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ORIGINAL PAPER

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Putrescine analogue cytotoxicity against *Trypanosoma cruzi*

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Abstract *Trypanosoma cruzi* is the etiological agent of American trypanosomiasis. Most of the available data on trypanosomatid parasites were obtained from African trypanosomes. Parasitic protozoa polyamine metabolism and transport pathways comprise valuable targets for chemotherapy. *T. cruzi* cannot synthesize putrescine, but its uptake from the extracellular milieu can promote parasite survival. Nevertheless, little is known about the cell biology of this diamine in *T. cruzi*. Here we notice that the putrescine analogue 1,4-diamino-2-butanone (DAB) inhibited *T. cruzi* epimastigotes' in vitro proliferation and produced remarkable mitochondrial destruction and cell architecture disorganization, as assessed by transmission electron microscopy. Mitochondrial damage was confirmed by MTT reduction. We decided to analyze the oxidative stress undergone by DAB-treated parasites. Thiobarbituric-acid-reactive substances were measured to assess lipid peroxidation. Analogue effects were dose-dependent; 5 mM DAB only slightly enhanced peroxidation, whereas 10 mM DAB significantly (P<0.05) diminished it. These data indicate that putrescine uptake by this diamine auxotrophic parasite may be important for epimastigote axenic growth and cellular organization.

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Introduction

Trypanosoma cruzi is the etiological agent of Chagas disease or American trypanosomiasis, which affects 16–18 million people in South America and Central America and is emerging in the Brazilian Amazon (Coura et al. 2002). Patients may present cardiomyopathy, which has high morbidity and mortality. Treatment of Chagas disease is based on the use of nitroheterocyclic agents, such as nifurtimox and benznidazole (*N*-benzyl-2-nitro-1-imidazole acetamide), which are associated with considerable side effects and refractory cases (Coura 1988; Gorla et al. 1988). In addition, sterile cures are usually not achievable. Therefore, new antitrypanosomal agents are required; however, despite the high incidence and mortality of the infection, there has been little commercial interest in developing new trypanocidal compounds (Trouiller et al. 2002).

Polyamines are fundamental for growth, development, cellular differentiation, and macromolecule stabilization, among other processes (Pegg and McCann 1982). These polycation biosynthesis pathways in parasitic protozoa differ from those in mammalian cells and, therefore, comprise valuable targets for chemotherapy (Müller et al. 2001; Bacchi and Yarlett 2002). The ornithine decarboxylase (ODC) irreversible inhibitor D,L-α-difluoromethylornithine (DFMO) was shown to be effective against both experimental *Trypanosoma brucei* infections (Bacchi et al. 1980) and against sleeping sickness caused by *Trypanosoma gambiense* (Marton and Pegg 1995). The selectivity of DFMO on *T. brucei* and *T. gambiense* is due to the slow turnover (~6 h half-life) of ODC compared to that of the human enzyme (~15 min half-life).

T. cruzi is devoid of ODC activity (Ariyanayagam and Fairlamb 1997) and thus must incorporate polyamines either from the extracellular milieu or from the host cell by

means of a high-affinity diamine transport system (Le Quesne and Fairlamb 1996). Putrescine uptake from the host cell may promote *T. cruzi* intracellular proliferation (Freire-de-Lima et al. 2000; Peluffo et al. 2004).

Therefore, putrescine transport and metabolism are of great interest for the understanding of the infection and the chemotherapeutic drug design. The putrescine analogue 1,4-diamino-2-butanone (DAB) inhibits the in vitro proliferation of the trichomonad *Tritrichomonas foetus* (Reis et al. 1999), *Entamoeba invadens* (Calvo-Méndez et al. 1993), and *Entamoeba histolytica* (Arteaga-Nieto et al. 1996), as well as of *Leishmania amazonensis* (Valentim et al., unpublished) and fungi (Martinez-Pacheco et al. 1989; Ueno et al. 2004), comprising a tool to approach putrescine's role in pathogens. Here we report the effects of this compound on the putrescine auxotroph *T. cruzi*.

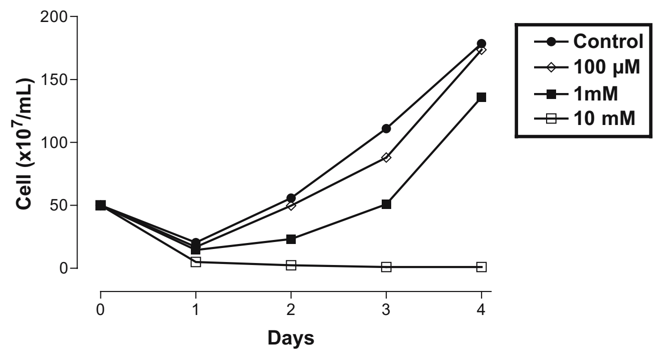

Fig. 1 Axenic proliferation of *Trypanosoma cruzi* epimastigotes in the absence or presence of increasing concentrations of DAB. Control (*filled circle*), 100 μM DAB (*diamond*), 1 mM DAB (*filled square*), 10 mM DAB (*square*)

Materials and methods

Chemicals DAB (1,4-diamino-2-butanone dihydrochloride), putrescine (1,4-diamino-butane or 1,4-tetramethylene-diamine), MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide], and thiobarbituric acid were purchased from Sigma (St. Louis, MO, USA). All reagents used were of analytical grade.

Parasites Epimastigotes of *T. cruzi* Y-strain were cultured at 28°C in plastic flasks containing 5 ml of liver infusion trypticase medium inoculated with 10^7 cells/ml and supplemented with 10% fetal calf serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Cells from the mid-log phase were harvested by centrifugation at 1,000×*g* and washed three times with phosphate-buffered saline (PBS) before the experiments. Parasite growth was assessed in hemocytometric chambers by daily counting under phase contrast microscopy.

Transmission electron microscopy Parasites were fixed in 2.5% glutaraldehyde (EM-grade; Sigma) and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer (pH 7.2); postfixed in 1% osmium tetroxide and 0.08% potassium ferricyanide; dehydrated in an acetone series; and embedded in Polybed resin (Polysciences, Inc.). Thin sections were stained with uranyl acetate and lead citrate, and then observed under a Zeiss CEM900 transmission electron microscope.

Lipid peroxidation Thiobarbituric acid-reactive substances (TBARS) were measured in parasites preincubated or not with the analogue for 24 h. After treatment, the cells were washed and resuspended in 200 μl of PBS, and then 200 μl of 1% thiobarbituric acid in acetic acid was added. After that, the samples presenting equal cell numbers were incubated at 95°C for 3–4 h and then cooled. Absorbance was determined at 532 nm.

MTT reduction Parasites (10^7 cells/ml) were treated as described above, washed, reinoculated in fresh culture medium containing 10% vol/vol MTT, and incubated for 16 h. Pellets were solubilized in DMSO and transferred to flat-bottom 96-well microtiter plates. Formazan precipitates derived from MTT reduction were determined

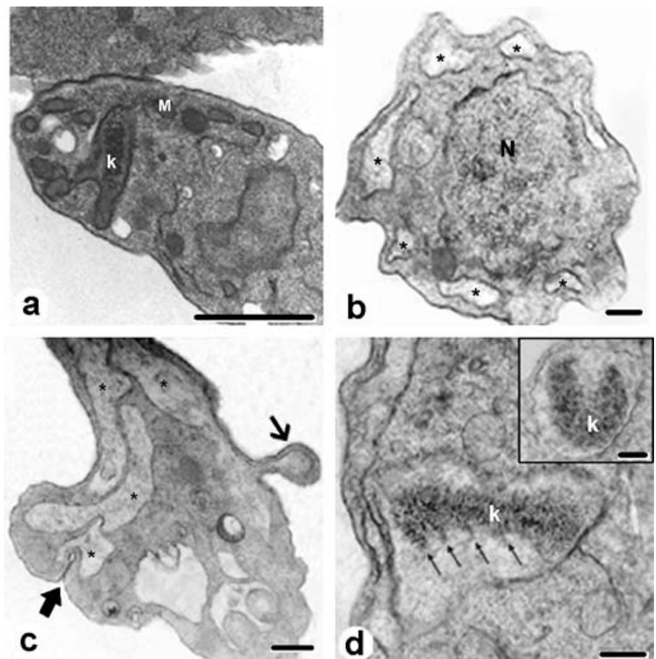

Fig. 2 Transmission electron microscopy of DAB effects on *T. cruzi* epimastigotes. **a** Low-magnification TEM showing the general appearance of untreated control parasites displaying normal mitochondria (*M*) and kinetoplast (*K*). Parasites incubated with 10 mM DAB in the culture medium for 24 h presented remarkable mitochondrial alterations. Transversally (**b**) and sagitally (**c**) sectioned DAB-treated epimastigotes displayed reduced mitochondrial electron density all over the cells. The single mitochondrion of the parasite was remarkably damaged and washed out (*asterisk*). Some parasites preserved the overall cytoplasmic architecture (**b**), but, eventually, tubular compartments left by the mitochondrion (**c**) appeared to be either collapsing (*wide arrow*) or protruding (*thin arrow*) at the cell surface, drastically affecting cell ultrastructure (**c**). DAB treatment induced kinetoplast disorganization. The parasite kDNA (*K*) was irregularly condensed, presenting disordered fibrils (**d**; *arrows*), or presented altered shapes (*inset*). **a**–**d** *Bars* represent 0.5, 0.25, 0.4, and 0.25 μm, respectively

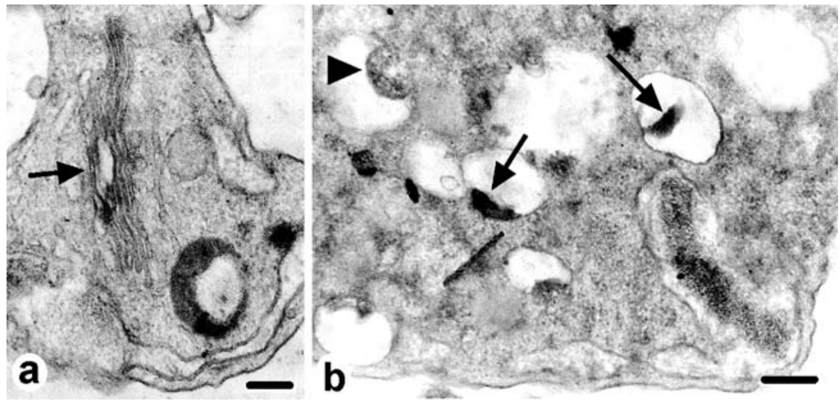

Fig. 3 Some cells presented distended Golgi apparatus cisternae (**a**; *arrow*). Many parasites showed numerous vesicles containing membrane-bound amorphous electron-dense materials (**b**; *arrows*), which sometimes displayed a granular appearance (*arrowhead*). **a**, **b** *Bars* represent 0.15 and 0.25 μm, respectively

spectrophotometrically at 570 nm. Wells containing solely MTT and DMSO were employed as controls.

Statistical analysis Presented data are representative of a minimum of three independent experiments, which yielded analogous results. Significant differences ($P<0.05$ and $P<0.001$) were statistically analyzed using ANOVA.

Results

Proliferation of *T. cruzi* epimastigotes in vitro was reduced in a dose-dependent manner by the putrescine analogue DAB. Significant ($P<0.05$) inhibition was achieved by 1 mM DAB treatment (with an apparent IC_{50} of 1.18 mM) and was totally blocked by 10 mM DAB (Fig. 1).

Transmission electron microscopy (TEM) revealed that DAB treatment resulted in profound ultrastructural changes in *T. cruzi* mitochondria, leading to a reduction of its electron density (Fig. 2a–d) as well as causing extensive damage and even complete destruction of the organelle (Fig. 2b,c). These effects ultimately resulted in a general ultrastructural disorganization (Fig. 2c). DAB also induced kinetoplast disorganization because the parasite kinetoplast DNA (kDNA) was irregularly condensed, presenting disordered kDNA fibrils (Fig. 2d, arrows), or presented altered shapes (Fig. 2, inset). Some cells presented distended Golgi apparatus cisternae (Fig. 3a) as well as numerous vesicles often containing amorphous electron-dense materials that were generally bound to the lining membrane (Fig. 3b, arrows), which sometimes displayed a granular appearance (Fig. 3, arrowhead).

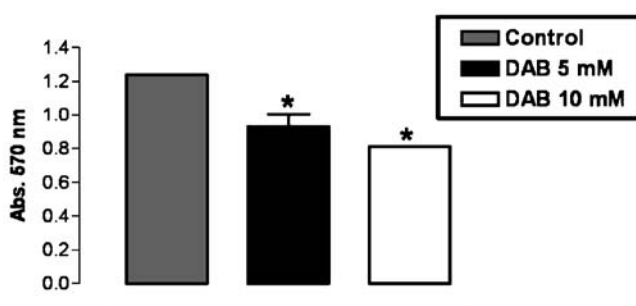

Fig. 4 Effect of DAB on mitochondrial function in *T. cruzi*. Epimastigotes were treated with either 5 or 10 mM DAB for 24 h and incubated with MTT, as described in "Materials and methods." Formazan precipitation was determined spectrophotometrically at 570 nm. Both concentrations significantly ($P<0.05$) diminished mitochondrial activity

Since the mitochondria seem to be the main target of DAB treatment on *T. cruzi* epimastigotes, we next evaluated possible mitochondrial damage by MTT reduction assay. Mitochondrial function was significantly ($P<0.05$) diminished by either 5 or 10 mM DAB (Fig. 4).

Since the mitochondria were the main sites of cellular reactive oxygen species (ROS) generation and since DAB treatment led to dramatic changes in this organelle, we decided to evaluate whether mitochondrial changes would be associated with oxidative stress in DAB-treated parasites. Measurement of TBARS revealed that DAB effects were dose-dependent. Whereas a concentration of 5 mM only slightly enhanced lipid peroxidation, a 10-mM concentration diminished it considerably (Fig. 5). Interestingly, coincubation of parasites with putrescine reverted the prooxidant effects of 5 mM DAB (Fig. 5).

Discussion

Devoid of de novo polyamine biosynthesis pathway, *T. cruzi* epimastigotes are obligate polyamine scavengers (Ariyanayagam and Fairlamb 1997). There is evidence that *T. cruzi* amastigotes and trypomastigotes, under polyamine shortage conditions, are able to synthesize putrescine from arginine via agmatine (reviewed in Peluffo et al. 2004); however, it seems that, most of the time, this protozoan relies on diamine incorporation from the hosts. The putrescine uptake by *T. cruzi* is about 50-fold higher than that in *Leishmania mexicana* (González et al. 1992). Conceivably, DAB would bind to the polyamine transporter at the parasite surface and dislocate the native diamine. However, it remains to be determined whether DAB enters the cell.

To approach the mode of action of this analogue, we employed TEM of parasites before and after DAB treatment. We noticed that DAB-treated epimastigotes displayed drastically altered or destroyed mitochondria (Fig. 2). Interestingly, this analogue also affected *Leishmania* mitochondria

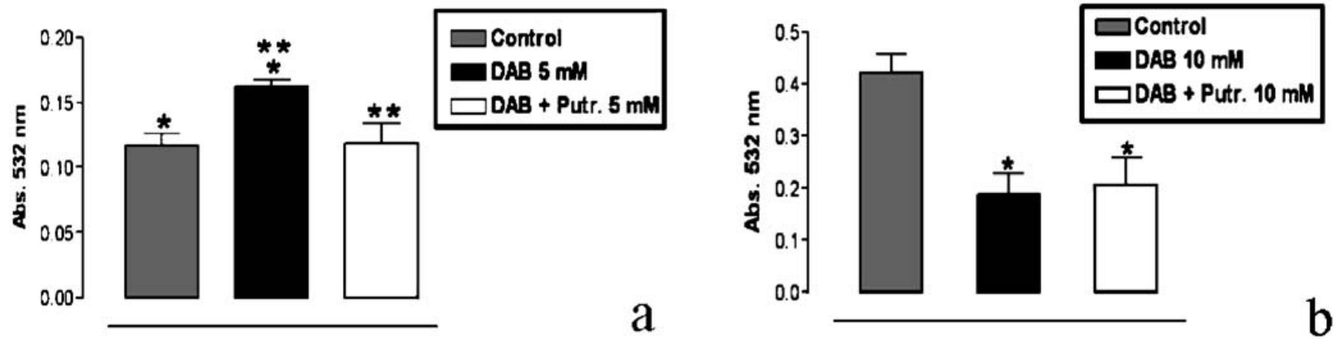

Fig. 5 Lipid peroxidation was assessed by the production of TBARS determined at 532 nm, as described in "Materials and methods." **a** A concentration of 5 mM DAB significantly ($P<0.05$) enhanced lipid peroxidation in epimastigotes, whereas putrescine reversed it to control levels. **b** Parasites incubated with 10 mM DAB presented a highly significant ($P<0.01$) decrease in lipid peroxidation, which was not reverted by putrescine

(Valentim et al., unpublished) and redox ATP-producing trichomonad hydrogenosomes (Reis et al. 1999).

Since mitochondrial swelling is associated with reduced state 3 respiration, which may trigger permeability transition, ROS generation, and, ultimately, apoptosis, and since ATP depletion impairs both organelle swelling and procaspase activation (Robertson and Orrenius 2002), it seems reasonable that DAB-treated epimastigotes undergo necrotic death before an apoptotic process could take place. Furthermore, polyamine depletion may shift drug-induced programmed cell death to necrosis (Monti et al. 2004), and the absence of nuclear fragmentation or chromatin condensation in our experiments reinforces the inference of a necrotic process rather than an apoptotic process. Nevertheless, bis(ethyl)-polyamine analogues produced mitochondrial swelling associated with a decreased ATP content in mammalian cells (Fukuchi et al. 1992; He et al. 1994), and the organelle was severely injured (Fukuchi et al. 1992; Snyder et al. 1994). DAB-induced mitochondrial dysfunction was confirmed by MTT reduction as that in *Leishmania* (Valentim et al., unpublished). At advanced stages of DAB treatment, the mitochondria were somewhat enlarged, but the organelle matrix was completely destroyed. In this regard, spermine was shown to inhibit calcium- and phosphate-induced mitochondrial swelling (Rigobello et al. 1993). In some parasites, mitochondrial space was either collapsed or protruded, remarkably distorting cell shapes (Fig. 2c). These morphological changes may be indicative of impaired calcium homeostasis, since sustained high cytoplasmic Ca^{2+} concentrations lead to cell surface blebbing by inducing cytoskeletal disorganization (Boobis et al. 1989) as well as oxidative stress in the mitochondria (Sousa et al. 2003). Since the trypanosomatid cell shape is maintained by subpellicular microtubules that run parallel underneath the plasma membrane (e.g., De Souza 2002; Vannier-Santos et al. 2002), it is reasonable to assume that parasite cytoskeleton is remarkably affected. Such calcium imbalance may be associated with the oxidation of sulfhydryl groups during oxidative stress (Boobis et al. 1989). These observations indicate that putrescine transport and, possibly, subsequent metabolism to spermidine and trypanothione are required for the maintenance of mitochondrial structure and function as well as for cell architecture. In this regard, Giffin et al. (1986) reported that putrescine, but not spermidine, was able to stabilize *T. brucei* mitochondrial membranes. In addition, putrescine was reported to protect *Escherichia coli* from oxidative stress (Chattopadhyay et al. 2003; Tkachenko et al. 2001). Mitochondrial permeability transition (MPT) was shown to be involved in both necrosis and apoptosis. ROS are among the factors that trigger MPT, and polyamines may protect the mitochondria from MPT (reviewed in Fairlamb and Cerami 1992; Tassani et al. 1995).

Putrescine depletion may lead to reduced trypanothione levels and, thus, oxidative stress by ROS. *T. cruzi* presents a mitochondrial trypanothione-dependent peroxidase acting as an important antioxidant defense in the vicinity of the kDNA, presumably to protect the mitochondrial genome from both endogenous and exogenous oxidative stresses (Wilkinson et al. 2000).

Parasite mitochondria comprise valuable targets for chemotherapy (Kita et al. 2001), and the crystal or gentian violet used to prevent the spread of Chagas disease by blood transfusion exerts its effects on the *T. cruzi* mitochondria (Gadelha et al. 1989). The kDNA is the site of action of several compounds used against trypanosomatid parasites (Shapiro and Englund 1990). Benznidazole metabolites were reported to bind to nuclear and kinetoplast DNA (De Toranzo et al. 1988), producing strand breaks (Goijman et al. 1985) and extensive mitochondrial alterations in intracellular *T. cruzi* (Andrade and Freitas 1987). Polyamine deficiency in DAB-treated parasites could result in remarkable alterations observed in *T. cruzi* kDNA organization. This analogue may prevent putrescine uptake by the mitochondria since this organelle transports spermine, spermidine, and putrescine in mammalian cells (Toninello et al. 1992). It is noteworthy that spermine prevents glutathione release by the rat liver mitochondria and thus is presumably involved in oxidative stress defense (Toninello et al. 1992). In trypanosomatid protozoa, most thiol groups are found in N_1,N_8 (glutathionyl) spermidine or trypanothione (Fairlamb and Cerami 1992).

Parasitic protozoa may rely on glycolytic metabolism for energy supply for long periods, but in trypanosomatid

parasites, the glycolytic pathway depends on the mitochondria because of a cyanide-insensitive glycerol-3-phosphate oxidase system that is localized in this organelle (Clarkson et al. 1989). Therefore, mitochondria-impairing compounds may comprise important candidate agents in parasitic disease chemotherapy (e.g., Kita et al. 2001; Nihei et al. 2002). In this regard, ergosterol biosynthesis inhibitors may lead to pronounced mitochondrial damage in *T. cruzi* (Lazardi et al. 1990) and *L. amazonensis* (Vannier-Santos et al. 1995; Rodrigues et al. 2002). Several microbicidal agents, including nitrofurans such as nifurtimox, act as redox cycling agents, producing oxidative stress and generating $O_2^{\cdot -}$ and H_2O_2 (Docampo and Stoppani 1979; Docampo et al. 1981). The dose dependence of DAB effects may be due to the complete mitochondrial destruction observed in parasites treated with a higher concentration, since this organelle is the main source of ROS.

Enzymes such as trypanothione reductase constitute rather promising targets for chemotherapy (Austin et al. 1999; Augustyns et al. 2001). Interestingly, trypanothione is involved in the detoxification of nifurtimox and benznidazole in *T. cruzi* (Repetto et al. 1996). These compounds decrease glutathione and trypanothione levels in *T. cruzi* (Maya et al. 1997), and metabolites of nitroimidazoles, such as megazole, are efficient trypanothione scavengers (Maya et al. 2003). Thus, polyamines may play a fundamental role in *T. cruzi* resistance to the drugs of choice and may therefore provide important targets for rational drug design.

Putrescine was reported not to be required in vitro for *Leishmania donovani* promastigotes devoid of ODC genes. Interestingly, trypanothione concentration was reduced, although spermidine levels were relatively unaffected. Although spermidine was both required and sufficient for *L. donovani* growth, it was unable to circumvent the lethal Δ*odc* mutation achieved by the addition of putrescine for both axenic growth and trypanothione production (Jiang et al. 1999). These data seem to indicate that putrescine itself may have a role in the biology of these parasites. In this regard, putrescine activates *T. cruzi S*-adenosylmethionine decarboxylase (Clyne et al. 2002). Furthermore, putrescine was shown to be required for the proliferation of carcinoma cells (Farriol et al. 2001) and insect neuroblasts (Cayre et al. 1997). In addition, hypoxic rat smooth muscle cells specifically require increased putrescine uptake for p38 mitogen-activated protein kinase activation (Ruchko et al. 2003), and this diamine can regulate the function of several genes (Pastorian et al. 2000; Fujimoto et al. 2001).

Many DAB-treated parasites displayed vesicles presenting electron-dense materials bound to the compartment membrane, resembling acidocalcisomes. Similar acidocalcisome-like compartments were observed in *L. amazonensis* cells incubated with ergosterol biosynthesis inhibitors. These structures presumably comprised autophagic vacuoles (Vannier-Santos et al. 1995, 1999). Some parasites presented distended Golgi apparatus cisternae. Golgi apparatus swelling and dysfunction were previously noticed in different polyamine-deficient mammalian cell types (Sakamaki et al. 1989).

The consumption of the arginine pool for putrescine synthesis by macrophages may be associated with reduced nitric oxide (NO) production (Mills 2001), enhancing *T. cruzi* intracellular proliferation (Freire-de-Lima et al. 2000; Piacenza et al. 2001; Peluffo et al. 2004). Similarly, *T. brucei* arginine uptake for polyamine synthesis reduces NO production by macrophages (Gobert et al. 2000). Therefore, antagonists of polyamine synthesis/transport may promote microbicidal activity by providing multifunctional chemotherapeutic agents.

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