Acta Tropica 134 (2014) 1–9

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

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

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ARTICLE INFO

Article history:
Received 29 July 2013
Received in revised form 17 January 2014
Accepted 11 February 2014
Available online 19 February 2014

Keywords:
Trypanosoma cruzi
Chagas' disease
Polyamine transport
Pentamidine

ABSTRACT

Pentamidine is an antiprotozoal and fungicide drug used in the treatment of leishmaniasis and African trypanosomiasis. Despite its extensive use as 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* trypomastigotes, 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.

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 (Hotez 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, buthionine 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

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http://dx.doi.org/10.1016/j.actatropica.2014.02.012
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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-diylbis(oxy)]dibenzenecarboximidamide), 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, was reported that pentamidine blocks the polyamine transporter LmPOT1, which is found in *L. major*. LmPOT1 has approximately 55% structural similarity with TcPAT12 (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-glutathionyl spermidine), which is founded 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). Trypomastigotes were obtained from infected VERO cells (*Chlorocebus sabaeus* kidney fibroblasts, obtained from ATCC, ATCC number: CCL-81). Cells were exposed to trypomastigotes (Y and Dm28c strains) at a 3:1 density (trypomastigote:cell). Trypomastigotes were allowed to infect cells for 24 h, after which the supernatant was extracted. Trypomastigotes 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) epimastigote 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 [^{14}C]-putrescine or 1 μM [^{3}H]-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 assessed 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 0.01 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 trypomastigotes (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 FACSAria-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 FACSDiva 6.1 software.

2.5. DAPI stain and intracellular amastigote quantification

VERO cells were exposed to *T. cruzi* trypomastigotes (Y strain) at a 5:1 ratio (trypomastigotes: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; Romanha 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 intrapeitoneal 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 (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.

Table 1
Effect of pentamidine on the viability of T. cruzi trypomastigotes. T. cruzi trypomastigotes of *T. cruzi* (Y and Dm28c strains), were exposed to pentamidine, nifurtimox and benznidazole for 24 h, and viability was measured by MTT assay. The data are presented as the means ± SD of three independent experiments.

	IC_{50} (μM)	
	Y	Dm28c
Pentamidine	2.8 ± 0.7	15.2 ± 3.2
Nifurtimox	0.9 ± 0.7	3.4 ± 2.3
Benznidazole	3.8 ± 1.8[a]	43.3 ± 21.3[a]

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

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 (TcII and TcI lineages, respectively) (Sturm et al., 2003) to pentamidine for 24 h and compared the effect with the standard antichagasic 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).

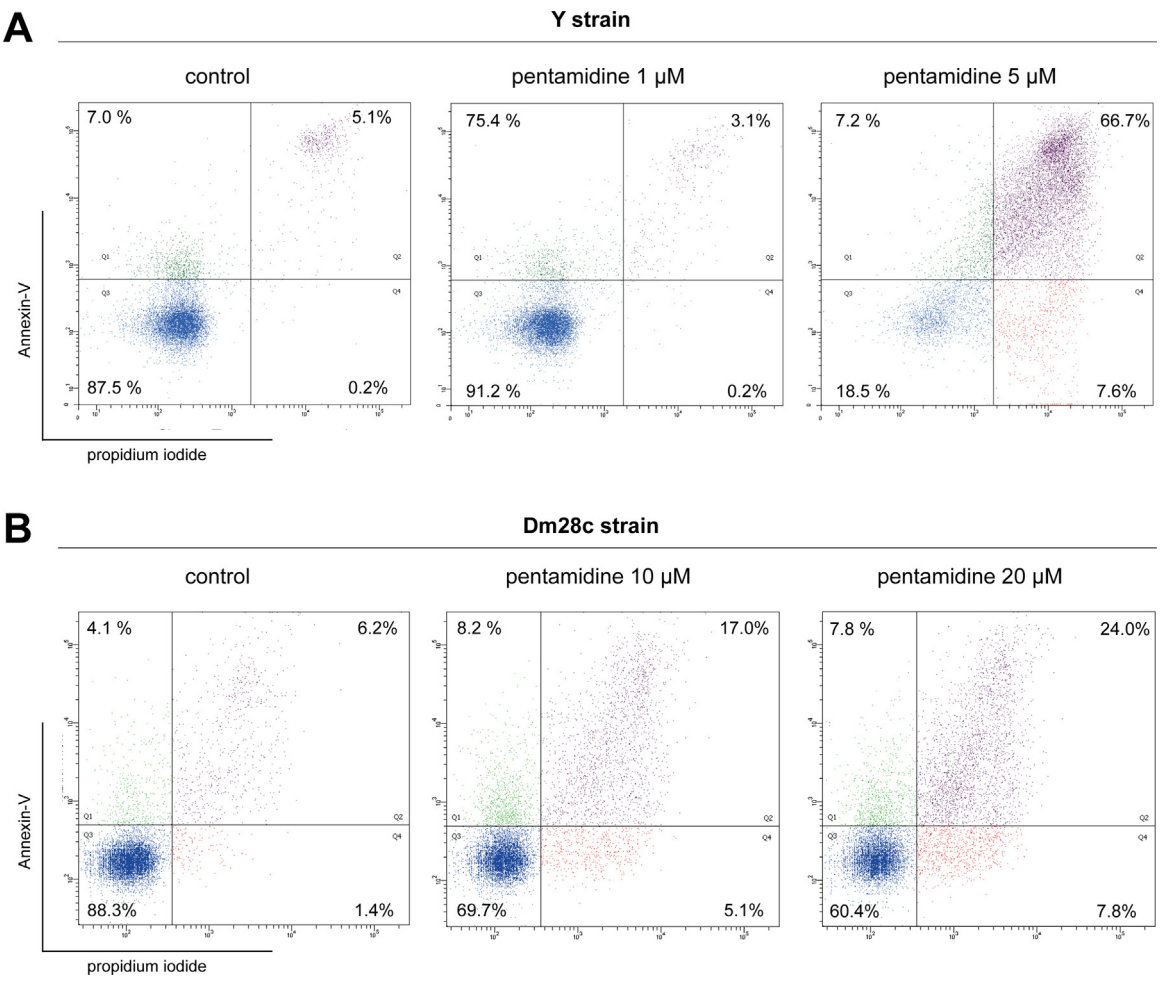

Fig. 1. *Pentamidine induces the cell death of isolated T. cruzi trypomastigotes. T. cruzi* trypomastigotes were exposed to pentamidine for 24 h. Flow cytometry analysis of viability was performed to follow death markers (Annexin-V and propidium iodide). (A) Viability of trypomastigotes Y strain (TcII lineage). Parasites were exposed to 1 or 5 μM of pentamidine. (B) Viability of trypomastigotes Dm28c strain (TcI lineage). Parasites were exposed to 10 or 20 μM of pentamidine. Representative plots of three experiments with similar results.

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).

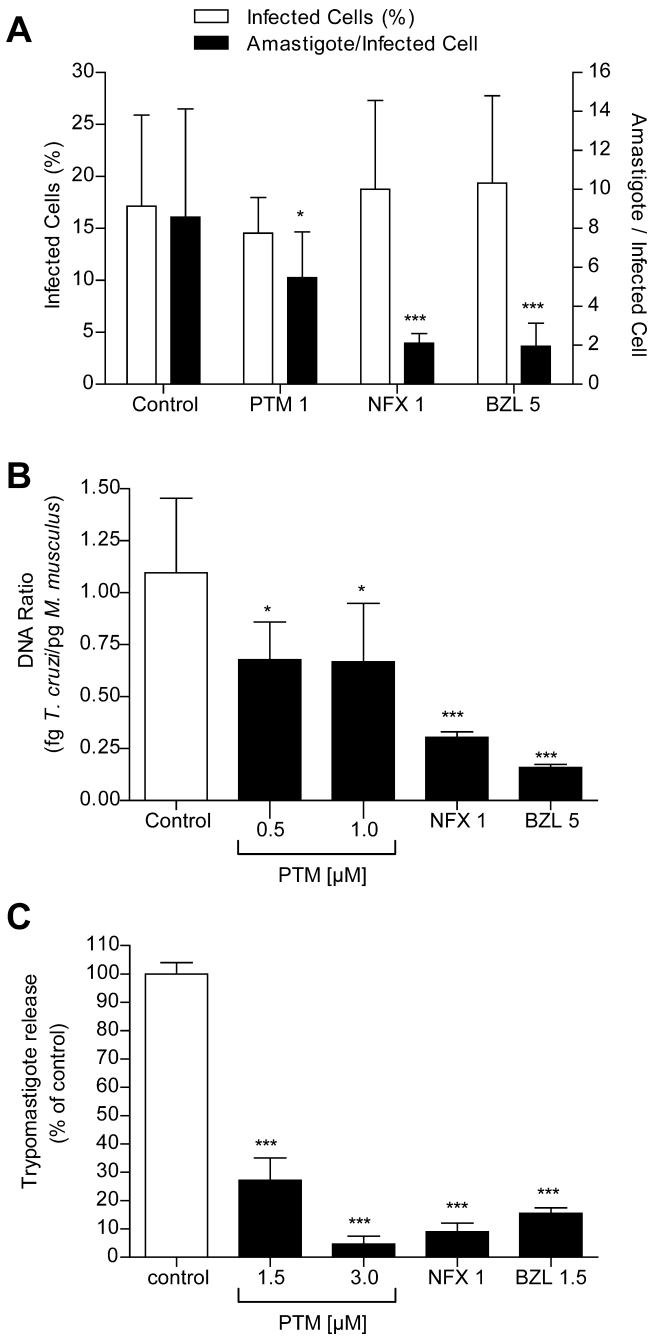

Fig. 2. *Pentamidine decreases the parasite burden of infected cells.* VERO or RAW cells were infected with *T. cruzi* (Y strain) for 24 h and treated with the different drugs. (A) Quantification of infected cells and intracellular amastigotes per infected cell after 48 h treatment with 1 μM pentamidine (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.

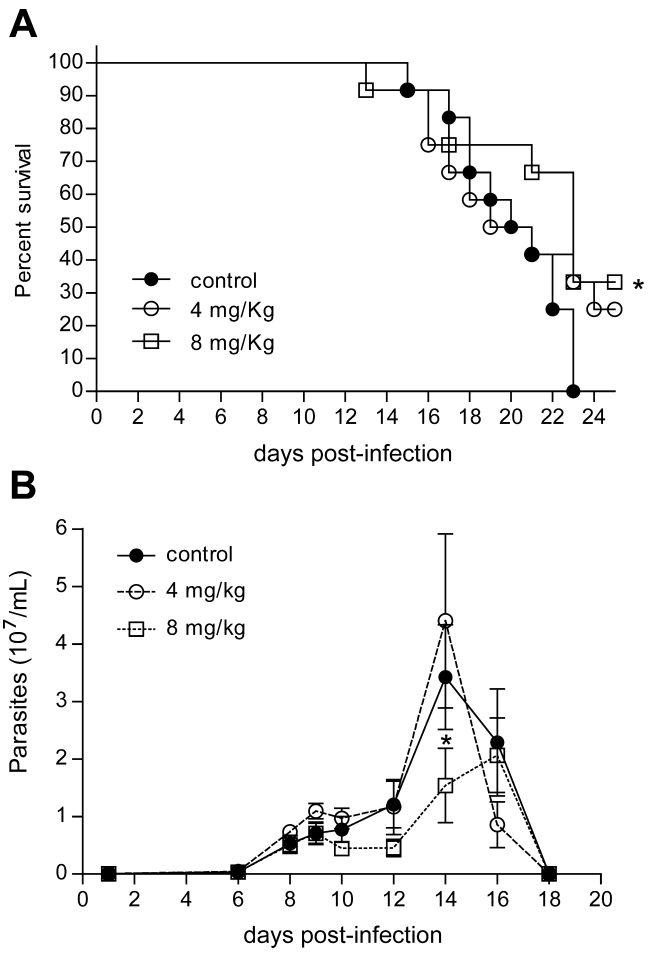

Fig. 3. *Pentamidine increases survival and decreases parasitemia in T. cruzi-infected mice.* BALB/c mice were infected with *T. cruzi* trypomastigotes (Y strain) and treated with 4 and 8 mg/kg/day pentamidine intraperitoneal for 10 days. (A) Survival rates of infected and treated mice. Survival was 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.

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

Table 2
Effect of pentamidine on the transport of radiolabeled polyamines. Transport of [^{14}C]-putrescine (5 μM), [^{3}H]-spermidine (1 μM), [^{3}H]-L-arginine (5 μM) and [^{3}H]-L-aspartate (32 μM) was measured at 10 min in the absence or presence of pentamidine at a 10-fold excess. The results are expressed as the mean ± SD of triplicate experiments.

	Polyamine uptake (pmol min^{-1} 10^{-8} cells)	
	Epimastigotes	Amastigotes
Putrescine	17.10 ± 0.97	3.34 ± 0.41
Putrescine + pentamidine	2.57 ± 0.41[a]	0.62 ± 0.06[b]
Spermidine	1.70 ± 0.15	1.95 ± 0.13
Spermidine + pentamidine	1.03 ± 0.12[c]	1.19 ± 0.10[d]
L-Arginine	1.12 ± 0.09	n.d.
L-Arginine + pentamidine	1.09 ± 0.11	n.d.
L-Aspartate	0.68 ± 0.14	n.d.
L-Aspartate + pentamidine	0.72 ± 0.12	n.d.

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.
[a] $p < 0.0001$.
[b] $p = 0.0004$.
[c] $p = 0.04$.
[d] $p = 0.001$ compared to the control group, calculated using a *t*-test

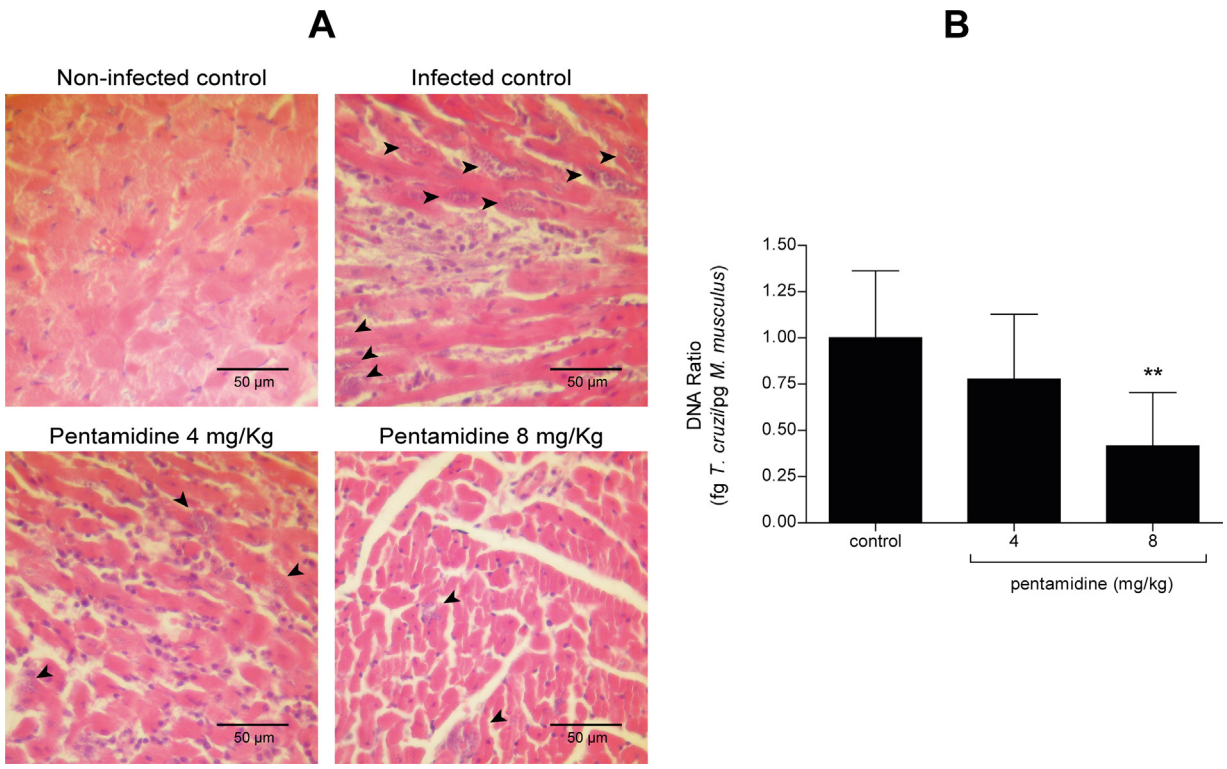

Fig. 4. *Pentamidine decreases inflammation and parasite burden in hearts from T. cruzi-infected mice*. BALB/c mice were infected with *T. cruzi* trypomastigotes (Y strain) and treated with 4 and 8 mg/kg/day pentamidine intraperitoneal for 10 days. (A) Histopathology of infected BALB/c mice with or without treatment at 25 days post-infection. The sections were stained with H&E. The arrowheads indicate amastigote nests. The images are representative of at least five mice in each group with similar results. (B) qPCR analysis of hearts from infected mice treated with 4 or 8 mg/kg/day pentamidine. Hearts from mice were extracted on the day of death or on day 25 for survivors. The graph data are expressed as the means ± SD of at least six mice per group. $^{**}p < 0.01$ vs. control, calculated by one-way ANOVA and Tukey post-test.

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 epimastigotes, and no significant inhibition was observed (Table 2). To further investigate the inhibition kinetics, we calculated the half maximal inhibitory concentrations (IC_{50}) 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 concentrations as pentamidine, representing the maximum inhibition that can be achieved. The calculated IC_{50} 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 previously demonstrated in trypanosomatids 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 pentamidine 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 trypanosomes), 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 diamidines failed in find a correlation with the trypanocide 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 transcription, ion channels, protein kinases, cell cycle, and maintenance 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., $H_2O_2/FeSO_4$ 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 inefficacy of DL-α-difluoromethylornithine (DFMO, a classic ODC inhibitor used against African trypanosomiasis), against isolated *T. cruzi* (Wilkinson and Kelly, 2009). However, intracellular amastigotes are unable to proliferate in host cells when mammalian ODC is inhibited by high concentrations of DFMO, indicating the dependence 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 membrane putative transporter sequence, previously reported in a *T. cruzi* genome database. This transporter has a high capacity to

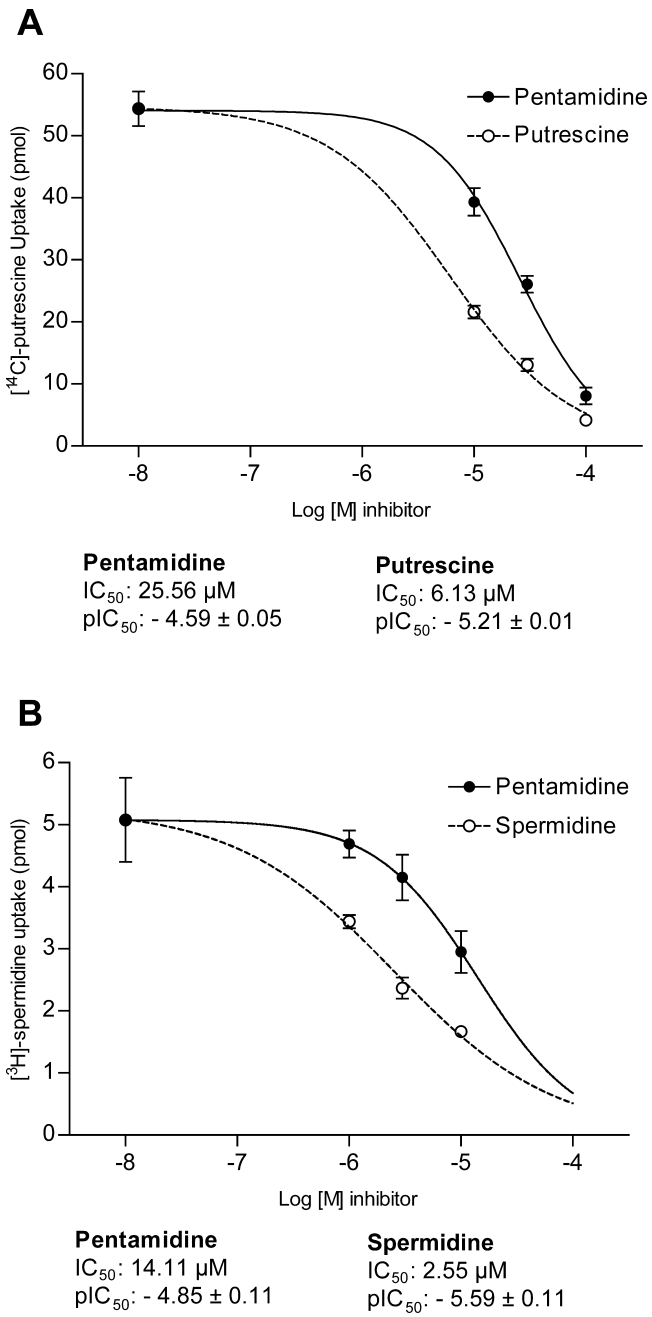

Fig. 5. *Pentamidine blocks the transport of putrescine and spermidine in T. cruzi.* (A) Measurement of the pentamidine IC_{50} for putrescine transport in epimastigotes. Transport of [^{14}C]-putrescine (5 μM) was measured at 10 min in the presence of pentamidine or unlabeled putrescine at a concentration range of 10–100 μM. (B) Measurement of pentamidine IC_{50} for spermidine transport in epimastigotes. Transport of [^{3}H]-spermidine (1 μM) was measured at 10 min in the presence of pentamidine or unlabeled spermidine at a concentration range of 1–10 μM. The results are expressed as the mean ± SD of triplicates.

transport spermidine (K_m = 14 μM). 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 (Soysa 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 *Lesihmania infantum* (Balana-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 potencially 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 anticha-gasic 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.

Acknowledgments

This work was supported by grants from the Consejo Nacional de Ciencia y Tecnología (CONICYT-Chile, grants FONDECYT 11110182 and FONDECYT 1130189), Vicerrectoría de Investigación y Desarrollo, Universidad de Chile (Grant U-INICIA 11/07), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP grants 2010-0685 and 2011-0263), Agencia Nacional de Promoción Científica y Tecnológica (FONCYT, PICT grants 2008-1209 and

2010-0289), Fundación Bunge y Born (grant 2012). M.R.M. and C.A.P. are members of the career of scientific investigator of CONICET (Argentina). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2014.02.012.

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