sterilized by filtration.

Effect on macromolecular synthesis : The incorporation of radioactive precursors into macromolecules following different exposure times of normal or transformed cells to Sinefungin was followed by a standard procedure (11).

[^{14}C]methyl group incorporation : To measure the extent of methylation, exponentially growing normal or transformed cells were labelled in the absence and in the presence of Sinefungin for various times at 37° with [^{14}C]CH_3 methionine 2 μCi/plate, in Ham F10 medium containing unlabelled

* The α carbon is L, the stereochemistry of the δ carbon is not yet known.

sodium formiate 20 mM, adenosine 20 μM and guanosine 20 μM to inhibit methyl incorporation into purine and thymine via the "one carbon" pool. After labelling, the radioactivity in the different fractions (acid soluble, nucleic acid and protein) was determined by the method of Schmidt and Thannhauser (12).

Preparation of cell-free extracts : Extraction was carried out at 4°. Monolayers were washed with phosphate buffered saline, pH 7.4 and scraped in buffer A (50 mM Tris-HCl pH 8, containing 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol) or in buffer B (10 mM potassium phosphate pH 7.2 containing 10 mM 2-mercaptoethanol). Cells were homogenized in a Dounce homogenizer, the extract centrifuged for 15 minutes at 12 000 x g and the supernatant fraction tested for tRNA and protein methylase activities.

tRNA methylase activity of normal and transformed CEF was measured as described previously (3).

Protein methylase activities of normal and transformed CEF : Protein methylase I (S-adenosyl-L-methionine protein (arginine) methyl transferase E.C.2.1.1.23) and protein methylase III (S-adenosyl-L-methionine : protein (lysine) methyl transferase E.C.2.1.1.25) were measured by the method of Paik et al. (13).

Protein concentration was determined using crystalline bovine serum albumin as standard (14).

RESULTS AND DISCUSSION

In earlier work, we described the inhibitory effect of SIBA on RSV induced cell transformation. SAH is inactive under the same conditions, due probably to its rapid intracellular degradation (3, 15). SIBA is much more stable in cells than SAH (16).

Sinefungin represents a new type of structural analogue of SAH and SAM, in which the S atom of SAH (or the $>\overset{\oplus}{S}$-Me of SAM) is replaced by a $>CH\text{-}NH_3^{\oplus}$; it is a "carba-analogue" and was expected to be active as inhibitor of transmethylases and of viral cell transformation. We thus compared the effect of Sinefungin, SIBA and SAH on foci formation in RSV infected CEF. As shown in Table 1 SIBA and Sinefungin inhibit cell transformation to about the same extent whereas SAH has no effect even at 1000 μM. In a recent publication Pugh et al. (9) showed that 100 μM Sinefungin inhibits vaccinia virus plaque formation in mouse L-cells to about 78 %. In their experiments plaques were counted after a 72 hours contact of the compound with infected cells. In our tests the inhibitor remains with infected cells for only 48 hours, then it is eliminated from the cultures by the renewal of the medium, and foci are counted 5 days later. So, Sinefungin seems to be at least as active against oncogenic RSV than against vaccinia virus.

TABLE 1 : Comparison of the effect of L-SAH, SIBA and Sinefungin on RSV induced transformation of CEF.

Compound	Concentration (μM)	% Inhibition
L-SAH	1000	20
SIBA	500	100
SIBA	250	97
Sinefungin	500	100
Sinefungin	250	90

The inhibitors were added to the cells one hour after infection for 48 hours. Then the medium was renewed and foci counted after 5 days growth in an inhibitor-free medium. The concentrations used were cytostatic but not cytotoxic for normal CEF.

The inhibitory effect of Sinefungin on various methylases: mRNA (guanine-7) methyl transferase, mRNA (nucleoside 2') methyl transferase (9) as well as on norepinephrine N-methyl-transferase, histamine-N-methyl transferase, catechol-O-methyl transferase (17) was measured and compared to that of SAH. It was shown that Sinefungin is a much stronger inhibitor than SAH in the case of the two mRNA methylating enzymes of vaccinia virus (9). We show in Table 2 that the same is true for crude tRNA methylases of normal and RSV transformed cells. The affinity of Sinefungin for tRNA methylases of transformed cells is 11 times higher than that of SAH. Protein methylases I and III are inhibited by Sinefungin and SAH to about the same extent in both cell types. The inhibition by SIBA is much weaker. We have also measured the effect of 500 μM Sinefungin on labelled methyl group incorporation into normal and transformed cells. Table 3 shows that the methylation of nucleic acids and proteins is inhibited more strongly by SIBA than by Sinefungin, in both cell types. SAH, as shown before (15), has only a slight inhibitory effect on methyl incorporation and only in normal cells.

As the inhibition of the methylation of nucleic acids and proteins may affect nucleic acid and protein biosynthesis, some preliminary experiments were performed to study the effect of Sinefungin on labelled leucine, uridine and thymidine incorporation into normal and transformed cells after 24 and 48 hours exposure time of the cells to the compound. Our results indicate that protein synthesis is not affected by Sinefungin, but RNA and DNA synthesis is inhibited after 48 hours by 54 and 32 % respectively, but only in normal cells.

TABLE 2 : Comparison of kinetic constants of L-SAM , L-SAH, SIBA and Sinefungin for three methylases of CEF.

Enzyme	L-SAM KmμM	L-SAH KiμM	SIBA KiμM	Sinefungin KiμM
Normal CEF				
tRNA methylase	7. 9	4. 6	2190	1. 2
Protein methylase I	12	1. 5	182	3. 5
Protein methylase III	59	5. 5	1328	4. 9
RSV-transformed CEF				
tRNA methylase	8. 7	11. 2	4143	0. 9
Protein methylase I	24. 7	4. 9	395	11. 1
Protein methylase III	101	5. 8	3117	7. 1

TABLE 3 : Comparison of the effect of SIBA and Sinefungin on [^{14}C]-methyl incorporation into normal and RSV transformed CEF, after 24 hours of incubation.

	% Inhibition in comparison to untreated cells	
	Sinefungin 500 μM	SIBA 500 μM
Normal cells		
TCA soluble	22	31
Nucleic acids	16	74
Proteins	16	56
Transformed cells		
TCA soluble	5	50
Nucleic acids	16	36
Proteins	6	51

Thus, Sinefungin seems to be a very good inhibitor of transformation induced by oncogenic RSV, and a very good inhibitor of various methylases in vitro. Its effect on methyl group incorporation in whole cells is, however much weaker.

The possibility that one or several individual tRNA methylases are inhibited by Sinefungin more strongly than the crude preparations used above is now under study in our laboratory.

To our knowledge, this is the first time that an antibiotic is shown to be a potent inhibitor of tRNA and protein methyltransferases. It is quite possible that the antifungal activity of Sinefungin is due to an inhibition of methyltransferases: SIBA has, however, been tested on the same strains and was found inactiv (C. R. Gordee, Lilly Research Laboratories, personal communication).

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