Pentamidine uptake in *Leishmania donovani* and *Leishmania amazonensis* promastigotes and axenic amastigotes

Mireille BASSELIN, Françoise LAWRENCE and Malka ROBERT-GERO

Institut de Chimie des Substances Naturelles, C.N.R.S., Avenue de la Terrasse, 91198 Gif-sur-Yvette, Cedex, France

A transport system for pentamidine in *Leishmania donovani* and *Leishmania amazonensis* promastigotes and axenic amastigotes has been identified and characterized. Pentamidine is not metabolized by these parasites. Its uptake process is saturable, carrier-mediated and energy-dependent. This drug does not inhibit purine or pyrimidine uptake, whereas it inhibits uptake of several amino acids non-competitively and that of putrescine and spermidine competitively. The results suggest that pentamidine shares polyamine-carrier systems in these parasites.

INTRODUCTION

The aromatic diamidine pentamidine is widely used for the treatment of African trypanosomiasis, antimony-resistant leishmaniasis and *Pneumocystis carinii* pneumonia in AIDS patients [1,2]. Although pentamidine has been used for 40 years, its mode of action remains unknown. It has been reported that in various Kinetoplastidae pentamidine inhibits *S*-adenosyl-L-methionine decarboxylase [3], uptake of radioactive putrescine, interferes with polyamine synthesis [4], mitochondrial topoisomerase II [5,6], mitochondrial membrane potential [7], calcium transport [8], thymidylate synthetase [9] and lysine-arginine transport [10].

African trypanosomes are reported to accumulate pentamidine via a high-affinity transport system which is not inhibited by either lysine or arginine [11].

The aim of the present work was to determine whether promastigotes and amastigotes of *Leishmania donovani* and *Leishmania amazonensis* also have a pentamidine transport system, and if so, to analyse its kinetics and substrate specificity.

Our data demonstrate the existence of a transporter for pentamidine in both strains of *Leishmania* and show that its uptake occurs via polyamine-carrier systems.

These results also show the wide diversity of pentamidine transport mechanisms among Kinetoplastidae and even among *Leishmania* species.

EXPERIMENTAL

Materials

Pentamidine isethionate was a generous gift from Roger Bellon Laboratories (France). [2-^{3}H]Adenosine (23 Ci/mmol), [5,6-^{3}H]uridine (39.70 Ci/mmol), [*methyl*-^{3}H]thymidine (84.20 Ci/mmol), L-[U-^{14}C]arginine (300 mCi/mmol), L-[U-^{14}C]lysine (300 Ci/mmol), DL-α-difluoromethyl[5-^{14}C]ornithine (60 mCi/mmol), L-[U-^{14}C]phenylalanine (450 mCi/mmol), L-[2,3,5,6-^{3}H]tyrosine (114 mCi/mmol), L-[1,2-^{3}H]tryptophan (6 Ci/mmol), L-[3-^{3}H]serine (29 Ci/mmol), L-[4,5-^{3}H]isoleucine (93 Ci/mmol), L-[U-^{14}C]aspartic acid (220 mCi/mmol), [1,4-^{14}C]putrescine dihydrochloride (105 mCi/mmol) and [*diaminobutane*-1,4-^{14}C]spermidine (90 mCi/mmol) were purchased from Amersham and Dupont–NEN (France).

2,4-Dinitrophenol, *N*-ethylmaleimide, unlabelled putrescine and spermidine were purchased from Sigma (France). 1-Heptanesulphonic acid sodium salt (98 %) and tetramethylammonium chloride (97 %) were purchased from Aldrich (France). Coomassie Blue reagent for protein assays was from Bio-Rad Laboratories (France). Culture medium components were from Gibco (France) and serum was from Flobio (France).

Stains and culture conditions

Leishmania donovani (strain MHOM/IN/80/DD8) originating from the strain collection of the World Health Organization's (WHO) reference centre in the London School of Hygiene and Tropical Medicine (U.K.) was provided by Dr. D. Evans.

Leishmania amazonensis (strain MHOM/BR/76/LTB-012) also originating from the strain collection of the WHO was provided by J. L. Lemesre (ORSTOM, Montpellier, France).

Promastigotes were grown at 26 °C in a semi-defined RPMI-1640 medium containing 2 mM glutamine, 25 mM Hepes (pH 7.4), 10 % (v/v) heat-inactivated fetal-calf serum, streptomycin at 5 μg/ml and penicillin at 5 units/ml.

Axenic amastigotes were obtained after transformation of promastigotes to amastigotes by J. L. Lemesre and were grown in a special medium (J. L. Lemesre; patent pending).

Analysis of intracellular pentamidine pools by HPLC

Intracellular pentamidine pools were determined by a modification of the method of Berger et al. [12]. Exponential-phase promastigote and amastigote cultures (100 and 50 ml respectively), treated or not with pentamidine, were harvested and washed twice with large volumes of cold PBS. The washed pellets were suspended in 5 % perchloric acid. After 30 min of incubation at 4 °C, the acid-soluble components were separated from cell proteins by centrifugation for 5 min at 11000 ***g***. The supernatant was removed, filtered through a 0.45 μm pore-size filter (Nalgene) and used directly for HPLC. The pentamidine pools were analysed on a Novapack C18 column (3.9 mm × 150 mm) kept at 40 °C. Chromatography was performed using a system consisting of a Waters 600 E gradient pump and a 990 photodiode-array detector with a flow rate of 1 ml/min. For the mobile phase, a linear gradient of 20–45 % acetonitrile in water containing sodium heptanesulphonate (10 mM), tetramethylammonium chloride (10 mM) and orthophosphoric acid (4.2 mM), over 30 min, was used. Cell volumes (22 μm^3 for *L. donovani* promastigotes and 100 μm^3 for *L. amazonensis* promastigotes) were determined respectively by Phelouzat et al. [13] and Berman et al. [14] taking 1 mg of protein, equivalent to 2.72×10^8 cells for *L. donovani* and 1.43×10^8 cells for *L. amazonensis*.

Protein determination

Protein concentration was measured by the dye-binding method, with BSA as standard [15].

Uptake of nucleosides, amino acids and polyamines

Centrifugation through oil was used to separate labelled cells from the medium or PBS and to determine the radioactivity associated with nucleoside, amino acid or polyamine. Parasites preincubated with the labelled molecule 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 3 min in an Eppendorf microfuge [16]. The supernatant was removed by careful aspiration, the pellet was resuspended in 1 M NaOH, and hydrolysed for 1 h at 80 °C. One aliquot of the hydrolysate was mixed with liquid scintillation fluid and the radioactivity counted (total uptake) and another was used for protein determination.

RESULTS

Pentamidine uptake

The amount of drug in promastigotes as a function of time of treatment was measured, by the IC_{50} values of the molecule, in two strains (Figure 1). In *L. donovani* cultured with 10 μM pentamidine, the uptake increases up to 8 h to reach 325 pmol/mg of protein corresponding to 53.3 μM and then decreases slowly. This represents a 5-fold accumulation of the drug within these cells. Similar results were obtained with the strain *L. amazonensis* treated with 1 μM pentamidine. After 8 h, a plateau is reached which remains stable until 24 h. The intracellular concentration was estimated to be 6.2 μM, corresponding to a 6-fold accumulation.

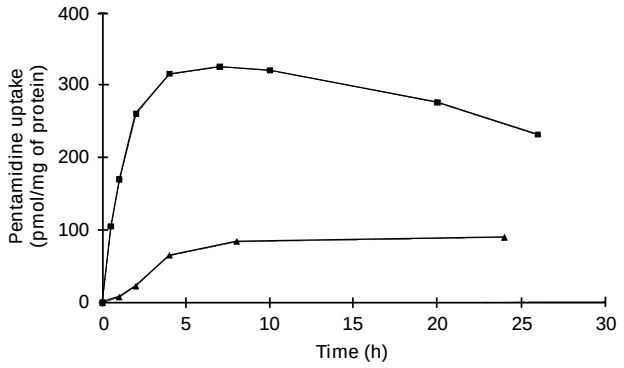

Figure 1 Pentamidine uptake by promastigotes as a function of treatment time

Promastigotes in exponential phase of growth (100 ml cultures) were incubated for various times with pentamidine, either 10 μM for *L. donovani* (■) or 1 μM for *L. amazonensis* (▲), and then processed as described in the Experimental section. Values are the means of two experiments.

Table 1 Kinetic parameters of pentamidine transporter in *Leishmania*

The apparent kinetic parameters were determined from Lineweaver–Burk plots [17]. The results (mean values for two different experiments) are expressed in pmol/mg of protein per 2 h (±20%) for V_{max} and in μM (±20%) for K_m.

Apparent kinetic parameters	*L. donovani*		*L. amazonensis*	
	Promastigotes	Amastigotes	Promastigotes	Amastigotes
K_m (μM)	73	322	8.3	167
V_{max} (pmol/mg per 2 h)	2000	333	1428	333

A plot of the rate of pentamidine uptake, as a function of its extracellular concentration, after 2 h treatment shows a typical saturation curve (results not shown), indicating a carrier-mediated transport mechanism. The apparent kinetic parameters of the transporter were determined from Lineweaver–Burk plots [17] (Table 1). The apparent V_{max} is similar for promastigotes of both strains, but the apparent affinity is different: 73 μM and 8.3 μM for *L. donovani* and *L. amazonensis* respectively.

Comparison of pentamidine uptake into promastigotes and amastigotes shows increased K_m and decreased V_{max} values in axenic amastigotes. The higher K_m value suggests a lower affinity for the specific substrate, and the lower V_{max} value can be interpreted as an indication of a reduced number of transport sites/cell.

Metabolism of pentamidine by *Leishmania*

Berger et al. [18–20] have demonstrated that pentamidine can be rapidly metabolized by rat liver cytochrome *P*-450-dependent mixed function oxidases to a number of metabolites. In extracts of pentamidine-treated promastigotes and amastigotes of *Leishmania*, no metabolites could be detected by HPLC, indicating that these parasites were unable to metabolize this molecule (results not shown). A similar observation was published by Berger et al. [21] on post-mitochondrial supernatant fractions from wild-type and pentamidine-resistant *Trypanosoma brucei brucei*.

Characterization of pentamidine uptake

In order to determine whether pentamidine uptake needs energy, the effect of metabolic inhibitors on its uptake into promastigotes was measured. A respiratory inhibitor such as potassium cyanide (1 mM), or an uncoupler of oxidative phosphorylation such as 2,4-dinitrophenol (1 mM) produced strong inhibition (90%) of the uptake into both strains, indicating that metabolic energy is required. *N*-Ethylmaleimide, a thiol-modifying reagent (0.5 mM), decreased the uptake (by 90%), suggesting that the presence of -SH groups on the drug transporter are important for the uptake process. However, it is not clear whether endo- or exofacial -SH groups, present on the carrier, are involved.

To determine the specificity of the pentamidine transport system, the effect of this drug on uptake of various molecules was analysed in promastigotes and amastigotes.

Adenosine transporter was shown to be implicated in pentamidine uptake in *Trypanosoma* [22]. In *Leishmania* the uptake of nucleosides (adenosine, thymidine, uridine) is not affected by 100 μM pentamidine for 1 h, indicating that pentamidine has no affinity and is not recognized by nucleoside carriers in this parasite.

Gutteridge [10] has suggested that pentamidine could enter *Crithidia fasciculata* by the lysine-arginine transport system. According to Kandpal et al. [23], pentamidine inhibits competitively arginine transport in *L. donovani* promastigotes.

In the presence of 100 μM pentamidine for 1 h, with a concentration ratio of pentamidine/amino acid of approx. 10, the uptake of arginine, lysine, ornithine, phenylalanine and aspartic acid into *L. donovani* and *L. amazonensis* promastigotes

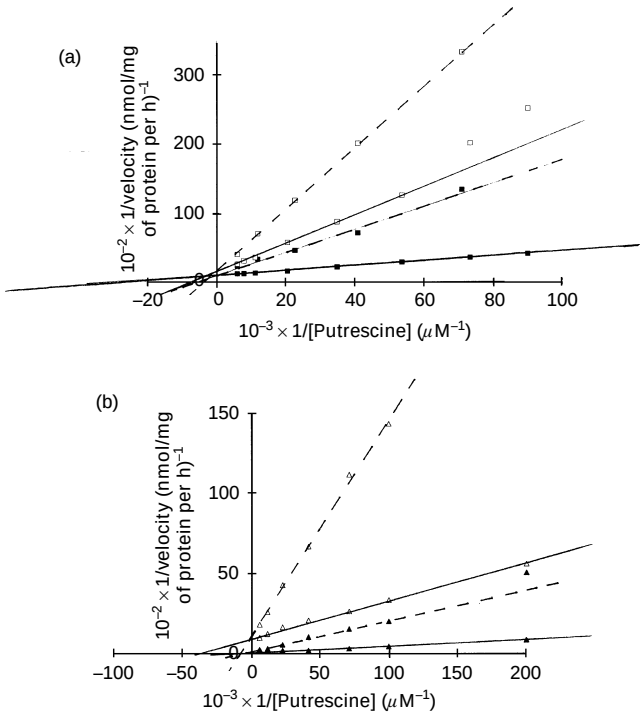

Figure 2 Uptake of putrescine as a function of its extracellular concentration and the effect of pentamidine

Cultures in exponential phase of growth (0.5 ml of culture in 24-well microplates) were incubated for 1 h at 26 °C with labelled putrescine (105 mCi/mmol; 1 μCi/ml) with or without pentamidine, and then processed as described in the Experimental section. Values are the means of two experiments. (**a**) *L. donovani*: putrescine alone in promastigotes (■—■) or in presence of 100 μM pentamidine (■ --- ■), putrescine alone in amastigotes (□—□) or in the presence of 200 μM pentamidine (□ --- □). (**b**) *L. amazonensis*: putrescine alone in promastigotes (▲—▲) or in presence of 100 μM pentamidine (▲ --- ▲), putrescine alone in amastigotes (△—△) or in the presence of 200 μM pentamidine (△ --- △).

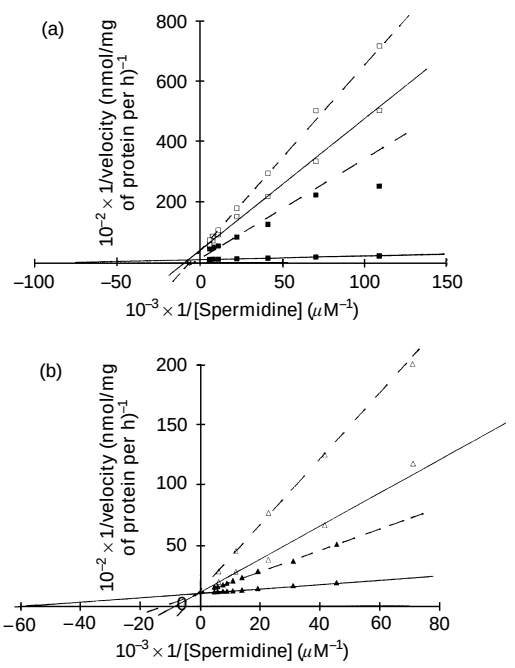

Figure 3 Uptake of spermidine as a function of its extracellular concentration and the effect of pentamidine

Cultures in exponential phase of growth (0.5 ml of culture in 24-well microplates) were incubated for 1 h at 26 °C with labelled spermidine (90 mCi/mmol; 1 μCi/ml) with or without pentamidine, and then processed as described in the Experimental section. Values are the means of two experiments. (**a**) *L. donovani*: spermidine alone in promastigotes (■—■) or in presence of 100 μM pentamidine (■ --- ■), spermidine alone in amastigotes (□—□) or in the presence of 100 μM pentamidine (□ --- □). (**b**) *L. amazonensis*: spermidine alone in promastigotes (▲—▲) or in presence of 100 μM pentamidine (▲ --- ▲), spermidine alone in amastigotes (△—△) or in the presence of 200 μM pentamidine (△ --- △).

is inhibited, non-competitively, by 80 %. With a concentration ratio of 1000, the uptake of tyrosine and isoleucine was reduced by about 50 %, but that of tryptophan and serine was not inhibited.

With amastigotes, addition of 200 μM pentamidine for 1 h, using a pentamidine/amino acid concentration ratio of 20 decreases the uptake of arginine and aspartic acid into both strains by 75 % and 57 % respectively. Thus, pentamidine affects the uptake of these amino acids but does not share their transporters.

As pentamidine and polyamines are cationic molecules having similar structures, the possibility that they share a common transport system was envisaged.

Preliminary experiments have shown that pentamidine, added before or at the same time as polyamine into the culture medium, inhibits, in a concentration-dependent manner, uptakes of putrescine and spermidine into *L. donovani* and *L. amazonensis* promastigotes. Plots of the rates of putrescine and spermidine uptake as a function of their concentrations show typical saturation curves (Figures 2 and 3). The apparent kinetic parameters of putrescine and spermidine uptakes in both strains are given in Table 2. These parameters are different for the two strains. K_m values obtained for putrescine uptake are in accordance with those found in the literature [24–26], except with those reported by Balana-Fouce et al. [27] who found a lower K_m value for *Leishmania infantum* promastigotes. Pentamidine competitively inhibits putrescine and spermidine uptakes in both strains, indicating a common carrier-mediated mechanism. In both strains, K_m values for putrescine and spermidine as well as the K_i for pentamidine are higher in axenic amastigotes than in promastigotes.

Table 2 Uptake of putrescine and spermidine in *Leishmania*

The apparent kinetic parameters were determined from the Lineweaver–Burk plot [17]. The results (mean values for two different experiments) are expressed in nmol/mg of protein per h (±20 %) for V_{max} and in μM (±20 %) for K_m and K_i^{Pent}.

Apparent kinetic parameters	*L. donovani*		*L. amazonensis*	
	Promastigotes	Amastigotes	Promastigotes	Amastigotes
Putrescine				
K_m^{Put} (μM)	43.5	100	14.1	35
V_{max} (nmol/mg per h)	10	8.33	62.5	12.5
K_i^{Pent} (μM)	21	85.7	12.7	42.4
Spermidine				
K_m^{Spd} (μM)	14.3	100	18	166
V_{max} (nmol/mg per h)	11.1	2.5	9.3	10
K_i^{Pent} (μM)	3	232.5	26	198

DISCUSSION

Our results show that *L. donovani* and *L. amazonensis*

promastigotes and amastigotes do not metabolize pentamidine but they accumulate the drug against a concentration gradient across the cell membrane. To our knowledge, this is the first report on pentamidine uptake by axenic amastigotes. The involvement of an active carrier in pentamidine uptake is indicated by saturation kinetics, and by strong inhibition by potassium cyanide, 2,4-dinitrophenol and *N*-ethylmaleimide. A similar conclusion was reported by Damper and Patton [11], for the blood-form trypomastigotes of *Trypanosoma brucei brucei*. On the contrary, Berman et al. [14] reported that pentamidine uptake is predominantly by simple diffusion in *Leishmania mexicana* promastigotes and in amastigotes isolated from macrophages.

The results concerning the specificity of the pentamidine transport system showed that contrary to what is reported for other protozoa, pentamidine does not share adenosine, pyrimidine and amino acid carriers in the promastigotes and amastigotes studied. However, the fact that pentamidine competitively inhibits polyamine uptake indicates a common carrier-mediated mechanism. Interestingly, in *L. infantum* promastigotes pentamidine and its analogues inhibited putrescine uptake non-competitively [26].

As Balana-Fouce et al. [27] in *L. infantum* and Gonzalez et al. [25] in *L. mexicana* have shown that promastigotes have independent transport carriers for putrescine and another for spermidine and spermine, pentamidine probably shares these two transporters in the promastigotes and amastigotes studied.

The inhibition of polyamine transport by this drug breaks the balance between extracellular and intracellular polyamine concentrations and may contribute to the growth arrest provoked by pentamidine. However, this effect is certainly not the only mechanism of anti-leishmanial activity of this molecule.

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