

The putrescine analogue 1,4-diamino-2-butanone affects polyamine synthesis, transport, ultrastructure and intracellular survival in *Leishmania amazonensis*

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Polyamines are important regulators of growth and differentiation in a variety of cells, including parasitic protozoa. Promastigotes of *Leishmania* species have high levels of putrescine and spermidine and their growth can be inhibited by polyamine biosynthesis antagonists. The putrescine analogue 1,4-diamino-2-butanone (DAB) is microbicidal against *Trichomonas foetus* and *Trypanosoma cruzi*, so we tested its effects on *Leishmania amazonensis* proliferation, viability, organization, putrescine transport and synthesis as well as *in vitro* infectivity. DAB impaired promastigote proliferation dose-dependently (IC₅₀ 144 µM) and the parasite putrescine concentration was reduced by nearly 50%. This analogue markedly inhibited both ornithine decarboxylase activity and [³H]putrescine uptake by promastigotes. Pre-treatment with DAB for 24 h led to compensatory enhancement of putrescine uptake, indicating an adaptive mechanism in DAB-treated parasites. Remarkably, DAB caused mitochondrial damage, assessed by transmission electron microscopy, and 3 h treatment with 1 mM DAB enhanced lipid peroxidation, whereas incubation with 10 mM DAB or for 24 h resulted in decreased peroxidation levels in the parasites. This effect was probably due to the loss of mitochondrial function, demonstrated by the diminished reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), not observed in macrophages. Murine macrophages infected with *L. amazonensis* amastigotes treated with DAB had parasite loads significantly ($P < 0.05$) lower than controls, presumably due to interference with putrescine uptake and/or synthesis. These results suggest that putrescine may be involved in leishmanial survival, possibly by maintaining the parasite's mitochondrial function. The use of analogues to interfere with polyamine/diamine synthesis and transport may shed light on its function in intracellular parasite survival and lead to identification of new targets for leishmaniasis chemotherapy.

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INTRODUCTION

Leishmaniasis is a vector-borne disease endemic in more than 88 countries, with 1 to 5 million new cases reported annually and at least 350 million people at risk of infection (Desjeux, 2004). Clinical manifestations may include single self-healing or disseminated cutaneous lesions, mucocuta-

neous destruction and visceral commitment, all resulting from *Leishmania* replication within mononuclear phagocytes (Vannier-Santos *et al.*, 2002). A growing interest by developed countries was prompted by increased frequency of leishmaniasis cases among HIV patients and overseas travellers (Desjeux, 2004).

Antileishmanial chemotherapy is currently limited to a few compounds such as the pentavalent antimonials glucantime (*N*-methylglucamine antimoniate) and pentostam (sodium stibogluconate), amphotericin B and pentamidine. Pentavalent antimonials have been the first-choice drugs for leishmaniasis treatment since the 1920s.

Abbreviations: AI, association index; DAB, 1,4-diamino-2-butanone; DFMO, DL- α -difluoromethylornithine; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ODC, ornithine decarboxylase; PA, polyamine; ROS, reactive oxygen species; TBA, thiobarbituric acid; TBARS, TBA-reactive substances.

Although they are generally effective against most leishmaniasis cases, severe side effects are often observed. Also, resistance to these compounds is a serious problem in many parts of the world such as India (Ouellette *et al.*, 2004). The high toxicity of the antimonial formulation has been implicated in the death of several patients under treatment (Silva, 2001; Ahasan *et al.*, 1996). Miltefosine was recently added to the therapeutic arsenal and is effective against Indian visceral leishmaniasis (Bhattacharya *et al.*, 2007). Nevertheless miltefosine cure rates in mucosal disease range from only 50 % (Soto *et al.*, 2004) to 70 % (Soto & Toledo, 2007), and 58 % in severe clinical forms. In addition, miltefosine is not as rapid as the antimonials (Soto *et al.*, 2008), not effective in HIV-infected patients (Troya *et al.*, 2008) and permits relapses in diffuse cutaneous forms (Zerpa *et al.*, 2007) as well as displaying limited efficacy against Brazilian cutaneous infection (Tuon *et al.*, 2008). Furthermore, the prolonged treatments and half-life of the compound, especially in an oral therapy with variable adherence, may promote resistance (Dupouy-Camet, 2004; Janvier *et al.*, 2008), and miltefosine-resistant parasites have been readily obtained *in vitro* (Ouellette *et al.*, 2004). Therefore, new chemotherapeutic agents are required.

The natural polyamines (PAs) putrescine, spermidine and spermine are low-molecular-mass organic cations found in up to millimolar concentrations in eukaryotic and prokaryotic cells and essential for cell growth and differentiation. PA biosynthesis is initiated by the rate-limiting decarboxylation of ornithine to putrescine by ornithine decarboxylase (ODC; EC 4.1.1.17). Successive transfers of aminopropyl groups from decarboxylated S-adenosylmethionine give rise to spermidine and then spermine.

PA analogues have been synthesized and tested for cancer and parasitic infection therapies. Distinct PA synthesis and transport mechanisms have been described in mammalian cells and micro-organisms, providing potential chemotherapeutic targets (Heby *et al.*, 2003; Müller *et al.*, 2001; Bacchi & Yarlett, 2002). Treatment of human *Trypanosoma gambiense* infection with eflornithine (DL- α -difluoromethyl-ornithine, DFMO), an irreversible ODC inhibitor, results in successful clearance of both early and late stages of the disease and also lacks severe side effects (Bitonti *et al.*, 1991; McCann *et al.*, 1987). However, the effectiveness of DFMO in *Leishmania* is controversial. It was reported not to inhibit replication of some species, but it is effective against *Leishmania infantum* both *in vitro* and *in vivo* (Gradoni *et al.*, 1989). The bis(benzyl)polyamine analogues also show significant activity against *Leishmania donovani* amastigotes *in vitro* and *in vivo* (Baumann *et al.*, 1990). We have previously shown that the putrescine analogue 1,4-diamino-2-butanone (DAB) blocks the proliferation of *Trichomonas foetus* (Reis *et al.*, 1999) and *Trypanosoma cruzi* (Menezes *et al.*, 2006). Here we tested the effects of DAB on *Leishmania amazonensis* proliferation, putrescine uptake and biosynthesis, ultrastructure and macrophage infection.

The implications of the findings for the parasite's biology are discussed.

METHODS

Chemicals. DAB, putrescine (1,4-diaminobutane or tetramethylenediamine dihydrochloride) and thiobarbituric acid (TBA) were purchased from Sigma. Stock solutions were made in PBS and stored at -20°C . $[1,4(n)-^3\text{H}]$ Putrescine dihydrochloride and $[1-^{14}\text{C}]$ ornithine hydrochloride were purchased from Amersham Pharmacia Biotech and stored at 2°C .

Parasites. The MHOM/Br/75/Josefa strain of *Leishmania amazonensis* was used throughout this study. It was isolated from a human case of diffuse cutaneous leishmaniasis in Brazil by Dr C. A. Cuba-Cuba (University of Brasília, Brazil) and has been maintained since then, in both axenic culture and hamster (*Mesocricetus auratus*) footpad inoculation. Promastigotes were maintained at 26°C in Warren's medium (brain and heart infusion, 20 mg folic acid l^{-1} and 20 mg haemin l^{-1}) supplemented with 10 % heat-inactivated fetal calf serum (FCS). Amastigotes were obtained from axenic cultures and cultivated as described elsewhere (Borges *et al.*, 1998). These cells were able to infect and induce lesion formation in hamsters.

Macrophages. Peritoneal macrophages from 6–8-week-old Swiss mice were collected in Hanks' balanced salt solution and plated on glass coverslips in Falcon 24-well tissue culture plates. Cells were allowed to adhere for 60 min at 37°C in a 5 % CO_2 atmosphere, after which the non-adhering cells were removed and fresh medium 199 plus 10 % FCS was added. Cells were cultivated overnight before the assays, as described previously (Borges *et al.*, 1998).

Proliferation assays. Mid-exponential-phase parasites were harvested and inoculated in fresh culture medium before or after addition of increasing concentrations of the analogue. Aliquots were collected and fixed every 24 h and parasite density was assessed by direct counting in a Neubauer chamber. The mean generation time, g , was determined according to the equation $g = (\log_{10} N_t - \log_{10} N_0) / \log_{10} 2$, where t is time and N is the cell number at a defined period of time. The IC_{50} was determined employing GraphPad Prism on the 5th day of axenic culture.

Transmission electron microscopy. Parasites were fixed in 2.5 % glutaraldehyde grade II (Sigma) in 0.1 M sodium cacodylate buffer pH 7.2, post-fixed in 1 % osmium tetroxide and 0.8 % potassium ferricyanide in the same buffer, dehydrated in an acetone series and embedded in Polybed resin (Polysciences). Thin sections were stained with uranyl acetate and lead citrate and observed under a Zeiss 900 transmission electron microscope.

MTT reduction. Parasites were inoculated (10^7 cells ml^{-1}) and treated as described under 'Proliferation assays' for different periods of time. Treated and untreated cells were washed, reinoculated in fresh culture medium containing 10 % (v/v) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated for an additional 16 h. Cell pellets were solubilized in DMSO, transferred to flat-bottomed 96-well plates and formazan precipitates derived from MTT reduction were determined spectrophotometrically at 540 nm. Wells containing solely MTT and DMSO were employed as controls. MTT reduction by macrophages was performed as above, but in samples with 10^5 cells incubated for 24 h.

Lipid peroxidation. Thiobarbituric acid-reactive substances (TBARS) were measured in parasites pre-incubated or not with the analogue for 24 h. After treatment, the cells were washed, resus-

ended in 200 μl PBS and 200 μl of 1% TBA in acetic acid was added. Then samples with equal cell numbers were incubated at 95 °C for 3–4 h, cooled, and the absorbance was determined at 532 nm.

Analysis of putrescine content. Putrescine content was determined as described elsewhere (Seiler & Lamberty, 1973), modified as follows (De Mello *et al.*, 1976). DAB-treated and untreated parasites were washed and incubated with 5% perchloric acid. Precipitated proteins were removed by centrifugation and 400 μl 112 mM dansyl chloride solution and 20 mg sodium carbonate were added to the supernatants. Samples were incubated overnight at room temperature protected from light and then the reaction was quenched by addition of 100 μl of an aqueous proline solution at 100 mg ml⁻¹. Dansyl derivatives were extracted in benzene and dansylated putrescine was separated by thin-layer chromatography in silica gel using benzene/cyclohexane/methanol (85:15:2, by vol.) as solvent. A second separation in the same direction was performed using ethyl ether/cyclohexane (90:10, v/v). Fluorescent dansylated PAs were identified under UV light. The putrescine spot was removed and extracted in dioxane and its concentration determined spectrofluorimetrically (365 nm excitation and 550 nm emission). This method accurately measured <10 nmol dansylated products.

Ornithine decarboxylase activity. Parasites were washed and stored at -20 °C in 20 mM Tris/HCl pH 7.1, 50 μM Na₄EDTA, 50 μM DTT and 50 μM pyridoxal phosphate until use. Samples were lysed by freeze-thaw cycles and incubated in the presence of L-[1-¹⁴C]ornithine hydrochloride (0.12 μCi ml⁻¹; 4.4 kBq ml⁻¹) for 15–90 min at 37 °C. Reaction was terminated by the addition of 10% trichloroacetic acid and the resulting ¹⁴CO₂ was adsorbed to 1 M benzethonium hydroxide (Sigma) at 37 °C for 30 min. ODC activity was determined by liquid scintillation. For reversibility assays the parasites were washed to remove the analogue before lysis.

Putrescine uptake. DAB-treated and untreated promastigotes were collected by centrifugation at 3000 g for 10 min, resuspended in PBS at a concentration of 10⁸ cells ml⁻¹ and incubated with 2.5 mM putrescine, containing [1,4(n)-³H]putrescine dihydrochloride (1 μCi ml⁻¹; 37 kBq ml⁻¹) for 15–60 min at 28 °C. Cells were then extensively washed in ice-cold PBS to remove non-incorporated putrescine, collected in MilliQ water and ruptured by three freeze-thaw cycles. Supernatant samples spotted onto fibreglass filters (Millipore AP25) were air-dried and the radioactivity measured by liquid scintillation. In all experiments the uptake of putrescine by cells was also measured at 0–4 °C. Under this condition the incorporation of the tritiated compound was inhibited by over 85% compared to the incorporation observed at 28 °C; this level of incorporation was considered as non-specific adsorption of the diamine to the parasite. The net putrescine taken up by the cells was calculated by subtracting the radioactivity observed at 0–4 °C from that at 28 °C.

Infection assays. DAB-treated and untreated parasites and macrophages were left in contact at 35 °C in a 10:1 parasite:macrophage ratio. Macrophage monolayers were incubated with 100 μM DAB prior to or after infection. Coverslips were collected after 1–96 h infection, rinsed in PBS, fixed in Bouin's fixative and stained with Giemsa. The percentage of infected macrophages was determined by counting 1000–2000 cells on each preparation. The association index (AI) was determined by multiplying the percentage of infected cells by the mean number of parasites per cell.

Statistical analysis. Comparisons between groups were done by the unpaired Student's *t*-test or one-way analysis of variance (ANOVA) and a posteriori Tukey's tests, by use of Prism 4.0 software (GraphPad). For all tests, differences of *P*<0.05 were considered significant.

RESULTS

Promastigote proliferation

The putrescine analogue DAB caused a dose-dependent growth inhibition of *L. amazonensis* promastigotes (Fig. 1), with an IC₅₀ of approximately 144 μM and up to threefold increase in the generation time. Addition of 10 mM exogenous putrescine or spermidine to the culture medium restored parasite growth at micromolar but not millimolar DAB concentrations, which led to the irreversible loss of viability of most cells (data not shown). Mass effects of excess DAB cannot be ruled out. Growth inhibition induced by DAB was also reversed by analogue removal up to 72 h (data not shown).

Parasite putrescine levels

Incubation of parasites with micromolar DAB concentrations for 24 h did not significantly alter (<5% reduction) the putrescine content, which was about 169.2 ng (mg protein)⁻¹, whereas 10 mM caused a significant reduction (46%, *P*=0.0009, ANOVA) in putrescine content (not shown).

The diminished intracellular putrescine pool could be explained by inhibition of its biosynthesis or transport. Therefore we assayed the parasite ODC activity and [³H]putrescine uptake in the presence or absence of DAB.

Parasite ODC activity

Promastigote ODC activity in homogenates was fully inhibited by 100 μM DAB; the inhibition was reversible, as removing the drug by washing the parasites entirely restored ODC activity (Fig. 2). Nevertheless, promastigotes treated with 100 μM DAB displayed putrescine contents quite similar to untreated cells, possibly due, at least in part, to exogenous putrescine uptake. Therefore we assayed putrescine uptake by parasite cells.

Putrescine transport

Promastigotes grown in Warren's medium and exposed to 10 mM DAB for 24 h displayed remarkably inhibited [³H]putrescine uptake (Fig. 3). However, promastigotes pretreated with 100 μM DAB for 24 h showed 40–50% enhanced putrescine uptake (Fig. 3A). Similarly, 10 mM DAB blocked putrescine uptake by axenic amastigotes, whereas preincubation of parasites with 100 μM DAB increased putrescine incorporation at least twofold (Fig. 3B). This compensatory effect was blocked by protein synthesis antagonists in both developmental forms (not shown).

Cellular organization

Transmission electron microscopy of promastigote cells revealed that compared to untreated controls (Fig. 4A), DAB-treated parasites showed severe damage of the

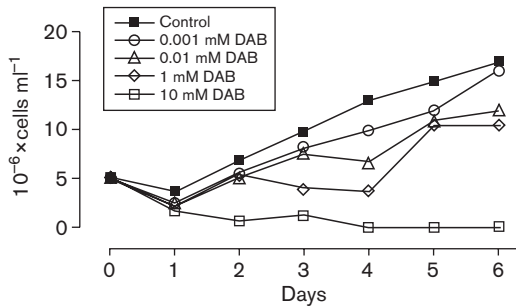


Fig. 1. Effects of DAB on *L. amazonensis* proliferation. Promastigotes were grown in the presence or absence of increasing DAB concentrations. Cell densities were determined daily by light microscopy.

mitochondrion-kinetoplast complex (Fig. 4B–F). The organelle displayed a fenestrated (Fig. 4B) or swollen appearance with a washed-out matrix and disarrangement of the cristae architecture (Fig. 4D, E), culminating in complete mitochondrial destruction. The kinetoplast sometimes showed disorganized k-DNA (Fig. 4C). Myelin-like figures were often observed within cytoplasmic vesicles (not shown) and the flagellar pocket in DAB-treated parasites (Fig. 4F). Vacuoles containing electron-dense material were often observed (not shown).

Lipid peroxidation

Since mitochondria are the main reactive oxygen species (ROS)-producing cellular compartment, we investigated whether the mitochondrial damage was associated with

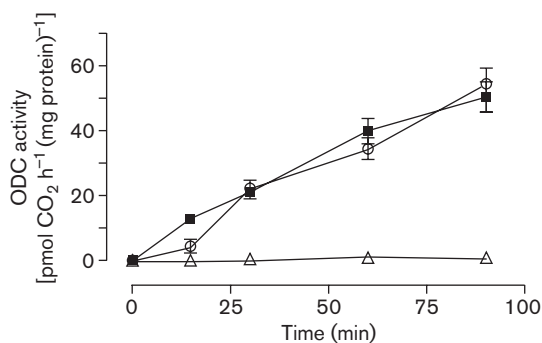


Fig. 2. ODC activity in DAB-treated and untreated parasites. Promastigote homogenates were incubated in the presence of [14 C]ornithine for 60 min at 37 °C and the resulting 14 CO $_2$ determined by liquid scintillation, as described in Methods. DAB (100 μ M) added during the assays with untreated parasite lysates totally blocked ODC activity (Δ). Parasites pretreated with 100 μ M DAB for 60 min and washed to remove the analogue resulted in enzymic activity (\circ) similar to control samples (\blacksquare), indicating that the inhibition is readily reversible. Data are presented as means \pm SD of at least three independent experiments.

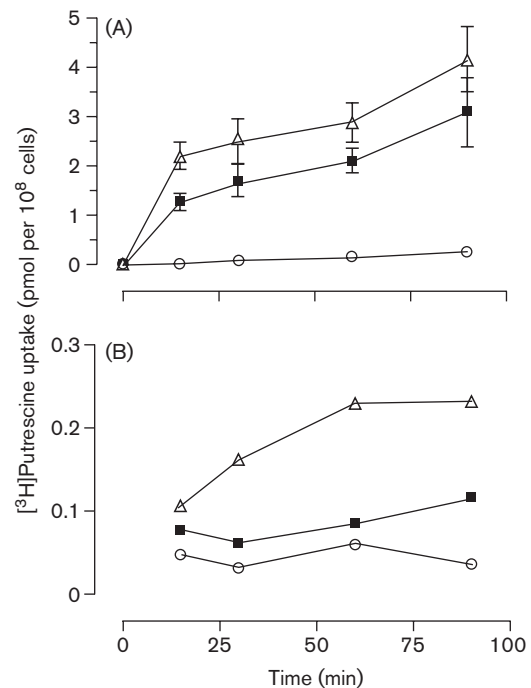


Fig. 3. Putrescine transport in *L. amazonensis* parasites. Promastigotes (A) and amastigotes (B) were incubated with [3 H]putrescine in the presence (\circ) or absence (\blacksquare) of 10 mM DAB for different periods of time at 28 °C and incorporated radioactivity was measured by liquid scintillation, as described in Methods. Parasites, particularly amastigote forms, pretreated with 100 μ M DAB for 24 h (Δ) showed increased putrescine uptake. Data in (A) are presented as means \pm SD of at least three independent experiments; data in (B) are representative of at least three independent assays.

increased production of free radicals. Lipid peroxidation, assessed by production of thiobarbituric acid reactive substances (TBARS), was remarkably enhanced following 3 h DAB treatment (Fig. 5A), whereas 24 h incubations led to significantly diminished peroxidation (Fig. 5B). Furthermore, putrescine addition was able to prevent short-term 1 mM DAB effects, but not 10 mM or 24 h-induced inhibition.

Mitochondrial function

In order to determine whether such differential effects could be attributed, at least in part, to destruction of mitochondria, we examined mitochondrial function by MTT reduction. DAB treatment for 24 h decreased mitochondrial function dose-dependently by up to nearly 80% (Fig. 6). MTT reduction by macrophages was not affected by DAB treatment (not shown).

Host cell infection

Aiming to verify whether putrescine uptake/synthesis are involved in leishmanial intracellular survival we incubated

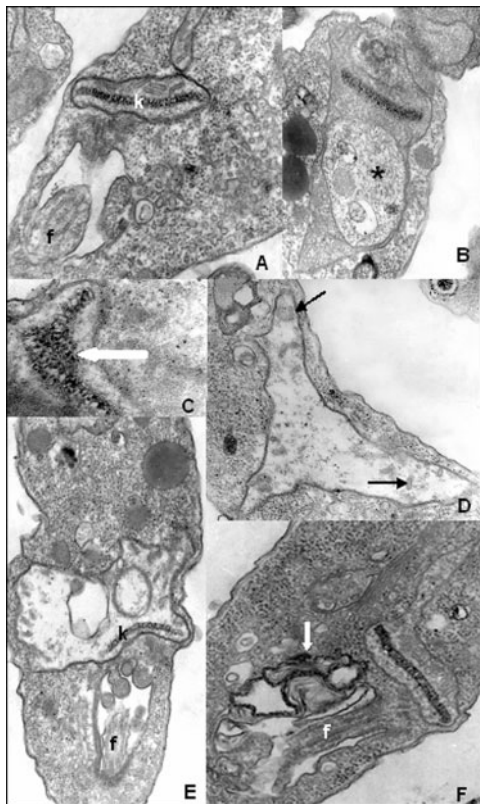


Fig. 4. Ultrastructural alterations induced by 24 h DAB treatment in *L. amazonensis* promastigotes. In contrast to untreated control *L. amazonensis* promastigotes (A), which displayed the usual appearance of organelles such as mitochondrion–kinetoplast complex (k) and flagellar pocket with flagellum (f), the DAB-treated parasites showed mitochondrial alterations (B–E) such as fenestration (B, *) and kinetoplast (k) DNA disorganization (C, arrow). In advanced stages, many parasites showed complete mitochondrial destruction (D, E) and the organelle was only recognized by the presence of scarce remains of the cristae (D, arrows) and k-DNA (E, k). Myelin-like figures were observed in the cytoplasm and associated with the flagellar pocket (F, arrow). Magnification: A and D, $\times 75\,000$; B $\times 40\,000$; C $\times 87\,000$; E, $\times 33\,500$; F, $\times 71\,000$.

infected macrophages with 100 μM DAB for 72 h. The putrescine analogue significantly ($P < 0.05$) reduced parasite load *in vitro*, as the AI of 583.0 in controls dropped to 127.9 after incubation with the analogue, but it did not affect parasite entry during the first 60 min (not shown). Interestingly, DAB concentrations of up to 10 mM did not affect spreading pattern or MTT reduction by macrophages (not shown), suggesting a selective *in vitro* action upon the host cell.

DISCUSSION

PA synthesis inhibitors have been shown to have antiparasitic (Bacchi & Yarlett, 2002) and particularly

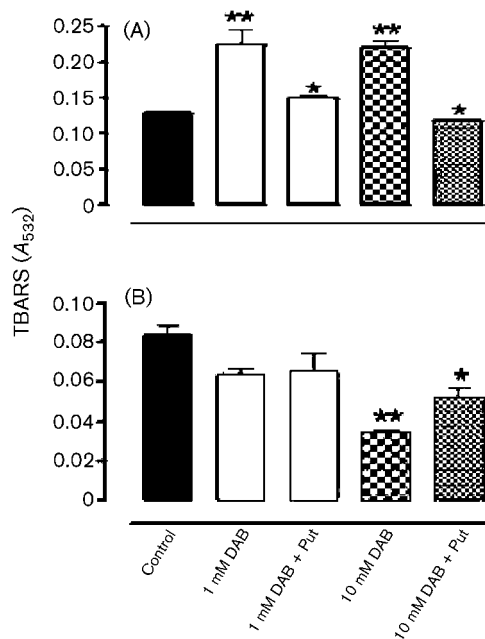


Fig. 5. Lipid peroxidation in untreated and DAB-treated *L. amazonensis*. Lipid peroxidation was measured by detection of thiobarbituric acid reactive substances (TBARS). (A) Incubations with 1 or 10 mM DAB for 3 h significantly enhanced TBARS concentration, and putrescine addition returned the peroxidation to control levels. (B) DAB incubations for 24 h significantly diminished peroxidation, and putrescine addition only partially reversed this effect. Data in (A) are presented as means \pm SD of at least three independent experiments; data in (B) are representative of at least three independent assays. * $P < 0.05$; ** $P < 0.001$.

antileishmanial activities (Baumann *et al.*, 1991; Balaña-Fouce *et al.*, 1991; Mukhopadhyay & Madhubala, 1995). The putrescine analogue DAB has been tested in different micro-organisms (Calvo-Méndez *et al.*, 1993; Arteaga-Nieto *et al.*, 1996; Reis *et al.*, 1999; Garcia *et al.*, 2005; Menezes *et al.*, 2006) and was effective against DFMO-resistant parasites (Calvo-Méndez *et al.*, 1993; Reis *et al.*, 1999). DAB has also been shown to be a powerful ODC inhibitor in a number of organisms (Calvo-Méndez *et al.*, 1993; Arteaga-Nieto *et al.*, 1996). In our study, DAB displayed a dose-dependent reversible antiproliferative activity *in vitro* that was competitively reversed by the addition of exogenous putrescine or spermidine. Moreover removal of the analogue readily restored ODC activity. Similarly PAs can modulate murine ODC activity post-transcriptionally (van Daalen Wetters *et al.*, 1989). Although 100 μM DAB completely blocked ODC activity in *L. amazonensis* it had no effect on cellular putrescine contents. Moreover, 10 mM DAB reduced the diamine concentration in parasite cells by only 46%. The different sensitivities of ODC and whole cells may be due, at least in part, to the presence of PAs in the culture medium and/or inefficient DAB uptake.

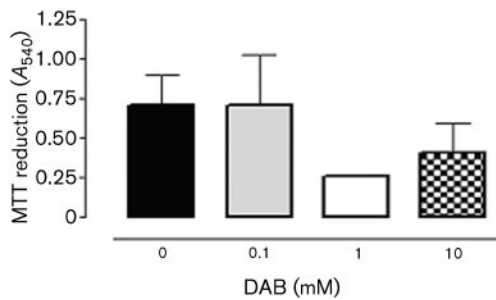


Fig. 6. Effect of DAB on mitochondrial function in *L. amazonensis*. Promastigotes ($5 \times 10^6 \text{ ml}^{-1}$) were treated with increasing DAB concentrations for 24 h and incubated with MTT. Formazan precipitation was determined spectrophotometrically at 540 nm, as described in Methods. DAB significantly decreased mitochondrial activity. Data are presented as means \pm SD of at least three independent experiments.

The antiproliferative effect of DAB may be due, at least in part, to destruction of mitochondria as reported for spermine analogues (Stevens *et al.*, 1977), consistent with the observed reduction in O_2 consumption in DFMO-treated *Trypanosoma brucei* (McCann *et al.*, 1981) and with gross mitochondrial ultrastructural alterations in DAB-treated *T. cruzi* (Menezes *et al.*, 2006). We have previously shown that DAB leads to the destruction of the trichomonad redox organelle (Reis *et al.*, 1999), suggesting a conserved mode of action of DAB in unrelated parasitic protozoa.

Antimicrobial compounds targeting mitochondria are valuable targets for chemotherapy (Kita *et al.*, 2003) and may be particularly relevant against trypanosomatid parasites, in which the glycolytic pathway depends on a cyanide-insensitive glycerol-3-phosphate oxidase system in the inner mitochondrial membrane (Clarkson *et al.*, 1989). In addition, pentamidine resistance in *Leishmania* involves drug extrusion from the mitochondria (Basselin *et al.*, 2002). In this regard, the PA transporter LmPOT1 of *Leishmania major* is inhibited by pentamidine (Hasne & Ullman, 2005). In addition putrescine may stabilize *T. cruzi* mitochondrial membranes (Giffin *et al.*, 1986).

PA analogues do inhibit leishmanial proliferation and cause mitochondrial potential changes and modifications compatible with apoptosis (Tavares *et al.*, 2005). Several lines of evidence indicate that oxidative stress is associated with mitochondrial disruption in several species. Interestingly the bis-naphthalimidopropyl putrescine analogue was the most active PA derivative against *Leishmania*, particularly in the amastigote form. Mitochondrial enlargement and disruption of internal membrane and cristae were also reported in DFMO-treated *Trypanosoma rhodesiense* (De Gee *et al.*, 1984). Therefore, ROS generation in the mitochondrion might explain the kDNA disorganization observed here, since these organic cations protect the DNA from oxidative species (Khan *et al.*, 1992). Moreover,

as PAs downregulate lipid peroxidation induced by pro-oxidant compounds (Tadolini, 1988; Bellé *et al.*, 2004) the enhanced peroxidation described above, assessed by TBARS production, probably results from increased ROS in the mitochondrion. The diminished lipid peroxidation observed after treatment with 10 mM DAB might be due to mitoptosis, or 'programmed mitochondrial destruction' a process in which the ROS-producing mitochondria are selected for destruction as an antioxidant defence (Skulachev, 1998). Interestingly, this DAB concentration did not significantly affect macrophage viability (not shown). The MTT reduction by mitochondria was markedly inhibited by DAB, corroborating the respiratory activity loss and the mitochondrial swelling observed here, which may lead to disruption of the organelle. After destruction, the mitochondria may be degraded by autophagy (reviewed by Skulachev, 1998) and therefore lipid peroxidation would not be detectable. Spermine and spermidine, but not putrescine, can reverse lipid peroxidation in *T. cruzi* (Hernandez *et al.*, 2006). Therefore the effects of DAB may be due, at least in part, to inhibition of the conversion of putrescine to spermidine, which is required for the synthesis of the major antioxidant molecule of trypanosomatid parasites, trypanothione [N^1, N^8 -bis(glutathionyl)spermidine].

The formation of myelin-like figures from endoplasmic reticulum cisternae may be indicative of autophagy. It is reasonable to suppose that parasites may undergo necrotic death before apoptosis, since no ultrastructural evidence of apoptosis was observed here. Another possibility is that accumulation of DAB by the parasite may lead to the production of H_2O_2 as a result of oxidation; nevertheless it is not known whether DAB can be a substrate for amine oxidases (Calcabrini *et al.*, 2002).

The appearance of vesicles with an electron-dense core, presumably acidocalcisomes, may result from microbicidal drug-induced autophagy (Vannier-Santos *et al.*, 1995, 1999) and it was reported that this organelle may have a lysosome-related origin in *Leishmania* amastigotes (Zhang *et al.*, 2005).

Putrescine is by far the most common PA/diamine in *Leishmania*, while trace amounts of spermine have been detected (Balaña-Fouce *et al.*, 1991). Its uptake is stage- and cell cycle-regulated (Gonzalez *et al.*, 1992), suggesting its importance in cell growth and differentiation. Interestingly, although not essential, addition of putrescine to ODC-deficient *L. donovani* mutants restores parasite growth more rapidly and to a greater extent than PAs such as spermidine (Jiang *et al.*, 1999). Furthermore the addition of this diamine leads to higher concentrations of trypanothione.

DAB significantly inhibited both ODC activity and putrescine uptake. This analogue acts as an ODC inhibitor in other parasitic protozoa (Calvo-Méndez *et al.*, 1993; Arteaga-Nieto *et al.*, 1996) but the mechanism has not been elucidated. It is known that, unlike mammalian cells, PAs

only slightly downregulate *Leishmania* ODC activity (Mukhopadhyay & Madhubala, 1995) and the enzyme was unaffected by up to 1 mM putrescine (Hanson *et al.*, 1992). *Leishmania* parasites not only synthesize putrescine and spermidine, but also accumulate extracellular PAs via specific transport systems. Energy-dependent putrescine and spermidine transporters have been described in *L. donovani*, *L. mexicana* (Basselin *et al.*, 2000) and *L. major* (Hasne & Ullman, 2005). PA transport in protozoa may furnish potential targets for parasitic disease chemotherapy (Reguera *et al.*, 2005). A PA permease was identified and characterized in *L. major* (Hasne & Ullman, 2005). These authors showed that the protein, termed LmPOT1, transports putrescine and spermidine, and transfected in *T. brucei* can stimulate putrescine uptake. Conceivably, DAB enters the cell via a putrescine transporter; this would explain inhibition of putrescine uptake by competition with an excess of the analogue. Nevertheless, long-term pre-treatment with DAB (24 h) increased the capacity of putrescine uptake twofold when cells were incubated with the amine after DAB removal, in a protein synthesis-dependent manner. Similarly, upregulation of PA transport was observed after DFMO treatment in *L. donovani* (Kandpal & Tekwani, 1997) and human cells (Redgate *et al.*, 2001) and may provide an alternative PA source in deprived cells. Interestingly DFMO induced a tenfold increase in putrescine transport in *L. infantum* (Balaña-Fouce *et al.*, 1991). Such compensatory homeostatic mechanisms must be considered in the search for PA analogues for antileishmanial chemotherapy.

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