Trypanosoma cruzi: Desferrioxamine decreases mortality and parasitemia in infected mice through a trypanostatic effect

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ABSTRACT

Desferrioxamine (DFO) is a potent iron chelator that is also known to modulate inflammation and act as an efficient antioxidant under normal conditions and under oxidative stress. Many *in vitro* and *in vivo* studies have shown the efficacy of DFO in the treatment of viral, bacterial and protozoan infections. DFO is known to reduce the intensity of *Trypanosoma cruzi* infections in mice even during a course of therapy that is not effective in maintaining anaemia or low iron levels. To further clarify these findings, we investigated the action of DFO on mouse *T. cruzi* infection outcomes and the direct impact of DFO on parasites.

Infected animals treated with DFO (5 mg/animal/day) for 35 days, beginning 14 days prior to infection, presented lower parasitemia and lower cumulative mortality rate. No significant effect was observed on iron metabolism markers, erythrograms, leukograms or lymphocyte subsets.

In the rapid method for testing *in vivo T. cruzi* susceptibility, DFO also induced lower parasitemia.

In regard to its direct impact on parasites, DFO slightly inhibited the growth of amastigotes and trypomastigotes in fibroblast culture. Trypan blue staining showed no effects of DFO on parasite viability, and only minor apoptosis in trypomastigotes was observed. Nevertheless, a clear decrease in parasite mobility was detected.

In conclusion, the beneficial actions of DFO on mice *T. cruzi* infection seem to be independent of host iron metabolism and free of significant haematological side effects. Through direct action on the parasite, DFO has more effective trypanostatic than trypanocidal properties.

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1. Introduction

Desferrioxamine (DFO) is a hexadentate iron chelator that complexes with iron in a 1:1 M ratio to yield the stable complex ferrioxamine (stability constant 1031). It is also known to modulate inflammation and to be an efficient antioxidant under normal conditions and under oxidative stress, functioning via free radical scavenging and lipid chain breaking (Minotti and Aust, 1987; Rachidi et al., 1994).

The utilisation of chelators has been proposed as a strategy to disrupt the progression of a multitude of diseases, including

atherosclerosis and cancer (Yu et al., 2006) as well as neurologic (Richardson, 2004) and infectious diseases (Spellberg et al., 2005).

Many *in vitro* and *in vivo* studies have demonstrated that DFO is effective in the treatment of protozoan infections. DFO leads to the inhibition of growth in *Plasmodium falciparum* (Hershko and Peto, 1988) and bloodstream forms of *Trypanosoma brucei* (Breidbach et al., 2002). It is also considered a promising drug for the treatment of *Toxoplasma gondii* in mouse models of toxoplasmosis (Mahmoud, 1999). Studies of patients with malaria showed that treatment with DFO alone or in combination with standard therapy enhanced the parasite clearance in both asymptomatic and severe malaria (Traore et al., 1991; Gordeuk et al., 1992; Mabeza et al., 1996).

In mice infected with the *Trypanosoma cruzi* Y strain, Arantes et al. (2007) showed that DFO treatment starting 14 days before infection and continuing until 21 days after infection led to lower parasitemia and reduced rates of mortality when compared to untreated animals, although there were no changes in host serum iron or haemoglobin levels. More recently, combination therapy with DFO and benznidazole (BZ), the only etiological treatment commercially available for Chagas disease in Brazil, showed high efficacy and a mortality rate of 0% in mice (Francisco et al., 2008). However, other authors have shown that a short-course treatment with DFO (until the 5th d.p.i.) did not lead to a less severe infection in Swiss mice infected with the Y and CL strains of *T. cruzi* (Pedrosa et al., 1990).

Despite clear evidence that DFO is able to reduce the intensity of murine *T. cruzi* infections, the mechanisms accounting for its beneficial effects are still unclear and may involve a mechanism that is independent of host iron depletion. In this paper, we try to explain some of the previous findings that were not fully understood, such as a lower parasitemia in the absence of anaemia or low iron levels. Here, we have investigated both parasite and host parameters to identify mechanisms underlying the impact of DFO on the course of experimental *T. cruzi* infections. This is the first study to suggest that DFO exerts trypanostatic action on *T. cruzi*.

2. Materials and methods

Ethical issues: This research was reviewed and approved in advance by the institutional Animal Care and Use Committee (CETEA 153/07 UFMG).

2.1. T. cruzi infections and DFO treatment

Experimental animals comprised 100 male Swiss mice approximately 20 days old. All experimental animals were submitted to the same conditions throughout the study period. The animals received a commercial diet in pellet form (Nuvilab CR1) together with sterilised water ad libitum.

2.1.1. T. cruzi mice infections

This work investigated both short- and long-term infections of mice with the *T. cruzi* Y strain. This strain was chosen to evaluate the infection *in vivo* because it is moderately resistant to BZ and it has been routinely used for both *in vitro* and *in vivo* studies of drug activity, which may ultimately be useful for comparing the efficacy of various compounds (Filardi and Brener, 1984, 1987; Oliveira et al., 2006; Romanha et al., 2010).

2.1.2. Short-term infections

In the short-term infection model, we used the assay standardised by Filardi and Brener (1984). It consists of a rapid method for testing the susceptibility of the circulating blood forms to various drugs. In our experiment, mice (*n* = 20) were infected with a high

inoculum (50,000 blood forms) of the Y strain of *T. cruzi*. At the parasitemia peak, which occurred seven days after infection, animals were treated with a single dose of 25 mg/animal of DFO (Desferal, Novartis, Basel, Switzerland) (*n* = 5), approximately 12.5 mg/animal of BZ (*n* = 5) or both (*n* = 5). Drug doses were established following protocols previously established by Brener (1962). The control untreated group (*n* = 5) was also evaluated. Parasitemia was determined immediately prior to treatment and 3 and 6 h after drug administration.

2.1.3. Long-term infections

For longitudinal studies, 80 animals were infected via i.p. injection of 500 bloodstream forms of the Y strain of *T. cruzi*, as described previously (Francisco et al., 2010).

Forty mice received a daily dose (5 mg/animal) of DFO by i.p. injection for 35 days, beginning 14 days prior to infection and continuing for 21 d.p.i. A second set of 40 animals received a daily i.p. injection of 0.05 mL of sterile water. Parasitemia and mortality studies were carried out in infected and treated animals (I/T; *n* = 10) and in infected but untreated animals (I/NT; *n* = 10). Parasitemia was checked and quantified daily according to the methodology described by Brener (1962), starting 4 days after inoculation. Mortalities were recorded on a daily basis and expressed as a cumulative percentage.

For the rest of the *in vivo* experiments, four groups of animals were examined: infected/treated (I/T; *n* = 15), infected/non-treated (I/NT; *n* = 15), non-infected/treated (NI/T; *n* = 15) and non-infected/non-treated (NI/NT; *n* = 15).

2.2. Biochemical analysis of iron metabolism (ferritin, serum iron, and total iron-binding capacity)

Serum ferritin was determined by ELISA (*Immunoperoxidase assay for determination of ferritin in mouse sera* Kit #E-90F, Immunology Consultants Laboratory, Newberg, OR, USA). Serum iron was determined in nonhaemolysed serum samples by spectrophotometric analysis using a commercially available kit (Ferrozine #38, Labtest, Lagoa Santa, Brazil) and employing an iron standard of 89.5 μmol/L. The iron binding capacity was determined in nonhaemolysed serum samples by spectrophotometric analysis using a commercially available kit (Ferrozine #41, Labtest) employing an iron standard of 170 μmol/L. Total iron-binding capacity (TIBC) was calculated by serum iron + latent capacity of iron binding.

2.3. Mouse blood cell counts

The blood cell counts were determined using an electronic haematology particle counter from ABC Vet (Horiba, ABX Diagnostics). Differential leukocyte counting was performed on Giemsa stained blood smears, and a total of 100 cells were counted. Blood was collected by orbital plexus puncture.

2.4. Flow cytometry immunophenotyping of cultured spleen cells

Suspensions of spleen cells were prepared as described by Taylor et al. (1987). The spleens were immersed in 5 mL of cold RPMI 1640 (GIBCO, Grand Island, NY, USA) in a Petri dish and placed on ice for maceration. Fragments were pressed using a blunt glass rod and then filtered through stainless steel gauze to obtain a single cell suspension. The cell suspension was washed twice in RPMI-1640 and resuspended at a concentration of 1×10^7 cells/mL.

The suspensions of spleen cells were incubated in the presence of 1 mL of RPMI-1640 in polypropylene tubes (Falcon, BD Pharmingen) for 12 h at 37 °C in a 5% CO_2 humidified incubator. This treatment was followed by incubation with Brefeldin A (BFA) (Sigma, St.

Louis, MO, USA) at a final concentration of 10 μg/mL for an additional 4 h.

At the end of the incubation period, cells were treated with EDTA (Sigma) at a final concentration of 20 mM for 10 min at room temperature, washed once with FACS buffer consisting of PBS with 0.5% bovine serum albumin, pH 7.4 (Sigma), by centrifugation at 600g for 7 min at room temperature, and resuspended to half of the original volume in FACS buffer. Samples of 400 μL of cell suspension from the cultures were dispensed into 5 mL polystyrene tubes (Falcon), each containing either 3 μL of anti-mouse CD4, anti-mouse CD8 and anti-mouse CD19 (Caltag Laboratories) or 3 μL of anti-mouse CD49 (BD Pharmingem), all labelled with FITC.

After incubation for 30 min at room temperature in the dark, cell surface-stained samples were treated with 2 mL of FACS Lyse/Fix Buffer (BD Pharmingen) and then immediately vortexed and re-incubated for an additional 3 min. Subsequently, the suspension was centrifuged at 400g for 10 min at room temperature, and the supernatant was removed. The cells were washed twice with 2 mL PBS (0.15 M phosphate buffered saline, pH 7.2) and then fixed with 100 μL of FACS FIX solution (10.0 g/L paraformaldehyde, 10.2 g/L sodium cacodylate and 6.65 g/L sodium chloride).

Flow cytometry acquisition was performed using a FACSCalibur flow cytometer (BD Pharmingen) examining a total of 30,000 events per tube. CELL QUEST software (Becton–Dickinson, San Jose, CA, USA) provided by the manufacturer was used for data acquisition and analysis.

2.5. In vitro T. cruzi growth inhibition assay (β galactosidase assay)

To evaluate the *in vitro* trypanocidal action of DFO, we used a modified protocol established by Buckner et al. (1996) that was recommended by the Experimental Models in Drug Screening and Development for Chagas Disease workshop, held in Rio de Janeiro, Brazil, on the 25th and 26th of November 2008 by the Fiocruz Program for Research and Technological Development on Chagas Disease and Drugs for Neglected Diseases Initiative (Romanha et al., 2010).

For this experiment, the Tulahuen *T. cruzi* strain expressing the *Escherichia coli* β-galactosidase gene was used. Infective trypomastigote forms were obtained through culture in monolayers of mouse L929 fibroblasts in RPMI-1640 medium without phenol red (Gibco BRL) containing 10% foetal bovine serum and 2 mM glutamine.

For the bioassay, 4000 L929 cells in 80 μL of supplemented medium were added to each well of a 96-well microtitre plate. After an overnight incubation, 40,000 trypomastigotes in 20 μL were added to the L929 cells and incubated for 2 h. Medium containing parasites that did not penetrate the cells was replaced with 200 μL of fresh medium, and the plate was incubated for an additional 48 h to establish infection.

After 48 h, the medium was again discarded and replaced with 180 μL of fresh medium with 20 μL of DFO. DFO was tested against the trypanosomes in triplicate in different concentrations (100, 50, 20, 10, 5 and 1 μg/mL). After 4 days of incubation cells were lysed by octyl phenoxylpolyethoxylethanol (Nonidet P-40®) (0.1% final concentration), in order to release intracellular amastigotes, and chlorophenol red β-D-galactopyranoside (CPRG) (100 μM final concentration) was added to the plates and incubated overnight at 37 °C.

The absorbance was measured at 570 nm in an automated microplate reader. BZ at its IC$_{50}$ (1 μg/mL = 3.81 μM) was used as positive control. Cell culture was morphologically checked routinely by microscopic examination in order to guarantee the cellular growth and survival as a quality control measure.

The results were expressed as the percentage of *T. cruzi* growth inhibition in the compound-tested cells as compared to the untreated cells (Oliveira et al., 2006; Romanha et al., 2010).

2.6. Mobility, viability of the parasites by trypan blue and apoptosis assays

The effect of DFO on parasite mobility, viability and apoptosis was assayed using Y strain trypomastigotes from the culture supernatant of L929 cells. L929 cells were maintained in our laboratory by serial passaging and kept frozen in liquid nitrogen. For the assays, 1×10^6 L929 cells were seeded in tissue culture flasks (75 cm^2, Falcon) with 10 mL of DMEM medium (GIBCO) containing 10% FBS and incubated at 37 °C in humidified air containing 5% CO_2. After 2 or 3 days, the monolayer was infected with 5×10^6 trypomastigotes of the *T. cruzi* Y strain obtained from experimentally infected mice. Cultures were maintained in DMEM with 10% FBS at 33 °C in 5% CO_2 at 95% humidity (Bertelli et al., 1977). After 5–6 days, trypomastigotes were harvested from the supernatant. Cell debris and amastigotes were removed by differential centrifugation at 100g for 10 min at room temperature. Supernatants containing most of the parasites were centrifuged at 1000g for 15 min at 4 °C. The pellets were washed three times in PBS supplemented with 10% FBS.

Cultures were performed under different drug concentrations depending on the specific assays.

2.6.1. Mobility assay and trypan blue staining

Trypomastigotes of the *T. cruzi* Y strain were cultured in the presence of different concentrations of DFO (1, 5, 10, 20, 50 and 100 μg/mL), gentian violet (50 μg/mL) or BZ (1 μg/mL) for 1, 3, 6, 9 or 12 h.

To determine trypomastigote mobility, 10 μL of culture supernatant containing 1×10^7 parasites was applied to a Neubauer chamber, and the percentage of mobile parasites was calculated under light microscopy.

To determine the viability of the trypomastigotes, 1×10^5 parasites were resuspended in PBS and incubated with 1 μL of 0.4% trypan blue for 10 min at room temperature before analysis by flow cytometry. A total of 10,000 events were acquired and analysed using Flow-Jo software. The viable parasites did not stain with trypan blue. The percentage of viable parasites was defined by a histogram of trypan blue content.

2.6.2. Annexin and PI staining

Trypomastigotes of the *T. cruzi* Y strain were cultured in the presence of different concentrations of DFO (1, 5, 10, 20, 50 and 100 μg/mL), gentian violet (50 μg/mL) or BZ (1 and 5 μg/mL) or a combination of DFO (1, 5 and 10 μg/mL) and BZ (1 μg/mL), for 0.5, 1 or 3 h.

For this experiment, 1×10^6 parasites were resuspended in a Ca^{2+}-enriched binding buffer (Apoptosis Detection Kit, Sigma, USA). Suspensions of the parasites were double stained with FITC-conjugated annexin V and PI for 10 min at room temperature and protected from light. They were immediately analysed on a flow cytometer in their staining solution. Annexin V and PI emissions were detected in FL-1 (band pass 530 nm, band width 30 nm) and FL-2 (band pass 585 nm, band width 42 nm) channels, respectively. Parasites showing no staining by either PI or annexin V-FITC were considered alive. Parasites that stained with annexin V-FITC alone were considered in the early apoptotic process. Parasites stained with both PI and annexin were considered in the late apoptotic process. Parasites stained with PI alone were considered dead.

2.7. Statistical analysis

Statistical analyses of the data were carried out using GraphPad Prism software (GraphPad Software 5.0, San Diego, CA, USA). Data were initially assessed by one-way analysis of variance (ANOVA) between days. When the interactions were significant, the Tukey test was used to determine the specific differences between mean values. The results of differences between groups were statistically evaluated using the unpaired Student t-test. Values are expressed as means ± standard deviation. Differences in mean values were considered significant at the $p < 0.05$ level.

3. Results

3.1. DFO decreases parasitemia in both short- and long-term infections

Short-term infection assays were used to evaluate the effects of DFO on highly infected animals. To test this, animals were infected with 50,000 parasites and treated 7 days after infection with DFO (25 mg/animal) and/or BZ (approximately 12,5 mg/animal) (Fig. 1A). Parasitemia was significantly reduced 3 h after treatment in DFO (65.11%), BZ (65%) and DFO + BZ (89.9%) treated mice. Nevertheless, 6 h after treatment, only BZ or DFO + BZ treated animals continued to exhibit lower parasitemia.

In a longitudinal study, we evaluated the capacity of DFO to reduce parasitemia and mortality in mice infected with 500 bloodstream forms of the Y strain of *T. cruzi*.

In animals treated with DFO from 14 days prior to infection to 21 d.p.i. (I/T), we observed a decrease in parasitemia and mortality
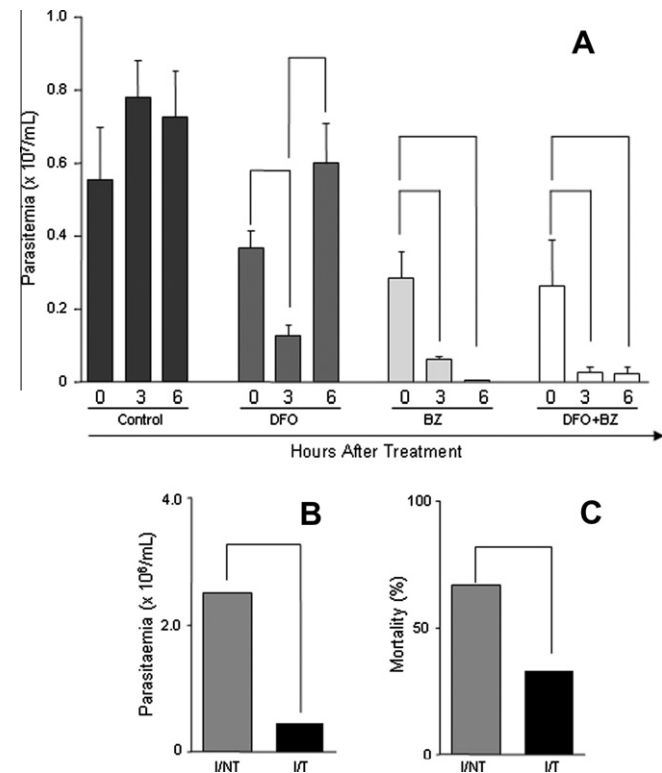
at 14 d.p.i. ($p < 0.05$). The average parasitemia value in I/T mice (46,444 trypomastigotes/0.1 mL of blood) was 5.4 times lower than in I/NT (250,444 trypomastigotes/0.1 mL of blood) ($p < 0.05$) (Fig. 1B).

Similarly, DFO was able to induce a decrease in mortality at 14 d.p.i. Mortality in the I/NT group was 67%, whereas the death rate in the I/T group was 33% ($p < 0.05$) (Fig 1C).

3.2. Impact of DFO on host biomarkers

To investigate the effect of DFO on host iron metabolism impairment, we analysed ferritin, serum iron and TIBC levels in infected and DFO treated animals. Surprisingly, we found that treatment with DFO did not interfere in host iron metabolism (Fig. 2), suggesting that the DFO effects on disease outcome were not dependent on the host's iron level.

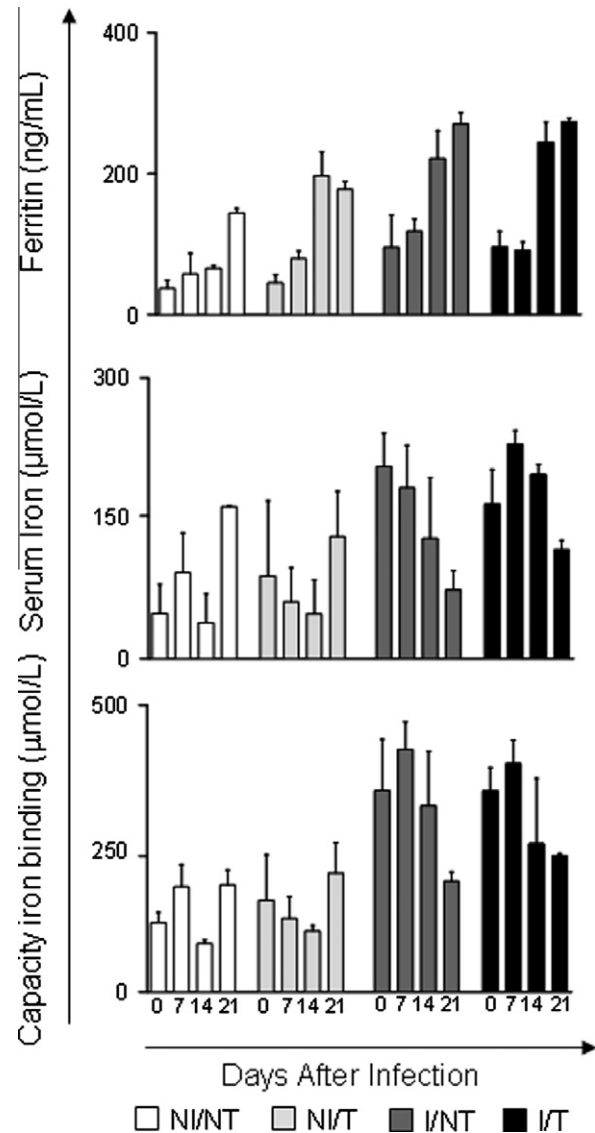The effect of DFO on blood cells was also investigated. No difference in erythrograms (erythrocytes, haemoglobin,

Fig. 1. (A) Swiss mice ($n = 20$) were inoculated with 50,000 *T. cruzi* Y strain trypomastigotes and received no treatment (control), 25 mg/animal DFO, approximately 12.5 mg/animal BZ or both. Parasitemia was estimated before treatment and 3 and 6 h after treatment. Lines mean significant difference ($p < 0.05$). (B and C) Swiss mice ($n = 20$) were inoculated with 500 *T. cruzi* Y strain trypomastigotes and received either no treatment (I/NT) or treatment with DFO (I/T). Parasitemia (B) and mortality (C) were observed 14 days after infection.

Fig. 2. Swiss mice ($n = 60$) were inoculated with 500 *T. cruzi* Y strain trypomastigotes and treated with DFO for 35 days or left untreated beginning 14 days prior to infection and continuing up to 21 d.p.i. As controls, mice were left uninfected or untreated. Levels of ferritin, serum iron and capacity iron binding were measured at 0, 7, 14 and 21 days after infection. Values shown are means ± SD.

haematocrit), leukocytes or platelet levels was observed (data not shown), indicating that DFO treatment was not accompanied by the evident impairment of erythroid, myeloid or megakaryocytic differentiation.

To further examine the potential unfavourable effects of DFO in lymphocyte subpopulations, we performed immunophenotypical analysis of spleen cells.

Phenotypic profiles of splenic lymphocyte populations from different experimental groups were presented (Fig. 3). NK cells (CD49^{+}), T lymphocytes (CD4^{+} + CD8^{+}), B cells (CD19^{+}) and T cell subpopulations (CD4^{+} and CD8^{+}) were analysed. We did not observe any differences between the groups.

Since these findings suggested that the sensitivity of T. cruzi to decreased iron levels could be greater than the sensitivity of the host cells, we hypothesised that DFO could act directly on the parasite.

3.3. Direct impact of DFO on T. cruzi

Using the protocol recommended by Experimental Models in Drug Screening and Development for Chagas Disease Workshop (Romanha et al., 2010), we evaluated the growth of the Tulahuen strain of T. cruzi in the presence of DFO.

This assay measures the activity of β-galactosidase expressed by either amastigotes or trypomastigotes, since culture cells are lysed
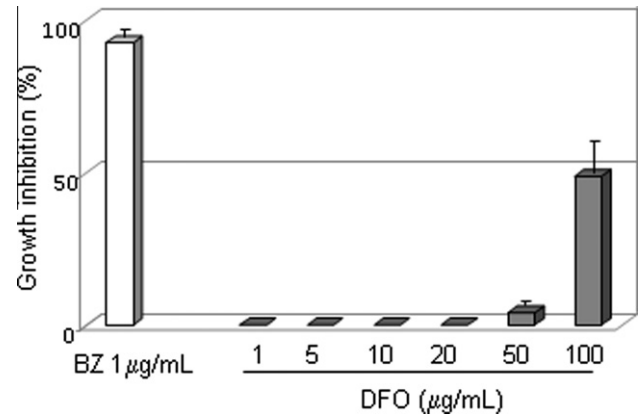
Fig. 4. The T. cruzi Tulahuen strain expressing Escherichia coli β-galactosidase gene was cultured for 4 days in L929 fibroblasts in the presence of different concentrations of DFO. The results are expressed as a percentage of growth inhibition in the supernatant of L929 cells infected with the Tulahuen strain of T. cruzi. BZ treatment was used as a positive control. Values shown are means ± SD.

before the measurement of enzyme activity. Using BZ as a positive control for growth inhibition, the activity of the DFO, shown by growth inhibition of amastigotes and trypomastigotes, is presented in Fig. 4. DFO was shown to be active only in high concentrations.
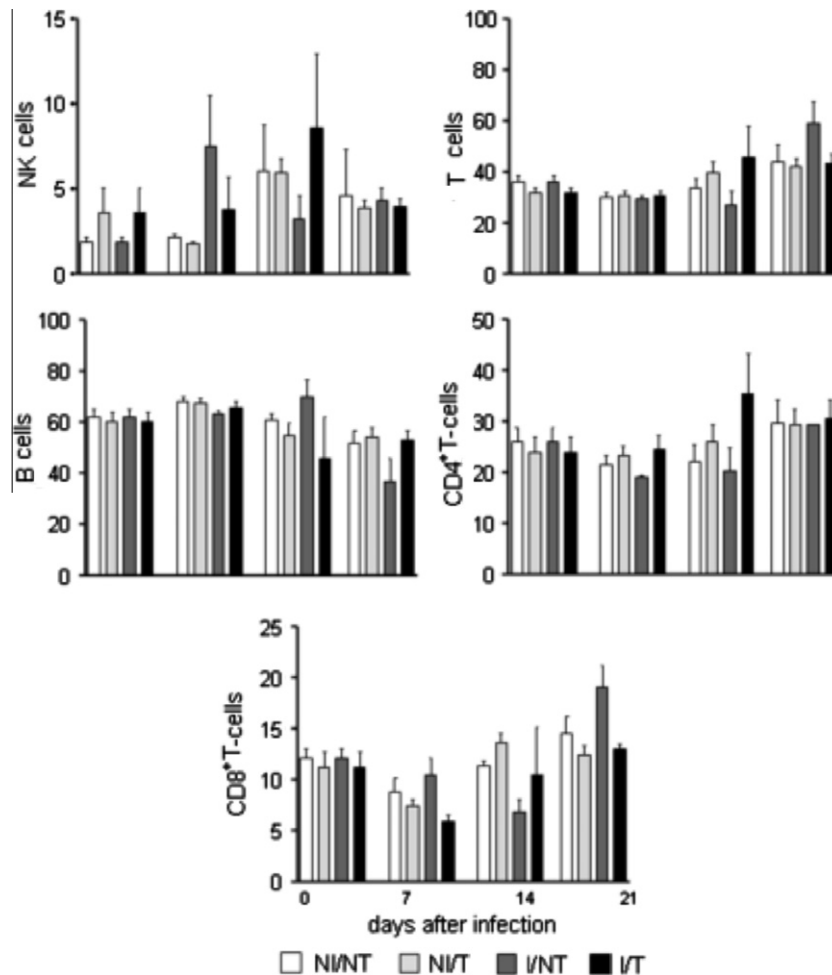
Fig. 3. Swiss mice (n = 60) were inoculated with 500 T. cruzi Y strain trypomastigotes and treated with DFO or left untreated. As controls, mice were left uninfected or untreated. Splenocytes were cultured for 12 h in culture medium and stained for the presence of NK cells (CD49), T cells (CD4^{+} CD8^{+}), B cells (CD19) and T-cell subpopulations with anti-CD4 and anti-CD8 markers. Results are expressed as a percentage of total splenocytes.

At a concentration of 100 μg/mL (IC$_{50}$), the percentage of growth inhibition was 49%, whereas treatment with BZ (1 μg/mL) caused a 93% growth inhibition of the parasites.

We also evaluated viability by trypan blue staining. The Y strain-infected L929 cells were cultured in the presence of different concentrations of DFO (1, 5, 10, 20, 50 and 100 μg/mL) or BZ (1 and 5 μg/mL). The parasites were incubated for 0, 0.5, 1, 3, 6, 9 and 12 h. Trypomastigotes from the supernatant were stained with trypan blue. In all tested concentrations of DFO or BZ, 70–90% of the parasites remained viable, with no differences between the groups (Table 1), suggesting a low trypanocidal effect *in vitro*.

Apoptosis induced by DFO was assessed by phosphatidylserine externalisation. Similar to the results of viability by flow cytometry, the majority of the trypanosomes treated with DFO showed no staining with either PI or annexin V-FITC after up to 3 h of incubation (Fig. 5), suggesting parasite preservation. Because PI is a membrane-permeable nuclear stain, these results indicate membrane integrity.

Because DFO did not considerably decrease *in vitro* parasite growth, we decided to investigate whether the compound had trypanostatic action. To test the effect of DFO on parasite motility, we counted the number of mobile trypanosomes in the supernatant of L929 cells after treatment with different concentrations of DFO (1, 5, 10, 20, 50 and 100 μg/mL) and times of incubation (1, 3, 6, 9 and 12 h).

We could observe a trypanostatic effect of DFO in sublethal concentrations. After 3 h of incubation, DFO at concentrations higher than 10 μg/mL showed more efficiency than 1 μg/mL of BZ in reducing parasite mobility.

Additionally, DFO at concentrations higher than 20 μg/mL showed more efficiency than 1 μg/mL of DFO, which was the lowest concentration of drug used ($p < 0.05$) (Fig. 6).

4. Discussion

Unfortunately, there is no current effective treatment for chronic Chagas disease, which is one of the world's most neglected tropical diseases. Two drugs have been clinically used, benznidazole and nifurtimox, but both were developed more than four decades ago (Coura and de Castro, 2002). They are far from ideal due to multiple side effects and limited efficacy, especially in patients with the chronic form of the disease (Rocha et al., 2007; Soeiro and De Castro, 2009). Side effects vary from discrete discomfort to peripheral polyneuropathy, hypersensitivity dermatitis, and haematologic disorders that lead to treatment discontinuation. In Brazil, only benznidazole is commercially available. The use of nifurtimox was halted owing to toxic effects.

Despite of the lack of interest from drug companies in developing novel compounds for Chagas disease, a few new drugs have
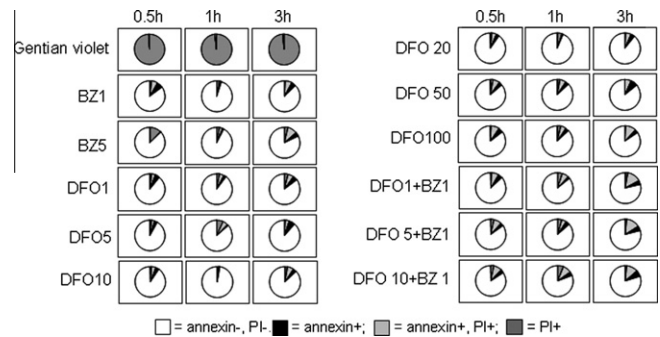

Fig. 5. Trypomastigote cells were cultured in L929 cells in the presence or absence of DFO. BZ and gentian violet were used as controls. A suspension of 1×10^6 parasites was double stained with FITC-conjugated annexin V and PI. Parasites showing no staining by either annexin or PI were considered alive (white). Parasites stained with annexin alone were considered in early apoptosis (black). Parasites stained by both PI and annexin were considered in late apoptosis (light grey). Parasites stained with PI alone were considered dead (dark grey).
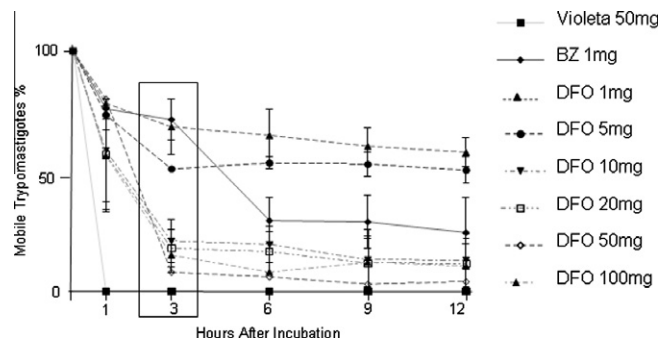

Fig. 6. Y strain trypomastigotes were cultured in L929 cells in the presence of different concentrations of DFO, and their motility was evaluated after 1, 3, 6, 9 and 12 h of incubation. BZ and gentian violet were used as controls. Value represent the percentage of mobile trypomastigotes. Each data point represents the mean ± standard error. Significant differences ($p < 0.05$) were observed between DFO in concentrations higher than 10 μg/mL and BZ 1 μg/mL as well as DFO in concentrations higher than 20 μg/mL and DFO 1 μg/mL (the lowest concentration of drug used).

undergone clinical trials, including allopurinol, itraconazole and fluconazole. The latter are last generation of anti-fungal therapies, and they act by preventing the synthesis of sterols (Coura, 2009). Nevertheless, more therapeutic options are necessary, not only as monotherapies but also for combined therapies that could be more effective and less toxic.

Table 1
Kinetic evaluation of viability (% of viable parasites unstained by trypan blue) of trypomastigotes cultured in the presence of DFO and or Bz (means ± SD).

Experimental groups (μg/mL)	0.5 h	1 h	3 h	6 h	9 h	12 h
Gentian violet	4.5 ± 1	9.6 ± 8	6.4 ± 2	6.4 ± 4	3.3 ± 0	3.0 ± 0
BZ 1	86.8 ± 7	80.9 ± 6	75.3 ± 9	81.4 ± 3	81.9 ± 4	74.3 ± 1
BZ 5	85.2 ± 0	84.6 ± 2	81.2 ± 6	79.6 ± 3	75.2 ± 6	75.6 ± 4
DFO 1	84.8 ± 7	82.6 ± 3	86.1 ± 6	78.2 ± 1	80.8 ± 0	80.4 ± 5
DFO 5	77.6 ± 8	88.0 ± 4	79.9 ± 6	87.4 ± 3	77.2 ± 2	87.8 ± 5
DFO 10	90.7 ± 1	86.5 ± 5	74.1 ± 1	79.3 ± 1	72.8 ± 9	75.9 ± 7
DFO 20	86.9 ± 7	89.9 ± 2	82.9 ± 2	83.1 ± 3	79.3 ± 2	78.3 ± 4
DFO 50	84.1 ± 6	82.3 ± 3	82.9 ± 4	82.3 ± 4	74.6 ± 3	78.7 ± 5
DFO 100	82.6 ± 5	79.4 ± 7	76.2 ± 4	70.8 ± 3	69.8 ± 8	78.9 ± 2
DFO 1 + BZ 1	87.0 ± 6	79.6 ± 8	74.2 ± 2	78.7 ± 5	72.2 ± 1	75.1 ± 1
DFO 5 + BZ 1	88.5 ± 5	82.1 ± 2	83.8 ± 8	70.8 ± 9	76.1 ± 1	76.6 ± 9
DFO 10 + BZ 1	81.9 ± 3	79.9 ± 4	77.1 ± 5	74.3 ± 3	71.6 ± 1	82.6 ± 1

DFO is known to reduce the intensity of murine *T. cruzi* infections. Our previous data indicated beneficial effects *in vivo* despite obvious interference in the host iron burden (Arantes et al., 2007; Francisco et al., 2008). Therefore we decided to further investigate the activity of DFO on *T. cruzi* infections.

In the present study, we used different *in vivo* and *in vitro* approaches. The use of a rapid *in vivo* test was supported by the fact that this methodology detects activity against circulating blood forms using a drug concentration five times higher than the recommended daily dose. Brener was the first to investigate the susceptibility of *T. cruzi* blood-stream forms to active compounds. Brener claimed that *T. cruzi* stout blood forms persist for some hours in the bloodstream without penetrating the host tissues (Brener, 1969, 1971; Filardi and Brener, 1984). In this rapid method, active drugs are supposed to induce a fast decline in the number of circulating parasites. The methodology we use here is considered efficient for characterising susceptibility to active drugs and for screening active compounds against *T. cruzi*. This approach may complement data relating to the effects of drugs on different *T. cruzi* intracellular stages (Maria et al., 1972). This methodology has been utilised recently by others (Alves et al., 1999; Aguirre-Ivarado et al., 2007).

As expected, the results we obtained with this method correlated with those obtained by prolonged treatment schedules. Both showed lower infection intensity in the presence of DFO treatment. DFO was able to induce lower parasitemia in both short- and long-term infections and diminished mortality in long-term infection.

Although DFO was able to early reduce parasitemia in the rapid test, this was an unsustained effect. Six hours after treatment, parasite levels were similar to that found in untreated animals. This finding is probably due to the short half-life in plasma and the rapid metabolism of DFO (Aouad et al., 2002). Nevertheless, this limitation was overcome by combination therapy with BZ.

Despite the clear effect of DFO on parasite growth, no interference in host iron metabolism or hematopoietic and lymphopoietic differentiation was observed. This result is supported by previous findings. In the work carried out by Pedrosa et al. (1990), mice infected with Y, CL and YuYu strains of *T. cruzi* and treated with DFO exhibited no reduction of serum iron or increase in anaemia. Likewise, Lalonde and Holbein (1984) demonstrated no significant changes in serum iron levels of either infected or uninfected mice treated with DFO. Similarly, Arantes et al. (2007) observed no decrease in the serum iron or haemoglobin levels of mice infected with *T. cruzi* and treated with DFO. Francisco et al. (2008) showed that serum iron concentrations were even higher in animals treated with DFO or with both DFO and BZ when compared to the untreated group.

These findings suggest that the sensitivity of *T. cruzi* to the unavailability of iron is greater than that of the host cell.

In the evaluation of the impact of DFO on *T. cruzi*, we assessed parasite growth inhibition, viability by trypan blue, apoptosis and motility assays.

Similarly to BZ, DFO did not affect the viability measured by trypan blue staining. But DFO was able to inhibit amastigote and trypomastigote growth in fibroblast culture, but only when the drug was at high concentrations (100 µg/mL). This is in contrast to BZ, which was highly effective at concentrations of 1 µg/mL.

The ability of DFO in reducing parasite growth has been reported previously by Loo and Lalonde (1984). The authors have shown that DFO reduced the rate of amastigotes replication in treated cell cultures in a dose dependent manner by depleting host cell iron.

All of our data suggest low trypanocidal *in vitro* effects of DFO, which contrasted with the beneficial effects observed in mouse infections. This led us to investigate the effects of DFO on parasite motility.

A decrease in motility was observed when trypomastigotes were cultured in the presence of DFO. This result is of great impor-

tance because it suggests a putative trypanostatic action. Little is known about DFO activity on *T. cruzi*, and this trypanostatic effect may be a major step in the action of DFO on parasite control since parasite motility is necessary for cell invasion and consequently parasite multiplication (Sibley, 2011). It is already described that one putative pathway for trypomastigote entry into mammalian cells involves activation of calcium signalling in the host cell and the recruitment of host cell lysosomes to the site of entry, where they fuse with the vacuole formed by the entering parasite (Rodriguez et al., 1996; Tardieux et al., 1992). The extremely active motility of the parasite likely contributes to this by causing local breaks in the plasma membrane and hence lysosomal recruitment is triggered as part of the wound healing response, akin to what happens in mammalian cells that have been damaged (McNeil and Kirchhausen, 2005). Andrade and Andrews (2004, 2005) reported that trypomastigotes invasion occurs either by direct recruitment and fusion of lysosomes at the plasma membrane, or through invagination of the plasma membrane followed by intracellular fusion with lysosomes. The lysosome-like parasitophorous vacuole is essential for preventing these highly motile parasites from exiting host cells and for allowing completion of the intracellular life cycle. They showed that when lysosome-mediated *T. cruzi* invasion is blocked, a significant fraction of the internalised parasites are not subsequently retained inside host cells for a productive infection.

Effects on motility have been previously observed in parasites with the use of certain drugs. Kamau et al. (2001) demonstrated that allopurinol had a leishmaniostatic effect in flow cytometry analysis. Nevertheless, the mechanism underlying this action is not fully understood.

The involvement of apoptosis as a possible mechanism of DFO action in *T. cruzi* was also tested. Previous work has shown that apoptosis can also be induced in unicellular parasites, including in *T. cruzi* (Ameisen et al., 1995; Piacenza et al., 2001). Infective forms of *T. cruzi* and *Leishmania* spp. display extracellular phosphatidylserine, a marker of apoptotic cells (de Freitas Balanco et al., 2001; Damatta et al., 2007). Apoptosis of a proportion of *Leishmania* parasites is required for the successful establishment of infection in the vertebrate host (van Zandbergen et al., 2006). In our data, we observed that DFO induces only minor apoptosis in trypomastigotes, indicating that this is not the major mechanism of DFO action and further studies should be conducted to better elucidate this possible mechanism.

Based on these results, we believe that DFO has more effective trypanostatic than trypanocidal properties and this effect may lead to lower infectivity by decreasing cellular invasion. It is possible that *T. cruzi* presents greater sensitivity to the unavailability of iron than the host cell and therefore DFO is apparently free of major haematological side effects.

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