Sinefungin shares AdoMet-uptake system to enter *Leishmania donovani* promastigotes

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The involvement of a carrier for sinefungin (SF) uptake in *Leishmania donovani* promastigotes is indicated by saturation kinetics, competition studies and SF accumulation against a 270-fold concentration gradient across the cell membrane. Whether SF uptake occurs via nucleoside- or AdoMet-carrier systems was investigated by competition experiments and com-

parison of the uptake of various molecules in wild-type and SF-resistant cells. Results show that SF did not inhibit purine or pyrimidine uptake whereas it competitively inhibited AdoMet uptake. Furthermore, the uptake of nucleosides in SF-resistant cells is similar to that in wild-type cells, whereas uptake of SF and AdoMet is lower.

INTRODUCTION

Sinefungin (SF), a nucleoside antibiotic produced by *Streptomyces griseolus* [1] and *Streptomyces incarnatus* (Rhône Poulenc Industries, France, Patent no. 761114, April 1976), is structurally related to *S*-adenosylmethionine (AdoMet), a key metabolite in the cell. This molecule, in which the methyl sulphonium group of AdoMet is replaced by an amino-substituted methylene unit, exhibits strong antifungal and antiparasitic activity both *in vitro* and *in vivo* (reviewed in [2]).

The trypanosomatid protozoan, *Leishmania*, is very sensitive to SF *in vitro* [3–6] and *in vivo* [5,7,8]. SF-mediated effects on cell growth and morphology can be antagonized by AdoMet, indicating that the mechanism of action of the antibiotic is based on competition with AdoMet for some targets [5,9–12]. In cultured promastigotes, SF has been shown to be a competitive inhibitor of carboxymethyltransferases [13,14].

The aim of the present work was to understand how SF enters promastigotes and ascertain whether the lower penetration of SF in resistant promastigotes is partly responsible for their lack of response to the drug.

Our data confirm the existence of a specific transporter for AdoMet in *Leishmania donovani* promastigotes, which is also used by SF to enter the cells. Furthermore, we demonstrate that SF-resistance is partly based on modification of the kinetic properties of AdoMet permease leading to a decrease in V_{max} and drug recognition.

EXPERIMENTAL

Materials

S-Adenosyl-L-[methyl]-³H]methionine (2.5 µCi/ml, 550 mCi/mmol), [³H]adenosine (5 µCi/ml, 23 Ci/mmol), [³H]thymidine (5 µCi/ml, 85 Ci/mmol), [³H]uridine (5 µCi/ml, 46 Ci/mmol), [¹⁴C]cytidine (1 µCi/ml, 50 mCi/mmol) and [¹⁴C]guanosine (1 µCi/ml, 53 mCi/mmol) were purchased from Amersham. Unlabelled AdoMet, *S*-adenosylhomocysteine (AdoHcy), methionine and adenosine were purchased from Sigma; Coomassie Blue reagent for protein assays was from Bio-Rad Laboratories. SF and its cyclic derivative were generously given by Dr Lombardi

(Rhône-Poulenc Santé, Vitry, France), dehydro-SF was a gift from Dr. R. Nagarajan (Eli Lilly Research Laboratories, Indianapolis, IN, U.S.A.) and SF-thymidine a gift from Dr. B. Quiclet-Sire (CNRS, ICSN, Gif-sur-Yvette, France). SF epimer (*R*) and SF-uridine were synthesized as described by Barton et al. [15]. MDL 73811 {5'-[(4-amino-2-butenyl)methylamino]-5'-deoxyadenosine} was kindly provided by Dr. P. McCann (Marion Merrell Dow Inc., Kansas City, KS, U.S.A.).

Culture medium components were from Gibco and serum was from Flobio.

Strains and culture conditions

L. donovani (strain LRC L52), originated from the strain collection of the World Health Organization's International Reference Center for leishmaniasis (WHO-LRC), was provided by Dr. L. F. Schnur (the Kuvn Center for the Study of Infectious and Tropical Diseases, Jerusalem, Israel). *L. donovani* (strain MHOM/IN/80/DD8), originated from the strain collection of the WHO reference centre in the London School of Hygiene and Tropical Medicine, was provided by Dr. D. Evans.

SF-resistant clones were obtained by increased drug pressure from these two wild-type cells [16].

Promastigotes were grown as described previously [12] in a semi-defined RPMI 1640 medium containing 2 mM glutamine, 25 mM Hepes, pH 7.4, 10% heat-inactivated fetal calf serum, streptomycin and kanamycin (5 mg/ml each) and penicillin (5 units/ml). The promastigotes were assayed in the exponential-phase of growth (20×10^6 – 35×10^6 cells/ml).

Analysis of Intracellular AdoMet and SF pools by h.p.l.c.

Intracellular pools of AdoMet and SF were determined by a modification of the O'Connor and Germain method [17] as described previously [16]. Briefly, exponential-phase promastigote cultures (100 ml at 20×10^6 cells/ml), treated or not with SF, were harvested and washed and macromolecules were precipitated in cold perchloric acid. The acid-soluble components were separated from promastigote proteins and used for h.p.l.c.

Abbreviations used: SF, sinefungin; AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; MDL 73811, 5'-[(4-amino-2-butenyl)-methylamino]-5'-deoxyadenosine.

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AdoMet and SF pools were analysed on an Ultrasil SCX column (4.6 mm $\times$ 25 cm) (Beckman). Chromatography was performed at a flow rate of 2 ml/min. The column, pre-equilibrated in buffer A (10 mM ammonium acetate, pH 4) was eluted for 10 min with the same buffer, then for 40 min with a linear gradient of 0–55% buffer B [0.5 M ammonium acetate/acetonitrile, pH 4 (7:3, v/v)]. AdoMet and SF concentrations were determined by area relative to standards.

Cell volume ($22 \mu\text{M}^3$ and $13 \mu\text{M}^3$ respectively for control and SF-treated cells) was determined as described by Darling et al. [18] on electron micrographs; 1 mg of protein is equivalent to 2.41×10^8 strain LRC cells or 2.72×10^8 strain DD8 cells.

Protein determination

Protein concentration was measured by the dye-binding method, with BSA as standard [19].

AdoMet uptake and incorporation

Uptake and incorporation of methyl groups were measured in both trichloroacetic-acid soluble and -insoluble fractions after cells had been recovered by centrifugation and washing in cold PBS.

In other experiments, centrifugation through oil was used to separate labelled cells from the medium and to determine the radioactivity associated with AdoMet. Parasites preincubated with labelled AdoMet were layered in Eppendorf tubes on top of a 9:1 mixture of dibutyl phthalate ($d = 1.045$) and liquid paraffin ($d = 0.88$), and centrifuged for 1 min in an Eppendorf Microfuge [20]. The supernatant was removed by careful aspiration, and the pellet was resuspended in 1 M NaOH, hydrolysed for 30 min at 80°C , mixed with liquid scintillation fluid and counted for radioactivity (total uptake). In some experiments the pelleted cells were resuspended in 5% cold trichloroacetic acid, the soluble fraction (uptake) was removed and the pellet hydrolysed in 1 M NaOH (incorporation) as described above.

RESULTS

Uptake of SF in wild-type and SF-resistant promastigotes

The intracellular drug concentration in promastigotes as a function of time of treatment was measured in wild-type and SF-resistant clones (Figure 1). In wild-type cells cultured in the presence of $0.26 \mu\text{M}$ SF, the intracellular amount of drug increases with the length of contact up to 6 h and then slowly decreases. The intracellular concentration of SF was estimated to be $42 \mu\text{M}$ in strain LRC and $63 \mu\text{M}$ in strain DD8 after an overnight treatment with SF at $0.26 \mu\text{M}$. This represents a 161–200-fold accumulation of the drug within wild-type cells. Addition of $100 \mu\text{M}$ AdoMet and $0.26 \mu\text{M}$ SF provokes a 50% decrease in the intracellular concentration of SF, indicating that AdoMet inhibits SF uptake (results not shown).

A plot of the rate of SF uptake (after 2 h treatment) as a function of extracellular SF concentration shows a typical saturation curve (Figure 2), indicating a finite number of sites on the cell surface involved in SF uptake. The apparent affinity for SF was similar for both wild-type strains (K_m $0.31 \mu\text{M}$) and the apparent $V_{\max}$ for DD8 and LRC strains respectively was estimated to be 1.25 and 2.10 nmol/mg of protein after 2 h treatment. These results indicate that SF enters by a carrier-mediated mechanism.

In the resistant strains the amount of intracellular SF (measured in the presence of $157 \mu\text{M}$ extracellular SF) is lower than in the respective wild-type strains cultured with $0.26 \mu\text{M}$. In DD8 SF-resistant cells a plateau is reached after 6 h (Figure 1a)

Figure 1 SF uptake in promastigotes as a function of the length of treatment

Promastigotes in exponential phase of growth (100 ml cultures) were incubated for various lengths of time with either $0.26 \mu\text{M}$ SF (wild-type cells) or $157 \mu\text{M}$ SF (SF-resistant strains), and then processed as described in the Experimental section. Results represent means $\pm$ S.E.M. of four independent experiments. (a) *L. donovani* DD8; $\circ$, wild-type; $\bullet$, SF-resistant. (b) *L. donovani* LRC: $\triangle$, wild-type; $\blacktriangle$, SF-resistant.

and in LRC SF-resistant cells the uptake is linear for up to 48 h (Figure 1b). Experiments carried out with resistant cells and $0.26 \mu\text{M}$ SF showed no detectable intracellular SF after 20 h of contact.

The intracellular concentration of SF was estimated to be

Figure 2 Uptake of SF as a function of its extracellular concentration

Promastigotes in exponential phase of growth (100 ml culture) were incubated for 2 h with various SF concentrations. Intracellular SF was determined by h.p.l.c. analysis of perchloric acid extracts as described in the Experimental section. $\triangle$, wild-type LRC strain; $\circ$, wild-type DD8 strain.

Figure 3 Time course of AdoMet uptake in *L. donovani* promastigotes

Promastigotes in exponential phase of growth (1 ml of culture in 24-well microplates) were incubated for various lengths of time with methyl-labelled AdoMet (85 Ci/mmol, 2 μ Ci/ml), and then processed as described in the Experimental section. ∇ , wild-type; $\blacktriangledown$, SF-resistant.

14 μ M in LRC SF-resistant cells and 22 μ M in DD8 SF-resistant cells after overnight treatment with 157 μ M SF.

These results indicate that SF is taken up by SF-resistant strains but at a lower rate and to a lower extent than by wild-type strains.

Characterization of SF uptake

The SF molecule is an adenosine moiety linked by a C–C bond to an ornithine residue. In order to discover whether SF is recognized by nucleoside transporters, we studied the uptake of purines and pyrimidines in the absence and presence of the antibiotic. Adenosine at a concentration of 100 μ M strongly inhibits the uptake of both purine and pyrimidine nucleosides (90% inhibition), whereas thymidine used at the same concentration is a weak inhibitor of adenosine uptake (25% inhibition). Thus, in this strain, adenosine is recognized by both purine and pyrimidine nucleoside transporters.

The addition of 0.26 or 260 μ M SF does not affect the uptake of purine and pyrimidine nucleosides after 1 h of treatment; this indicates that SF is not recognized by these nucleoside transporters. These results are further supported by the fact that uptake of these nucleosides in the resistant clone is not significantly changed compared with their respective wild-type cells, whereas, as demonstrated above, SF uptake is decreased. The uptake values in DD8 SF-resistant cells are for thymidine, cytidine, adenosine and uridine 91, 69, 102 and 92% respectively of those measured in the wild-type cells.

Addition of high concentrations of AdoMet (100 μ M) inhibits the uptake of pyrimidine nucleosides by 73–86% and purine nucleosides by 39–45%. However, these inhibitions are not observed at lower concentration (10 μ M). This suggests that, when present at high concentration, AdoMet may enter via nucleoside carriers in contrast with SF.

A typical time course of labelled AdoMet uptake in wild-type and SF-resistant clones is shown in Figure 3. The plateau is reached after 2–3 h. At the steady state the intracellular concentration of the labelled AdoMet in the wild-type cells is 9 μ M which is 350 times greater than the external AdoMet concentration (0.0256 μ M). In SF-resistant cells, AdoMet uptake is

Figure 4 Uptake of AdoMet as a function of its extracellular concentration and the effect of SF

Promastigotes in exponential phase of growth (1 ml of culture in 24-well microplates) were incubated for 30 min with methyl-labelled AdoMet (85 Ci/mmol, 2 μ Ci/ml) in the presence or absence of various SF concentrations, and then processed as described in the Experimental section. Results are expressed as nmol of AdoMet/mg of protein. (a) *L. donovani* wild-type: AdoMet alone (Δ) or in the presence of 0.76 μ M ($\diamond$) or 1.52 μ M ($\square$) SF. (b) *L. donovani* LRC SF-resistant: AdoMet alone ($\blacktriangle$) or in the presence of 76 μ M ($\square$) or 225 μ M (∇) SF. (c) *L. donovani* DD8 SF-resistant: AdoMet alone ($\bullet$) or in the presence of 76 μ M SF ($\square$).

slower; after 6 h the intracellular concentration is 1.5 μ M, which is 60 times greater than the external concentration.

Under other experimental conditions, after 1 h contact with 5 μ M AdoMet, the concentration of the label in promastigotes is estimated to 133 and 112 μ M respectively in LRC and DD8 cells indicating that AdoMet is concentrated inside the wild-type cells. This is not an artefact due to incorporation of methyl groups into macromolecules as the concentration in the soluble pool is also 12–16-fold higher than that in the incubation medium (77 and 67 μ M in LRC and DD8 cells respectively). All these data confirm the existence of an AdoMet permease in *L. donovani* promastigotes.

A plot of the rate of uptake as a function of AdoMet concentration shows a typical saturation curve (Figure 4). The results of a comparative study of the apparent kinetic parameters of AdoMet uptake in the two wild-type and two resistant clones are given in Table 1. A Lineweaver–Burk plot [21] gives an apparent K_m for AdoMet around 1 μ M for both wild-type strains. In the resistant clones, the apparent affinity for AdoMet is slightly increased, but has the same order of magnitude as that of the wild-type cells, and the apparent maximal rate of AdoMet

Table 1 AdoMet uptake in wild-type and SF-resistant strains of *L. donovani*

Promastigotes in exponential phase of growth were incubated for 30 min at 22 °C, with various concentrations of labelled AdoMet (from 9.2 μ M to 0.03 μ M) with or without SF. The reaction was stopped by centrifugation through oil and processed as described by Aronow et al. [20]. To characterize AdoMet uptake, the apparent affinities of the transport elements were calculated by the method of Lineweaver and Burk [21]. Results are means $\pm$ S.E.M. of six different experiments.

Strain	$V_{\max}$ (pmol/30 min per mg)	K_m for AdoMet (μ M)	K_i for SF (μ M)
LRC	6783 $\pm$ 2500	1.18 $\pm$ 0.43	0.36 $\pm$ 0.07
LRC SF-resistant	196 $\pm$ 127	4.50 $\pm$ 2.50	289 $\pm$ 122
DD8	2895 $\pm$ 1705	0.89 $\pm$ 0.16	0.27 $\pm$ 0.08
DD8 SF-resistant	1044 $\pm$ 616	2.50 $\pm$ 1.00	5.70 $\pm$ 1.20

Table 2 Effect of various SF analogues on AdoMet uptake

Promastigotes in exponential phase of growth were incubated for 30 min at 22 °C with various concentrations (from 0.96 to 0.03 μ M) of labelled AdoMet in the presence of various effectors. The reaction was stopped by centrifugation through oil and processed as described by Aronow et al. [20]. To characterize AdoMet uptake, the apparent affinities of the transport elements were calculated by Lineweaver–Burk plots [21]. ni, Not inhibitory at the highest concentration used: SF-uridine, 8 μ M; SF-thymidine, 8 μ M; adenosine, 145 μ M; L-methionine, 291 μ M.

Molecules tested	Apparent K_i (μ M)	
	LRC	DD8
AdoMet	1.23	1.10
Natural SF (S)	0.35	0.33
Epi-SF (R)	2.38	1.60
Dehydro-SF	3.57	3.46
SF lactame	4.73	4.95
MDL 73811	6.30	3.60
AdoHcy	10.5	22.00
SF-uridine	ni	99.00
SF-thymidine	ni	ni
Adenosine	ni	169.00
L-Methionine	ni	ni

uptake is 3 to 35 times lower than in the respective wild-type cells.

SF competitively inhibits AdoMet uptake in both resistant and wild-type cells (Figure 4), indicating a common carrier-mediated mechanism. As shown in Table 1, its apparent affinity is similar for both wild-type strains (0.3 μ M), whereas in both resistant cells the K_i for SF is one to two orders of magnitude higher (5.7–289 μ M).

Thus the uptake of AdoMet is modified in the SF-resistant strains compared with the wild-type cells: a lower $V_{\max}$ and decreased recognition of SF, although the affinity for AdoMet is not greatly changed.

In order to test the specificity of SF inhibition of AdoMet uptake in promastigotes, we studied the effect of various SF analogues on AdoMet uptake (Table 2). All the compounds tested that inhibited AdoMet uptake behaved as competitive inhibitors with respect to AdoMet in both *L. donovani* wild-type strains. The relative affinities of the major competitive SF analogues are shown by their K_i values which are greater than the K_i for SF.

The C-6' epimer (R) of SF is 5–7-fold less active than natural SF (S), indicating that the C-6' chiral centre contributes to the

binding of SF to the AdoMet carrier. Dehydro-SF, which differs from SF only by the presence of a double bond at the 5' end, and the cyclic derivative are both less effective than SF (10- and 15-fold respectively). The absence of a methyl group on the side chain (AdoHcy) also contributes to the decrease in affinity. The replacement of the adenine portion of SF by uracil or thymine results in a nearly complete loss of recognition. These results indicate that the adenine moiety is critical for binding, although adenosine is a very weak inhibitor. MDL 73811, an AdoMet analogue which is an enzyme-activated irreversible inhibitor of AdoMet decarboxylase in mammals and *Trypanosoma* [22,23], is as active as dehydro-SF and SF lactame in inhibiting AdoMet uptake.

DISCUSSION

The results presented here show that, in *L. donovani* promastigotes grown in culture, AdoMet and SF accumulate against a concentration gradient across the cell membrane. The involvement of a carrier-mediated transport system is well supported by the fact that the uptake of AdoMet and SF show saturation kinetics. In both wild-type strains studied, SF competes with AdoMet uptake and vice versa; furthermore, in the SF-resistant promastigotes, uptake of both AdoMet and SF is lower than in the respective wild-type cells. All these data are in favour of the existence of a common transporter. A similar conclusion has been reached for *Saccharomyces cerevisiae* for which SF is a good inhibitor of cell growth except in strains that are deficient in AdoMet permease [24]. In the protozoan, *Leishmania*, the acquired resistance to SF leads to modification of the kinetic properties of AdoMet permease: a decrease in $V_{\max}$ and drug recognition.

A K_m value of 0.125 μ M for AdoMet uptake and a $V_{\max}$ of 24 pmol/h per mg of protein were reported for American *Leishmania* [25]. The differences between these kinetic parameters and those reported in the present work could be explained by the difference in parasite species used or differences in experimental conditions, for example in the former work uptake was studied in cells incubated for 4 h with AdoMet.

We showed that AdoMet uptake in *L. donovani* is highly specific. The data obtained with AdoHcy indicate the importance of the methyl group and/or the significance of the positive charge on the sulphur for binding or positioning of the molecule. The replacement of S-CH₃ by CH-NH₂ at the same position is highly inhibitory. Binding activity is lost when the adenine ring is replaced by uracil, dihydrouracil or thymine. The conformation of the C-6' chiral centre is important because epi-SF (R configuration) is less effective than natural SF (S configuration). The inhibitory activity is very low when the 6'-amino group forms a lactame with the α -carboxyl group. Methionine and adenosine do not interfere with AdoMet uptake at a molecular ratio of two to three orders of magnitude greater than AdoMet. No information is available on the structure–activity relationship for AdoMet uptake in Trypanosomatids, although Avila and Polegre [25] reported inhibition of AdoMet uptake by high methionine concentrations and after 2 h preincubations followed by 6 h of labelling with AdoMet. In yeast the methyl group and the α -amino group of methionine, as well as the N⁶ amino group of the adenine, are required for AdoMet binding to the transport protein [26].

Our data should help in the understanding of the structure–activity relationship of SF as an AdoMet competitor, and contribute to the design of analogues with improved specificity. Assays for inhibition of AdoMet uptake could be a quick test for

screening new SF analogues, to predict if they are likely to enter promastigotes and thus be potential cell-growth inhibitors.

In conclusion, in SF-resistant promastigotes, uptake of both AdoMet and SF is lower than in the respective wild-type cells. We found a decrease in $V_{\max}$ and drug recognition.

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