Pentamidine exerts in vitro and in vivo anti Trypanosoma cruzi activity and inhibits the polyamine transport in Trypanosoma cruzi

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ABSTRACT

Pentamidine is an antiprotozoal and fungicidal drug used in the treatment of leishmaniasis and African trypanosomiasis. Despite its extensive use as an antiparasitic drug, little evidence exists about the effect of pentamidine in Trypanosoma cruzi, the etiological agent of Chagas’ disease. Recent studies have shown that pentamidine blocks a polyamine transporter present in Leishmania major; consequently, its might also block these transporters in T. cruzi. Considering that T. cruzi lacks the ability to synthesize putrescine de novo, the inhibition of polyamine transport can bring a new therapeutic target against the parasite. In this work, we show that pentamidine decreases, not only the viability of T. cruzi trypanomastigotes, but also the parasite burden of infected cells. In T. cruzi-infected mice pentamidine decreases the inflammation and parasite burden in hearts from infected mice. The treatment also decreases parasitemia, resulting in an increased survival rate. In addition, pentamidine strongly inhibits the putrescine and spermidine transport in T. cruzi epimastigotes and amastigotes. Thus, this study points to reevaluate the utility of pentamidine and introduce evidence of a potential new action mechanism. In the quest of new therapeutic strategies against Chagas disease, the extensive use of pentamidine in human has led to a well-known clinical profile, which could be an advantage over newly synthesized molecules that require more comprehensive trials prior to their clinical use.

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1. Introduction

American trypanosomiasis (Chagas’ disease) is a parasitic disease caused by the flagellate protozoan Trypanosoma cruzi (Chagas, 1909). Chagas’ disease is a major concern in Latin America, where it is the vector-borne disease with the second highest prevalence and mortality, after malaria (WHO/NTD et al., 2010). Regional initiatives and vector control strategies have succeeded in reducing the incidence of the disease. Nevertheless, nearly 8 million people are infected in South America, with approximately 40,000 new cases recorded per year (Reithinger et al., 2009; World Health Organization, 2013). Moreover, migration of people from endemic countries to developed countries has expanded the risk of infection, particularly through blood and organ donation, because these countries have no controls in place to detect the parasite in blood banks. As a result, it has been found in immigrant populations in countries such as Japan, Australia and, with a high prevalence, in Spain and the United States, transforming this disease into an emerging worldwide problem (Hotz et al., 2012).

The only drugs with proven efficacy against Chagas’ disease are nifurtimox and benznidazole. Benznidazole is commonly used as a first-line treatment for the disease; however, despite its long-lasting use, the first international multicenter trial was only recently initiated to study the safety and efficacy of this drug during the chronic phase (Marin-Neto et al., 2009).

Novel synthesized molecules and many approved drugs with potential trypanocidal action have been tested, both alone and in combination with the classic antichagasic drugs. These molecules include nitroheterocyclic derivatives, quinines, bithionine sulfoximine, and clinically used drugs such as allopurinol, phenothiazines, imidazole antifungals and analogs such as ketoconazole (Cerecetto and Gonzalez, 2008; Faundez et al., 2005, 2008; Maya et al., 2007). Some of them, for reasons such as natural resistance, solubility, toxicity, and inefficacy, have shown to be no better than nifurtimox or benznidazole. Others, such as posaconazole and...
itraconazole, have good perspectives in the safety and efficacy profile in experimental [Diniz Lde et al., 2013] or human Chagas disease [Apt et al., 2013]. However, the first multi-center clinical trial to assess the efficacy of posaconazole in human Chagas disease is just in recruitment status (Merck Sharp and Dohme, 2011). Thus, studies focusing on new trypanocidal strategies are still necessary.

Pentamidine (4,4’-[(pentane-1,5-diyli)bis(oxy)]dibenzencarboximide), an aromatic diamidine, is classified as a broad-spectrum antiparasitic drug and has been used for decades against several trypanosomatids, such as Leishmania major and Trypanosoma brucei and some fungi such as Pneumocystis jirovecii (Wilkinson and Kelly, 2009). Yorke and co-workers discovered this drug in the late 1930s. In Yorke’s work, pentamidine was reported to be inactive against T. cruzi (Yorke, 1940). Despite its extensive use as an antiparasitic drug, very little evidence exists about the potential anti-T. cruzi activity of pentamidine. In addition, most of the available evidence is based only on studies of epimastigote viability (Chan-Bacab et al., 2009; Gonzalez et al., 2007; Navarrete-Vazquez et al., 2011). Thus, evidence about the effect of this drug in the human pathological forms of the parasite is lacking.

Pentamidine is associated with several alterations in polyamine transport in trypanosomatids. Recently, it was reported that pentamidine blocks the polyamine transporter LmPOT1, which is found in L. major. LmPOT1 has approximately 55% structural similarity with TcPOT1 (also called TcPOT1), its T. cruzi ortholog, which transports putrescine and spermidine. Within the similar sequences, there are two conserved residues critical for putrescine recognition (Carrillo et al., 2006; Hasne et al., 2010).

In trypanosomes, polyamines are involved in crucial cellular processes, including the synthesis of the antioxidant compound trypanothione (bis-gluthionyl spermidine), which is found exclusively in trypanosomatid protozoa. Due the lacking of ornithine decarboxylase (the rate-limiting step in the eukaryotic synthesis of putrescine) (Carrillo et al., 1999), the intracellular availability of putrescine in T. cruzi depends exclusively on transport processes. Thus, the inhibition of this process can alter the viability of the parasite.

In this work, we evaluated the activity of pentamidine in in vitro and in vivo models of chagasic infection and its effect on polyamine transport in T. cruzi and assessed its potential role in antichagasic therapy.

2. Materials and methods

2.1. Parasites

Stock cultures of T. cruzi epimastigotes (Y and Dm28c strains, DTUs: T. cruzi II and I, respectively) were maintained in axenic conditions at 28 °C in BHT (brain-heart–tryptose) media (pH 7) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 mg/L streptomycin (Camargo, 1964). Trypanostigotes were obtained from infected VERO cells (Chlorocebus sabaensis kidney fibroblasts, obtained from ATCC, ATCC number: CCL-81). Cells were exposed to trypanostigotes (Y and Dm28c strains) at a 3:1 density (trypanostigote:cell). Trypanostigotes were allowed to infect cells for 24 h, after which the supernatant was extracted. Trypanostigotes and amastigotes were released from VERO cells after 4 and 10 days of infection, respectively. The parasites were harvested and collected for viability assays. By microscopic examination, we ensured that the culture media was enriched with at least 80% of the respective forms indicated in the text. Each culture-derived parasite stage was also confirmed by different criteria such as motility, cell morphology and the position of the kinetoplast respect to the nucleus (Camara et al., 2013).

2.2. Polyamine transport assays

Aliquots of T. cruzi (Y strain) epimastigotes or amastigote cells (10^7 parasites) were centrifuged at 8000 × g for 30 s and washed once with phosphate-buffered saline supplemented with 2% (w/v) glucose (PBS-G). The cells were then resuspended in 0.2 mL of PBS—G containing 5 μM [14C]-putrescine or 1 μM [3H]-spermidine (NEN/DuPont, Boston, MA, USA; 0.4 μCi). Following incubation for 5 min at 28 °C, the cells were centrifuged at 8000 × g for 30 s and washed twice with 1 mL of ice-cold PBS—G. The pellets were then resuspended and radioactivity was measured using an UltimaGold XR liquid scintillation cocktail. Non-specific transport and carry over were measured in transport mixtures containing 10 mM putrescine or spermidine (Pereira et al., 1999). Pentamidine competition assays were performed via parasite incubation with the standard transport mixture containing polyamine concentrations close to the previously reported K_m values, as it is the most variable range of concentrations in terms of transport velocity, and a 10-fold excess of pentamidine, as previously described (Le Quesne and Fairlamb, 1996). All assays were run at least in triplicate, and cell viability was assessed by direct microscopic examination and flow cytometry with propidium iodide staining, under the same conditions as the transport experiments (data not shown).

2.3. Viability measurement by tetrazolium reduction assay

The effect of the drug on parasite viability was evaluated through the tetrazolium salt (MTT) reduction assay (Mosmann, 1983). Briefly, 10 μL of 5 mg/mL MTT dye (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) plus 0.22 mg/mL phenazine methosulfate (electron carrier) were added to each well containing 10^6 parasites (Y or Dm28c strain) in 100 μL of RPMI 1640 without phenol red. After incubation for 4 h at 37 °C, the generated formazan crystals were dissolved with 100 μL of 10% (w/v) SDS in 0.1 M HCl. The plates were incubated overnight at 37 °C, and the optical density (OD) was determined using a microplate reader (Labsystems Multiskan MS, Finland) at 570 nm. Under these conditions, the OD is directly proportional to the viable cell number in each well. All experiments were performed at least three times, and the data are shown as the means and their standard deviations from triplicate cultures.

2.4. Flow cytometry cell death assays

For these assays, 10^7 trypanostigotes (Y or Dm28c strain) were exposed to pentamidine for 24 h in RPMI at 37 °C and 5% CO_2. Then, the cells were washed in PBS, resuspended and incubated with propidium iodide (PI) and Annexin-V labeled with Alexa Fluor 488. The incubation was performed using the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Molecular Probes, USA) following the manufacturer’s instructions. The fluorescence of the samples was measured using a FACSArray-III flow cytometer (BD Biosciences, USA), using 530/30 and 630/22 filters to detect Annexin-V and PI, respectively. For each sample, 5000 events were recorded, and the data were analyzed using FACSData 6.1 software.

2.5. DAPI stain and intracellular amastigote quantification

VERO cells were exposed to T. cruzi trypanostigotes (Y strain) at a 5:1 ratio (trypanostigotes:cells) for 24 h. Then, the cells were washed and treated with different compounds for 48 h. The cells were washed and fixed in cold methanol (70%) overnight. The fixed cells were then washed, and 1 mL of PBS (pH 7.4) was added. The DNA was stained with DAPI (NucBlue, Molecular Probes, USA) following the manufacturer’s instructions. The cells were photographed using a Nikon Eclipse 400 fluorescence microscope, using
358 nm (excitation) and 461 nm (emission) wavelengths. Five pictures were obtained per well, and each picture was counted by two independent researchers.

2.6. Effect of drugs on infected RAW 264.7 cells

The effect of drugs on T. cruzi-infected RAW 264.7 cells was assessed by the number of trypomastigotes released to the culture supernatants. Twenty-four hours after infection treatments were started at concentrations described in the results section. Every 24 h culture media was removed and fresh medium was added together with the drugs at the same concentrations. Cell culture medium was harvested at the third day of treatment and centrifuged at 500 × g during 5 min. Supernatants were discarded, pellets resuspended in 1 mL of fresh RPMI 1640 and trypomastigotes were counted using direct microscopy (Freire-de-Lima et al., 2000; Nunes et al., 1998).

2.7. In vivo infection model

All animal handling protocols were performed according to the “Guide for the Care and Use of Laboratory Animals,” from the National Institutes of Health, USA (National Research Council (US), Committee for the Update of the Guide for the Care and Use of Laboratory Animals et al., 2011), and were approved by the Institutional Ethical Committee at the Faculty of Medicine, University of Chile (Protocol CBA# 0448 FMUCH) in association with FONDECYT-Chile grant number 11110182. All efforts were made to minimize animal suffering.

Adult male BALB/c mice (20–25 g) were obtained from the Animal Facility at the Faculty of Medicine, University of Chile. The animals were first infected intraperitoneally with 30,000 T. cruzi bloodstream trypomastigotes (Y strain). Thereafter, the animals were randomized to receive the different treatments. T. cruzi infection was assessed by parasitemia through the direct microscopic visualization of circulating trypomastigotes in the peripheral blood, as previously described (Bustamante et al., 2007; Huang et al., 2002). This model of acute infection implies the evaluation of the rate of survival as main endpoint, as previously described (Croft and Brun, 2003; Romana et al., 2010). Mice were monitored for survival daily, and signs of distress were recorded following a rated surveillance protocol approved by the Institutional Ethical Committee at the Faculty of Medicine, according to NIH, USA guidelines (National Research Council (US), Committee for the Update of the Guide for the Care and Use of Laboratory Animals et al., 2011). Animals obtaining the highest scores (15–20 points) were euthanized. For this, mice were anesthetized with and intraperitoneal administration of a mixture of ketamine (85 mg/kg) and xylazine (30 mg/kg) and euthanized by cervical dislocation. Those animals reaching a score equivalent to 10–14 points, were placed on acetaminophen treatment to ameliorate suffering. To complete the qPCR and histology analyses, surviving mice at day 25 post-infection had to be euthanized.

2.8. Real-time PCR

Infected RAW cells (murine macrophages, ATCC number: TIB-71) and hearts from infected animals were homogenized, and DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, USA) following the manufacturer’s instructions. DNA was quantified through 280 nm absorbance measurements using a Varioskan spectrophotometer (Thermo Scientific, USA). Parasite DNA quantification was performed using the primers TCZ-1 and TCZ-2, which were designed to amplify a 195 bp Satellite-DNA sequence of T. cruzi (Cummings and Tarleton, 2003). We used the TNFα-5241 and TNFα-5411 primers, which amplify a 170 bp sequence of the Mus musculus TNF-α gene as a loading control.

### Table 1: Effect of pentamidine on the viability of T. cruzi trypomastigotes

<table>
<thead>
<tr>
<th>IC₅₀ (µM)</th>
<th>Y</th>
<th>Dm28c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentamidine</td>
<td>2.8 ± 0.7</td>
<td>15.2 ± 3.2</td>
</tr>
<tr>
<td>Nifurtimox</td>
<td>0.9 ± 0.7</td>
<td>3.4 ± 2.3</td>
</tr>
<tr>
<td>Benznidazole</td>
<td>3.8 ± 1.8a</td>
<td>43.3 ± 21.3a</td>
</tr>
</tbody>
</table>

*a p < 0.05, when compared with nifurtimox, p value was calculated by one-way ANOVA and Tukey post-test.

(Caldas et al., 2012; Cummings and Tarleton, 2003). PCR amplifications were carried out in a 7300 Real-Time PCR System (Applied Biosystems, USA). All reactions were performed using 10 ng of DNA and the SensiMix SYBR Hi-ROX Kit (Bioline, UK) at a final volume of 20 µL.

2.9. Cardiac tissue histology

Hearts were extracted at the moment of death from those mice dead before the end point. Surviving mice were euthanized at day 25 p.i. and their hearts were extracted. Hearts were longitudinally sectioned to further analysis by histopathology and qPCR. Samples were fixed in 10% formaldehyde in 0.1 M phosphate buffer (pH 7.3) for 24 h, dehydrated in alcohol, clarified in xylene, and embedded in paraffin. Then, 5 µM sections were obtained and stained with hematoxylin–eosin for routine histological analysis and to evaluate the presence of T. cruzi amastigote nests and inflammation of the myocardium (Duaso et al., 2010; Faundez et al., 2008).

2.10. Statistical analysis

For all experiments, the statistical significance was established at p < 0.05. Results represent mean ± SD of triplicates. All statistical analyses were performed using GraphPad Prism (5.0) software. Normal distribution of data was assessed using D’Agostino-Pearsons analysis. One-way ANOVA (with Tukey post-test) or t-test analysis were performed when required. For survival analysis, the log-rank (Mantel Cox) test was performed.

3. Results

3.1. Pentamidine decreases viability in isolated trypomastigotes

To assess the effect of pentamidine on the viability of the infectious form of T. cruzi, we exposed isolated trypomastigotes from Y and Dm28c strains (TeI and Tcl lineages, respectively) (Sturm et al., 2003) to pentamidine for 24 h and compared the effect with the standard antischagics drugs nifurtimox and benznidazole. As shown in Table 1, pentamidine was active against the two T. cruzi strains assayed, with similar potency compared with nifurtimox and benznidazole. In this setting, in both strains pentamidine was more potent than benznidazole, but less than nifurtimox. To corroborate the effect of pentamidine on parasite viability, we followed death markers by flow cytometry after exposing trypomastigotes of Dm28c and Y strain to pentamidine for 24 h. For Y strain parasites, pentamidine 5 µM decreased cell viability from 88.7 ± 1.5% (control) to 18.2 ± 0.4% (Fig. 1A). On the other hand, in agreement with the MTT experiments, Dm28c parasites were more resistant to pentamidine. Pentamidine 20 µM decreased the viability of these parasites from 85.4 ± 4.2% (control) to 63.2 ± 4.0% (Fig. 1B).
3.2. Pentamidine decreases the parasite burden in infected cells

We assayed the effect of pentamidine on two models of in vitro infection: VERO cells (monkey fibroblasts) and RAW 264.7 cells (murine macrophages) infected with Y strain trypomastigotes. We treated infected VERO cells with pentamidine, nifurtimox or benznidazole and evaluated the outcome of the infection through fluorescence microscopy, using nuclei stain with DAPI probe. After 48 h of treatment, pentamidine (1 µM), nifurtimox (1 µM), and benznidazole (5 µM) were unable to decrease the percentage of infected cells (Fig. 2A). However, all drugs significantly decreased the number of amastigotes per infected cell (Fig. 2A). We also explored the effect of the drugs upon intracellular amastigotes in infected murine macrophages. By the fourth day of RAW cell infection, trypomastigotes began to be released into the medium, and these parasites could be counted via direct microscopy (Lopez-Munoz et al., 2010). At 1.5 and 3 µM, pentamidine decreased trypomastigote release by 72.8% and 95.5%, respectively. In the same model, benznidazole (1.5 µM) and nifurtimox (1 µM), used as positive controls, decreased trypomastigote release by 84.4% and 90.9%, respectively (Fig. 2C). In addition, in infected RAW cells, we measured the content of parasite DNA per cell using qPCR, after 48 h of treatment, before the parasites started to emerge into the medium. At 0.5 and 1.0 µM, pentamidine significantly decreased the T. cruzi DNA content in RAW cells (Fig. 2B).

3.3. Pentamidine increases the survival of T. cruzi–infected mice

Contrary to the results reported by Yorke (1940), pentamidine demonstrated an interesting effect on mice infected with T. cruzi. Treatment for 10 days with 8 mg/kg/day pentamidine increased the median survival of the infected mice, with a p value of 0.033 with respect to controls calculated by log-rank (Mantel-Cox) test (Fig. 3A). In addition, 8 mg/kg/day pentamidine significantly decreased the peak of parasitemia, characteristic for the day 14 post-infection (Fig. 3B). When we evaluated the cardiac structure of mice (Fig. 4A), infected mice exhibited severe inflammatory infiltration, edema, and amastigote nests. Mice treated with 4 mg/kg/day pentamidine did not present changes in cardiac histology compared with the infected controls, whereas mice treated with 8 mg/kg/day presented less inflammation and edema, and their heart tissue histology appeared normal. In addition, pentamidine 8 mg/kg/day significantly decreased the parasite burden in cardiac tissue (Fig. 4B).

3.4. Pentamidine blocks polyamine transport by T. cruzi

To explore a potential mechanism of action of pentamidine, we assessed whether pentamidine affects polyamine transport in the parasite. Epimastigotes and amastigotes were first assayed for putrescine and spermidine transport in the presence of a single, sub-lethal dose of pentamidine (10-fold molar excess).
Transport of putrescine and spermidine by the different *T. cruzi* stages was found to increase linearly with time (initial velocities) up to 30 min. As shown in Table 2, pentamidine inhibited the transport of putrescine by approximately 85% in epimastigotes and 81% in amastigotes. In addition, pentamidine also inhibited the spermidine uptake by approximately 39% in epimastigotes and

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**Table 2**

<table>
<thead>
<tr>
<th>Polyamine uptake (pmol min⁻¹ 10⁻⁸ cells)</th>
<th>Epimastigotes</th>
<th>Amastigotes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine</td>
<td>17.10 ± 0.97</td>
<td>3.34 ± 0.41</td>
</tr>
<tr>
<td>Putrescine + pentamidine</td>
<td>2.57 ± 0.41</td>
<td>0.62 ± 0.06</td>
</tr>
<tr>
<td>Spermidine</td>
<td>1.70 ± 0.15</td>
<td>1.95 ± 0.13</td>
</tr>
<tr>
<td>Spermidine + pentamidine</td>
<td>1.03 ± 0.12</td>
<td>1.19 ± 0.10</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>1.12 ± 0.09</td>
<td>n.d.</td>
</tr>
<tr>
<td>L-Arginine + pentamidine</td>
<td>1.09 ± 0.11</td>
<td>n.d.</td>
</tr>
<tr>
<td>L-Aspartate</td>
<td>0.68 ± 0.14</td>
<td>n.d.</td>
</tr>
<tr>
<td>L-Aspartate + pentamidine</td>
<td>0.72 ± 0.12</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d.: not determined.

Transport of putrescine and spermidine by the different *T. cruzi* stages was found to increase linearly with time (initial velocities) up to 30 min.

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**Fig. 2.** Pentamidine decreases the parasite burden of infected cells. VERO or RAW cells were infected with *T. cruzi* (Y strain) for 24 h and treated with the different drugs. (A) Quantification of infected cells and intracellular amastigotes per infected cell after 48 h treatment with 1 μM pentamidine (PTM), 1 μM nifurtimox (NFX) or 5 μM benznidazole (BZL). (B) qPCR quantification of parasite load in infected RAW cells treated for 48 h with pentamidine (PTM), nifurtimox (NFX) or benznidazole (BZL). Graph data are expressed as the means ± SD of three independent experiments. *p < 0.05 and **p < 0.001 vs. control, calculated by one-way ANOVA and Tukey post-test. (C) Trypomastigote release by infected RAW cells treated for 72 h with pentamidine (PTM), nifurtimox (NFX) or benznidazole (BZL). Graph data are expressed as the means ± SD of three independent experiments. **p < 0.001 vs. control, calculated by one-way ANOVA and Tukey post-test.

**Fig. 3.** Pentamidine increases survival and decreases parasitemia in *T. cruzi*-infected mice. BALB/c mice were infected with *T. cruzi* trypomastigotes (Y strain) and treated with 4 and 8 mg/kg/day pentamidine intraperitoneal for 10 days. (A) Survival rates of infected and treated mice. Survival was recorded until day 25 post-infection. The graph summarizes two independent experiments with n = 6 each. *p < 0.05 vs. control, calculated by log-rank (Mantel Cox) test. (B) Parasitemia of infected mice (n = 6) treated with 4 or 8 mg/kg/day pentamidine for 10 days. The bars indicate the standard deviation of the means. *p < 0.05 compared with control, calculated by ANOVA.
amastigotes (Table 2). To determine whether pentamidine non-
specifically inhibits all uptake processes, L-arginine and L-aspartate 
transport were evaluated in the presence of pentamidine in epis-
mastigotes, and no significant inhibition was observed (Table 2). 
To further investigate the inhibition kinetics, we calculated the 
half maximal inhibitory concentrations (IC₅₀) of pentamidine, over 
the transport of putrescine and spermidine. The obtained values 
for putrescine and spermidine transport were 25.6 and 14.1 μM, 
respectively (Fig. 5A and B). These results were compared with 
putrescine and spermidine isotopic dilutions using the same con-
centrations as pentamidine, representing the maximum inhibition 
that can be achieved. The calculated IC₅₀ values were 6.1 and 
2.6 μM for putrescine and spermidine, respectively (Fig. 5A and 
B). It is worth to mentioning that pentamidine intake was previ-
ously demonstrated in trypansomadits and mammalian cells by 
De Koning (2001) and Ming et al. (2009), respectively.

4. Discussion

The mechanism of action of pentamidine is not fully understood.
The most frequent action described thus far is the binding of pen-
tamidine to the DNA minor groove of T. brucei kinetoplast, which 
disrupts parasite kDNA. However, the natural and drug-induced 
generation of viable parasites lacking kDNA (dyskinetoplastic try-
panosomes), indicates that the loss of kDNA does not explain the 
killing mechanism of this drug (Schnaufer et al., 2002). In addition, 
assays with large series of diamines failed in find a correlation 
with the trypanocidal activity and the kDNA-binding ability of these 
drugs (Daliry et al., 2011). Thus, additional mechanisms must be 
involved in the trypanocidal action of pentamidine.

Polyamines are biomolecules present in all living cells. In 
mammals, the natural polyamines are putrescine (butane-1,
4-diamine), spermidine [N-(3-aminopropyl)butane-1,4-diamine] 
and spermine [(N,N′-bis(3-aminopropyl)butane-1,4-diamine]. 
These molecules have a wide variety of functions affecting cell 
growth and development, including the regulation of gene tran-
scription, ion channels, protein kinases, cell cycle, and mainte-
nance of nucleic acids and membrane structure and stability. Due 
to this complex spectrum of actions, polyamines are essential for 
cell growth and survival (Pegg and Casero, 2011). In T. cruzi, 
spermidine and putrescine inhibit DNA fragmentation and restore 
proliferation in parasites exposed to human serum (Piacenza 
et al., 2001). In epimastigotes, spermidine has a role in protecting 
the parasite against lipoperoxidation when T. cruzi is exposed to 
oxidative systems, e.g., H₂O₂/FeSO₄ or nitroheterocycle drugs such 
as nifurtimox (Hernandez et al., 2006).

Polyamines can be obtained by synthesis de novo from ornithine, 
in some cases from L-arginine (via agmatine), or by transport from 
the extracellular medium. In contrast with other protozoa, T. cruzi 
is unable to synthesize putrescine de novo, due to the lack of L-
arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) 
(Carrillo et al., 1999). The lack of ODC in T. cruzi explains the 
efficacy of dl-α-difluoromethylornithine (DFMO, a classic ODC 
inhibitor used against African trypanosomiasis), against isolated T. 
cruzi (Wilkinson and Kelly, 2009). However, intracellular amastig-
otes are unable to proliferate in host cells when mammalian ODC is 
inhibited by high concentrations of DFMO, indicating the depend-
ence of this parasite on host polyamines (Kierszenbaum et al., 
1987).

At the molecular level, the polyamine transporter TcPAT12 
has been described and characterized (Carrillo et al., 2006). 
This transporter was expressed and characterized from a mem-
brane putative transporter sequence, previously reported in a T. 
cruzi genome database. This transporter has a high capacity to
transport spermidine ($K_m = 14 \mu M$). In addition, it has the ability to transport putrescine and L-arginine, but at rates 6.7- and 5.4-fold slower, respectively, indicating the high specificity of this transporter for spermidine (Carrillo et al., 2006). This system correlates with the further description of the TcPOT1.1/1.2 transporter, which is encoded by the same gene as TcPAT12 and transports putrescine at high rates (Hasne et al., 2010). In addition, recently was described an approach to the 3D structure of the TcPOT1.1 transporter (Soyza et al., 2013). However, there is not yet crystalline structures that allow know in detail the molecular interactions between the transporter and its ligands or inhibitors.

Aromatic diamidines have been previously studied in its ability to block the polyamine transport in *Leishmania infantum* (Balaña-Fouce et al., 1989; Reguera et al., 1994). In the study made by Reguera et al., 7 diamidine derivatives were studied, of which pentamidine was the most active (Reguera et al., 1994). Our findings correlate with these descriptions and indicate that pentamidine blocks this transport system, inhibiting the internalization of both polyamines but with stronger putrescine inhibition (Table 1). A competitive inhibition mechanism of the polyamine transporter by pentamidine could not be discarded, as we shown in Fig. 5. Under this hypothesis, pentamidine could be co-transported with polyamines presenting a dual mechanism of action; inhibiting polyamine transport and acting as an antimetabolite inside the cell. In fact, pentamidine also inhibit reversibly the AdoMetDC of *T. brucei* (Bitonti et al., 1986), this protein have a 96% of identity with AdoMetDC of *T. cruzi*. Thus, it is probable that the effect of pentamidine is due to more than a specific target.

The use of pentamidine in several parasitic models has led to active research on aromatic diamidines and analogs of pentamidine, which have been demonstrated to be active in vitro against trypanosomatids, including *T. cruzi* (Soeiro et al., 2008). However, our result shows that pentamidine is more potent than some of those novel derivatives assayed (Batista et al., 2010; Borges et al., 2004; de Souza et al., 2011). Furthermore, our results show that pentamidine is similar or better than new aromatic diamidines assayed in *T. cruzi*-infected mice (Batista et al., 2011; da Silva et al., 2008; de Souza et al., 2006; Zhu et al., 2012).

Pentamidine is able to induce adverse events of variable severity, i.e., injection-site pain, abdominal pain, nausea, vomiting, leucopenia, renal and liver abnormalities (Barrett and Croft, 2012; Oliveira et al., 2011). Despite these known adverse effects, it is interesting to notice that this well-known toxicity profile is given by the extensive use of pentamidine in patients since the 1940s decade, in comparison to new molecules from synthesis, that need comprehensive and expensive clinical trials prior to their use in patients. Moreover, in our model, pentamidine decreased parasite viability (Fig. 1) and intracellular growth (Fig. 2), decreased in vivo parasitemia, and most importantly, increased mice survival. Thus, it could be interesting to consider this drug and potentially useful analogs, to be used in a therapeutic approach of Chagas disease treatment, either alone or in combination with the most traditional drugs nifurtimox and benzidazol.

In conclusion, we have shown that pentamidine has antichagasic effects, both in vitro and in vivo, that are likely related to its capacity to block polyamine transport. In this setting, the inhibition of polyamine transport in a parasite auxotroph for putrescine not only suggests a new potential therapeutic target but also makes it possible to study new therapeutic schemes based on new drugs with high synergistic potential.

Transparency declarations

None declared.

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References


