TETRADENTATE POLYAMINES AS EFFICIENT METALLODRUGS FOR CHAGAS DISEASE TREATMENT IN MURINE MODEL

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About us

The collaboration established around the Consolider Ingenio 2010 project, awarded by the Government of Spain, has helped to join different groups of chemistry and parasitology to work together in strategies of drug discovery against trypanosomatids. Our teams have been developing new compounds and procedures to evaluate their activity in order to find

new compounds to be included in the list of prospective candidates to fight against the *Trypanosoma cruzi* and *Leishmania* spp infections. We currently are testing compound, of different chemical background, in vitro and in vivo. Our teams are integrated by: doctoral and postdoctoral student, associate researchers and technicians; all them supervised by the PI's of each discipline. So, we have built a solid structure that have been working hard in the last past few years obtaining a good output of results that we are currently sharing now with the scientist community through regular meetings and conferences, as well as upcoming publications.

Conflict of interest

None of the authors has any conflict of interest to disclose

Acknowledgments

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Abstract

A series of tetraamine-based compounds was prepared and their trypanocidal effects against *Trypanosoma cruzi* and cytotoxicity were determined through the determination of IC_{50} values. *In vivo* assays were performed in mice, where parasitaemia levels were quantified by fresh blood examination and the assignment of a cure was determined by PCR and reactivation of blood parasitaemia levels after immunosuppression. The mechanisms of action were elucidated at metabolic and ultra-structural levels, by ^{1}H NMR, Fe-SOD inhibition, and TEM studies. The high-selectivity indexes observed *in vitro* were the basis of promoting one of the tested compounds to *in vivo* assays. Compound 6 induced a remarkable decrease in the reactivation of parasitaemia after immunosuppression and curative rates of 33%. The experiments allowed us to select Compound 6 as a promising candidate for treating Chagas disease, but a further high-level study should be considered to obtain an improved efficiency.

Keywords

American Trypanosomiasis; chemotherapy; tetradentate polyamines; murine model.

Introduction

The World Health Organisation (WHO) lists Chagas disease as one of the most neglected tropical diseases. Chagas disease is endemic in Latin America; it is estimated that 10 million people are infected worldwide and more than 25 million are living at risk of infection.[1] The main drugs used for the treatment of Chagas disease are benznidazole (Bz, LAFEPE) and nifurtimox (Lampit®, Bayer), which present significant side effects and cure <20% of patients with chronic Chagas disease.[2] Thus, many researchers have combined efforts to discover drugs against new targets, looking for lower toxicity and a greater tolerance in patients, aiming not only for efficiency in the acute phase, but also in the chronic phase.

One of the self-defence strategies of *Trypanosoma cruzi* (the etiological agent of Chagas disease) is its highly active and exclusive iron superoxide dismutase (Fe-SOD), which differs from Cu–Zn-SOD or Mn-SOD operating in mammals. Fe-SOD is an extremely efficient enzyme for preventing any oxidative damage from the host in combination with peroxidases. So this enzyme is a parasitic molecule involved in protozoan protection against oxidative stress, which is now considered a virulence factor of *T. cruzi*.[3]

In previous work, we designed iron and manganese coordination complexes containing polyamine ligands, which were capable of generating highly oxidising species.[4–6] Highly reactive Mn(IV)=O and Fe(IV)=O species embedded in these ligand scaffolds have been prepared and their oxidative reactivity studied. Furthermore, these polyamine ligands bind very strongly to iron and manganese ions, forming coordination complexes that are stable under highly acidic and oxidative conditions.

Herein, we report on the *in vitro* and *in vivo* anti-trypanosomal properties of Mn-based polyamines and polyamine complexes (Fig. 1), which represent a class of compounds

that have, so far, only rarely been explored for Chagas disease chemotherapy. Finally, we analyse the possible mechanism of action over the parasite structure and function as well as the protective enzymes mentioned above.

Materials and methods

Chemistry

Comp 4 was purchased from Catexcel. Compounds 1,[7] 3,[8] 5,[7] 6,[9] were prepared as described in the literature.

Synthesis of comp 2: Comp 4 (47.1 mg, 0.14 mmols) was dissolved in acetonitrile (1 mL). A solution of $FeCl_2$ (36.2 mg, 0.28 mmols) in acetonitrile (2 mL) was prepared under inert atmosphere. The solution of $FeCl_2$ was added dropwise at room temperature to the solution of **4**, the mixture turned pale yellow and was stirred at room temperature for 1 hour. Then, $AgClO_4$ (114.7 mg, 0.55 mmols) was added and the formation of a white precipitate was observed. The mixture was stirred over 30 minutes. Afterwards a white powder and a purple solution were clearly distinguished. The purple solution was filtered through syringe filters (25 mm Ø) and the white precipitate was washed with CH_3CN until the filtrates become colorless. 121 mg of purple crystals (0.11 mmols, 80 %) were obtained after diffusion with ethyl ether. Molecular structure was confirmed by single crystal X- ray diffraction. Crystals loose acetonitrile upon drying under vacuum, but color is instantaneously recovered upon solution in acetonitrile. ESI/MS (CH_3CN): 749.1 $[M - 6CH_3CN - ClO_4]^+$, 685.0 $[Fe^{II}_2(ClO_4)_2(OH)(H_2O)(TACN2)]^+$. ^{1}H-NMR ($CD_3CN$): 110.7, 103.3, 65.8, 38.4, 36.0, 33.1 ppm. Elemental analysis: Anal Calcd. for $[(C_{18}H_{40}N_6)Fe(CH_3CN)_3](ClO_4)_4$: C, 29.62 %; N, 12.95 %; H, 5.07%. Found: C, 28.9 %; N, 12.95 %; H, 5.08 %.

Biological activity: In vitro assays

Antiprotozoan properties of the compounds were firstly evaluated *in vitro* against epimastigote, amastigote and trypomastigote forms of *T. cruzi*, and Vero cells were used for the determination of unspecific cytotoxicity, according to the methodology

described by us.[10] *Cytotoxicity*: Vero cells (EACC number 84113001), originally obtained from monkey kidneys, were grown in RPMI (Gibco) supplemented with 10% inactivated fetal bovine serum in a humidified 95% air/5% CO_2 atmosphere at 37ºC for 2 days. The compounds and the reference drug (Bz) were dissolved in DMSO (Panreac, Barcelona, Spain) at a concentration of 0.01% (v/v). The compounds were added to the culture medium at dosages of 100, 50, 25, 10, l, 0.5, 0.25, and 0.1 μM. After 72 h of treatment, the cell viability was determined by flow cytometry. Thus, 100 μL per well of propidium iodide solution (100 mg/mL) was added and incubated for 10 min at 28°C in darkness. Afterwards, 100 μL per well of fluorescein diacetate (100 ng/mL) was added and incubated under the same conditions. Finally, the cells were recovered by centrifugation at 400 *g* for 10 min and the precipitate was washed with phosphate-buffered saline (PBS). Flow cytometric analysis was performed with a FACSVantage flow cytometer (Becton Dickinson). The percentage viability was calculated in comparison with the control culture. The IC_{50} was calculated using linear regression analysis from the K_c values of the concentrations employed (0.1–100 μM). *Epimastigote assays*: The compounds were added in the same μM concentration than used before for Vero cells. The effects of each compound against epimastigote forms of *T. cruzi* were tested at 72 h using a Neubauer hemocytometric chamber. The antitrypanosomatid effect is expressed as the IC_{50}, that is, the concentration required to give 50% growth inhibition, calculated by linear regression analysis from the K_c values of the concentrations employed. *Amastigote assays:* Vero cells were grown as described above in RPMI medium (Gibco). Cells were seeded at a density of 1×10^4 cells per well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultured for 24 h. Afterwards, the cells were infected *in vitro* with metacyclic forms at a ratio of 10:1 over 12 h. The nonphagocytosed parasites were removed by washing, and then the drugs (at

100, 50, 25, 10, 1, 0.5 0.25, and 0.1 μM) were added. Vero cells with the drugs were incubated for 72 h at 37°C in 5% CO_2. The drug activity was determined on the basis of the number of amastigote forms in the treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The number of amastigote forms was determined by analyzing 200 host cells distributed in randomly chosen microscopic fields. The antitrypanosomatid effect is expressed as the IC_{50}; **Bz was included as reference drug**.

Trypomastigotes assay: compounds were also evaluated in blood trypomastigote forms of *T. cruzi*. BALB/c female mice infected with *T. cruzi* were used 7 days after infection. Blood was obtained by cardiac puncture using 3.8% sodium citrate as an anticoagulant in a 7:3 blood:anticoagulant ratio. The parasitaemia in the infected mice was about 1×10^5 parasites/mL. The compounds were diluted in phosphate-buffered saline solution (PBS) to give a final concentration 10, 25, and 50 μM for each compound. Aliquots (20 μL) of each solution were mixed in culture trays (96 wells) with 55 μL of infected blood containing the parasites at a concentration of approximately 1×10^6 parasites/mL. Infected blood with PBS, at the same concentrations as the products, was used as control. The plates were shaken for 10 min at room temperature and kept at 4°C for 24 h. Each solution was examined microscopically (Olympus CX41) for parasite counting using the Neubauer haemocytometric chamber (a dilution of 1:100 in PBS was necessary to get into the range of counting). The activity (percent of parasites reduction) was compared with that of the control.

These studies were complemented by infectivity assays on Vero cells with the most active compounds, representing a reproduction of the lifecycle *in vitro*. Briefly, Vero cells were grown under the same conditions expressed over 2 days. Afterwards, the cells were infected *in vitro* with metacyclic forms of *T. cruzi* at a ratio of 10:1. The drugs (IC_{25} concentrations) were added immediately after infection and were incubated for 12 h at

37°C in 5% CO_2; **Bz was included as reference drug.** The non-phagocytosed parasites and the drugs were removed by washing, and then the infected cultures were grown for 10 days in fresh medium. Fresh culture medium was added every 48 h. The drug activity was determined from the percentage of infected cells, the number of amastigote forms per infected cell, and the number of trypomastigote forms in the medium, in treated and untreated cultures, as well as in methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigote forms per infected cell were determined by analyzing 200 host cells distributed in randomly chosen microscopic fields every 48 h. The number of trypomastigote forms in the medium was determined using a Neubauer hemocytometric chamber every 48 h.

Biological activity: In vivo assays

The *in vivo* trypanocidal activities of the compounds were studied using a murine model from acute to chronic phases of Chagas disease, according to our previously published procedures.[11] Briefly, groups of six Balb/c female albino mice (6−8 weeks old, 25−30 g weight) maintained under a 12 h dark/light cycle (lights on at 07:30 h) at a temperature of 22 ± 3°C and provided with water and standard chow *ad libitum*, were inoculated through the intraperitoneal route with 5×10^3 blood trypomastigote forms of *T. cruzi* obtained from previously infected mice blood. The animals were divided as follows: **I** positive control group (6 mice infected but not treated) and **II** study group (6 mice infected and treated with the compounds under study). The administration of the tested compounds was initiated on the seventh day of infestation once the infection was confirmed, and doses of 15 mg/kg body weight per day were administered for 5 consecutive days (7−12 days pi) through the intraperitoneal route; **Bz was included as reference drug**. Peripheral blood was obtained from the mandibular vein of each mouse (5 μL samples) and diluted in 495 μL of PBS solution at a 1:100 dilution. The circulating parasite numbers were quantified

with a Neubauer's chamber for counting blood cells. This counting was performed every 3 days over 40 days (acute phase). The number of bloodstream forms was expressed as parasites/mL. After day 60, the mice entered the chronic phase of the experiment where parasitemia continued decreasing its levels progressively, independently of the treatment. So, on day 120, the parasitemia was checked and found to be undetectable by fresh blood microscopic examination; then, the mice received four intraperitoneal doses of 200 mg/kg·w of body mass cyclophosphamide monohydrate (CP) (ISOPAC®) on alternate days. The efficacy of such an immunosuppression procedure to assess cryptic infection was verified by the high parasitemias under microscopic examination and/or mortality close to the 100% of chronically untreated mice, having received the immunosuppression treatment. Within 1 week after the last CP injection, parasitemia was evaluated according to the procedure described for the acute phase to quantify the presence of blood trypomastigote forms as a reactivation rate. Finally, the mice were bled out under gaseous anaesthesia (CO_2) through a heart puncture and the blood was collected. Blood was incubated for 2 h at 37ºC and then over night at 4ºC in order to allow clotting and to obtain the serum from the samples after centrifuging the supernatant twice at 1000 and 2700 *g*, consecutively. The serum was divided into aliquots and used for ELISA and biochemical analyses, as explained below. Hearts were harvested and immediately flushed free of blood by gentle infusion of 10 mL of prewarmed PBS through the left ventricle in order to avoid contamination of collected tissue with blood parasites. Afterwards, they were frozen at –80ºC and stored until they were used for DNA extraction.

Toxicity testing was also included to determinate the potential damage that the compounds could cause for the host, which was measured by analysing different biochemical parameters as markers of the kidney, heart and liver profiles. A fraction of the obtained serum was sent to the Biochemical Service at the University of Granada, where a series of

parameters were measured according to the commercial kits acquired from Cromakit® by BS-200 Chemistry Analyser (Shenzhen mindray, Biomedical electronics co., Ltd.). With the levels obtained for different populations of sera (n = 15, n = 6), we calculated the mean value and standard deviation. Finally, we also calculated the confidence interval for the mean normal populations based on a confidence level of 95% [100 × (1–α) = 100 × (1.05)%]. The obtained ranges are shown in Table 2, which allows the comparison and analysis of the sera studied in this work.

Mechanism of action

In order to get an insight into the molecular mode of action of the compounds, a ^{1}H NMR study was conducted, according to a previously published procedure,[10] and the changes in the nature and quantity of the excreted metabolites were examined to gain information about the effect of the compounds on the energetic metabolism of parasites. Shortly, cultures of *T. cruzi* epimastigote forms (initial concentration of 5 x 10^5 cells/mL) received IC_{25} of the compounds (except for the control cultures). After incubation for 96 h at 28°C, the cells were centrifuged at 400 g for 10 min. The supernatants were collected in order to determine the excreted metabolites through ^{1}H NMR, and the chemical shifts were expressed in parts per million (ppm), using dimethyl sulphoxide (DMSO) as the reference signal. One-dimensional ^{1}H NMR spectra were acquired on VARIAN DIRECT DRIVE 400 MHz Bruker spectrometer with AutoX probe using D_2O. The assignments of metabolites were based on 1D NMR spectrum. The chemical shifts used to identify the respective metabolites were consistent with those described previously by our group[10]. In addition, the human metabolome database (http://www.hmdb.ca/) was also used for this purpose. The spectral region of 1.0 to 5.5 ppm was bucketed into a frequency window of 0.1 ppm. The region corresponding to water (4.5 to 5.5 ppm) was excluded during binning to avoid artefacts due to pre-saturation of water, and the region corresponding to glucose

(3.4 to 3.8 ppm) was also excluded. The aromatic region was excluded because the signal to noise ratio in this region was poorer compared to that of the aliphatic region. The peak (2.6 ppm) corresponding to DMSO was removed before binning. The resulting integrals were normalised to the working region (1.0 to 3.4) ppm of the spectrum to correct for inter-sample differences in dilution. The binning and normalisations were achieved using Mestrenova 9.0 software. The matrix obtained in Mestrenova was imported to Microsoft Excel for further data analyses.

In addition, ultra-structural alterations of the parasites treated with the compounds were investigated using transmission electron microscopy (TEM), briefly, epimastigote forms of *T. cruzi* were cultured at a density of 5×10^5 cells/mL in each corresponding medium containing the compounds tested at their IC_{25} concentrations. After 72 h, these cultures were centrifuged at 400 *g* for 10 min and the pellets produced were washed in PBS and then mixed with 2% (v/v) paraformaldehyde/glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 4 h at 4ºC. Following this, the pellets were prepared for TEM study using a previously described technique.[11].

The ability of the compounds to interfere with the antioxidant defence of the parasites was evaluated by studying them as putative inhibitors of Fe-SOD (in contrast to human Cu–Zn-SOD). In order to do that, epimastigote forms of *T. cruzi*, cultured as described above, were collected by centrifugation. The pellet was suspended in 3 mL of STE buffer (0.25 M sucrose, 25 mM Tris–HCl, 1 M EDTA, pH 7.8) and the cells were disintegrated through three cycles of ultra sonication, each for 30 s at 60 W. The homogenate was centrifuged for 5 min at 1500 *g* and 4ºC, and the pellet was washed three times in ice-cold STE buffer. This fraction was centrifuged (2500 *g* for 10 min at 4ºC) and the supernatant was collected. The protein concentrations in the supernatant were determined using the Bradford method. The Fe–SOD and CuZn–SOD activities were determined using the method described by

Beyer and Fridovich, which measures the reduction of nitrobluetetrazolium (NBT) by superoxide ions. Into each cuvette, 845 μL of the stock solution [3 mL of L-methionine (300 mg, 10 mL^{-1}), 2 mL of NBT (1.41 mg, 10 mL^{-1}) and 1.5 mL of Triton X-100 1% (v/v)] was added, together with 30 μL of the parasite homogenate fraction, 10 μL of riboflavin (0.44 mg, 10 mL^{-1}) and an equivalent volume of the of the compound solution. Five different concentrations were used for each compound (1–100 μM). In the control experiment, the volume was made up to 1000 μL with 50 mM potassium phosphate buffer (pH 7.8), whereas 30 μL of the parasite homogenate fraction was added to mixtures containing the compounds. Then, the absorbance (A_0) was measured at 560 nm in a spectrophotometer. Every cuvette was illuminated with a fixed lamp light for 10 min under constant stirring and the absorbance (A_1) was measured. The human CuZn–SOD, coenzymes, and substrates used in these assays were purchased from Sigma Chemical Co. The obtained data were analysed using the Newman–Keuls test.

Statistical analyses

Data were recorded on an Excel spread sheet (Microsoft, Redmond, WA, USA), and statistical analysis performed by using SPSS Statistics (V. 17) software (IBM, NY, USA). The t test for paired samples was used to verify whether there were differences between the assays used, with p-0.05 considered statistically significant and with a 95% confidence interval. Also statistical studies based on contingency tables (prevalence) were conducted, together with the x2 test of the relationship between variables.

Results and discussion

In vitro trypanosomicidal evaluation

Five out of seven of the polyamines showed better selectivity indexes (SI = IC_{50} Vero cells/IC_{50} extracellular and intracellular forms of *T. cruzi*) compared to the reference drug Bz. As summarised in Table 1, compound 6, with SI values 13, 17 and 21 times higher than those of Bz, was the best compound, independent of the parasite stage (epimastigote, amastigote and trypomastigote forms, respectively). This activity meets good criteria to consider it as a strong candidate to undergo *in vivo* studies.

To obtain more accurate information about the most active polyamine, 6, the development of the parasites in Vero cells was studied by measuring the rates of infection and the average number of amastigote and trypomastigote forms present during a ten-day treatment period. Vero cells were infected with metacyclic forms of *T. cruzi* and the gradual differentiation into amastigote forms was followed. During the observation time, in the control group, the rate of infection of the host cells gradually increased, reaching 99% invasion at the end of the experiment (Fig. 2A). The test was repeated in the presence of the reference drug and in the presence of compound 6, at their IC_{25} concentrations. It was found that the rate of infection decreased in all cases, with respect to the control, and compound 6 showed reductions in the infection rate of 81% thus with much a higher efficiency than Bz (19% reduction) (p-value = 0.031).

In terms of the average number of amastigote forms per Vero cell (Fig. 2B), the results were consistent with those mentioned above for infection rates. After treatment, compound 6 significantly reduced the number of amastigote forms per cell by 56%, whereas Bz showed reduction of only a 39%. The average number of amastigote forms increased to a maximum on the sixth day of culture, but decreased thereafter; the rupture of Vero cells

released trypomastigote forms. In terms of the number of trypomastigote forms found per millilitre of culture medium (Fig. 2C), a maximum was reached on day 10 (9.5×10^3 trypomastigote forms/mL) for the control, whereas this value was substantially reduced by the reference drug (41%), but even more so by compounds 6 (88%). In summary, the results of the parasite spreading in Vero cells are in agreement with those of the trypanocidal activity reported in Table 1 for intracellular and extracellular forms of *T. cruzi*, with compound 6 being much better than any of the other drug candidates tested.

In vivo activity of tetradentate polyamines

The promising *in vitro* results prompted us to study the *in vivo* activity of this polyamine in female BALB/c mice. As the effectiveness of the drugs currently in use against Chagas disease varies widely between the acute and chronic phase, we decided to evaluate the impact of our compound on both phases. For the acute phase experiments, we considered the first 40 days after infection (dpi), whereas the effect on the chronic phase was studied between days 40 and 120 after infection. The intraperitoneal (i.p.) doping route was preferred over the intravenous procedure, because it is well known that i.p. treatment substantially reduces animal mortality.[12] In fact, no mouse died in any of our experiments with either the control or with the compound assayed at the concentrations used (15 mg/kg of body mass). However, the survival percentage for the mice treated with Bz was only about 80%. The female mice were inoculated with trypomastigote forms, as described in the material and methods section, and i.p. treatment with the compounds was started five days post-infection and maintained for five alternated days. A group of mice (control group) was treated in the same manner, but using only the vehicle. During the study of acute-phase activity, the level of parasitaemia was determined every two days. Fig. 3 shows the number of trypomastigote forms found during the acute phase (1–40 dpi). On

the day of maximum parasite burden (20–22 days after infection), the tested compound greatly reduced the number of blood trypomastigote forms compared with the control group. Furthermore, compounds 6, lowered the level of parasitaemia on day 40 by 61%, being this effect significantly greater than that found for the reference drug (11%). Statistical tests revealed the effect of the comp 6 in decreasing the parasitemia levels with a p-value = 0.0017 < α = 0.05.

Therefore, this group of mice, treated with compound 6 where kept until 120 dpi (late chronic phase, in which there are no parasites remaining in the bloodstream that are quantifiable by fresh blood analysis) when the mice underwent an immunosuppression treatment as it was explained in the Material and Methods section. Fig. 4A shows the reactivation of parasitaemia after immunosuppression. It can be appreciated that the control group recovers half of its initial peak of parasitaemia, with a reactivation of 55 %. In contrast, the treated group show a parasitaemia load of 37.2% with respect to their burden peak when the mice have been administered with compound 6. To test this hypothesis χ^2 test we were performed using Fisher statistic (being a 2x2 table) to obtain a p- value of 0.021 < α = 0.05 . ELISA assays offer an alternative method for verifying the effectiveness of these three compounds in the chronic phase after being challenged with an immunosuppression cycle. Fig. 4B shows the total Ig-G levels of anti-*T. cruzi* and, according to previous reports, the titer of Ig-G in Balb/c becomes stable during chronic phase of the disease;[13] this is confirmed in fig. 4B. The control group showed an almost doubling in the Ig-G levels in response to the presence of the parasite in the bloodstream after immunosuppression. However, in the treated group, the differences between immunosuppressed (IS) and non-immunosuppressed (non-IS) groups were smaller and not significant in the case of compound 6. According to the Fisher's statistic, the equality between both treatments was rejected (p- values of 0.019 and 0.02). This means that the

parasite-specific Ig-G levels were not higher, just proportional, than the remaining amount of total unspecific Ig-G caused by the hypergammaglobulinaemia characteristic of the infection by *Trypanosoma cruzi*.[14]

Finally, fig. 4C shows the polymerase chain reaction (PCR) results after necropsy. The hearts were grinded and underwent a total DNA extraction and amplification of a fragment within the parasite SOD gene; the hearts of the control animals showed the expected presence of the parasite, since the cardiac tissue is the main target tissue for this particular strain of *T. cruzi*. In contrast, the hearts from mice treated with compound 6 were relatively clean (2 hearts out of 6) from parasites, thus confirming the partial curative effect of these compounds at this dosage. Biochemical clinical parameters were tested in different groups of BALB/c mice infected with *Trypanosoma cruzi* in different experimental situations (Table 2), showing that an increase in the dosage does not cause a toxicity risk. All biochemical parameters tested, regarding the kidney, heart and liver profiles, were not altered after compound administration.

In order to improve the effectiveness of compound 6, we must take into consideration an increase in the dosage for future experiments and modify the schedule of treatment for a better exposure of the compound in the bloodstream.

Metabolite excretion study

As trypanosomatids are unable to completely degrade glucose to CO_2, they excrete a considerable portion of their hexose skeleton as partially oxidised fragments in the form of fermented metabolites, whose nature and percentage depend on the pathway used for glucose metabolism.[15,16] The catabolism products in *Trypanosoma cruzi* were acetate and succinate, with smaller percentages of L-alanine and D-lactate, in agreement with the data in previously published reports.[17] Detection of large amounts of succinate as a major end

product is an usual feature, because it relies on glycosomal redox balance, enabling re-oxidation of the NADH produced in the glycolytic pathway. Succinic fermentation requires only half of the phosphoenolpyruvate produced to maintain the NAD^+/NADH balance, and the remaining pyruvate is converted inside the mitochondria and the cytosol is converted into acetate, D-lactate, L-alanine or ethanol, according to the degradation pathway followed by each species.[18] In order to obtain some information concerning the effect of the tested compounds on glucose metabolism in the parasites, we obtained ^1H NMR spectra of *T. cruzi* epimastigote forms after treatment with compound 6, from which the final excretion products were identified qualitatively and quantitatively (spectra not shown). The results were compared with those obtained from parasites maintained in a cell-free medium (control) for four days after inoculation with the parasite. The characteristic presence of acetate, succinate, D-lactate and L-alanine was confirmed in the control experiments. As expected, succinate and acetate were the most abundant end products identified. However, after treatment of the parasites with compound 6, the excretion of metabolites was slightly altered at the dosages employed. Fig. 5 displays these modifications with respect to the control at the height of the spectral peaks, corresponding to the most representative final excretion products. Marked differences in the catabolic pathways appeared, which seemed to be connected with the trypanocidal activity mentioned above. The most remarkable differences were the decreased the amount of succinate by 17%; in addition, an increase in the amount of acetate, D-lactate and L-alanine by 17, 18 and 21%, respectively, was found upon treatment with compound 6. A nonparametric test in each case was performed and a p- value $< \alpha = 0.05$ (p -values 0.045 , 0.087 , 0.0013 and 0.012) was obtained. So, in conclusion, even though the disturbances were quantifiable, we could not attribute the metabolism of the involved enzymes to the direct targets of the compounds.

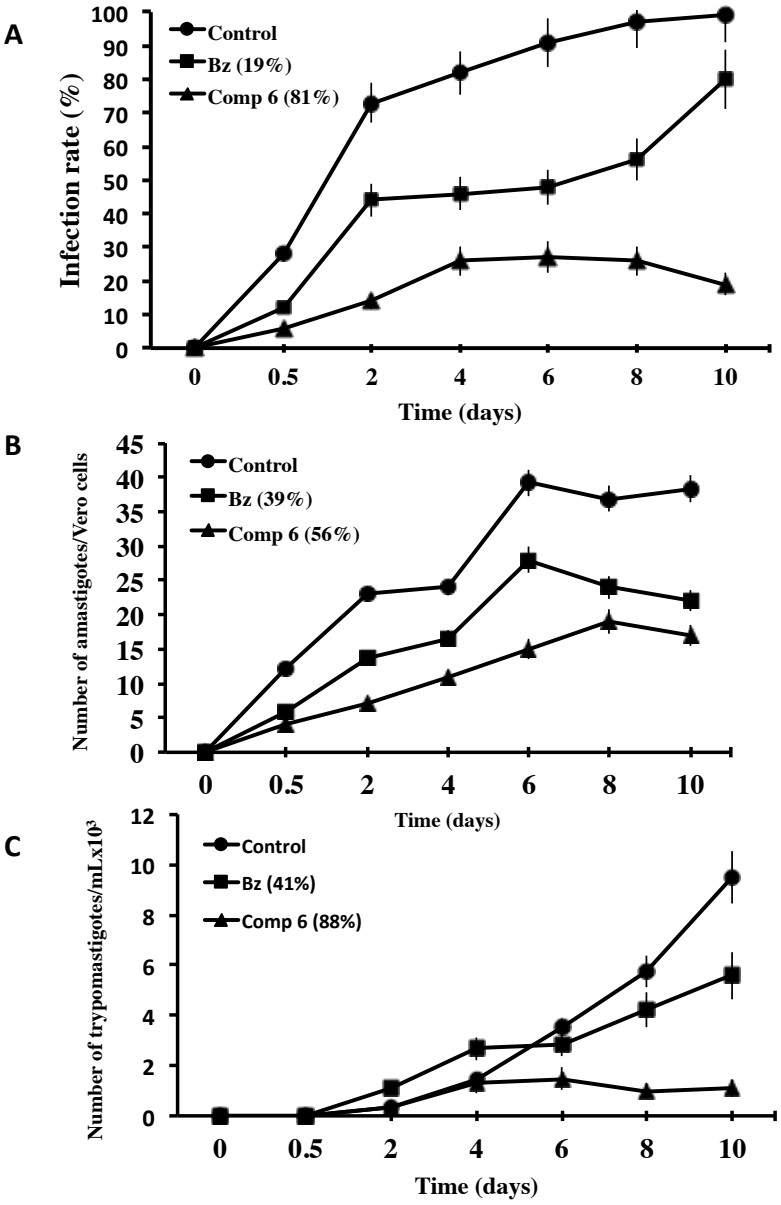

Figure 2. Effect of compound 6, and Bz on the infectivity of *T. cruzi* in Vero cells. (A) Rate of infection, (B) mean number of amastigote forms per infected Vero cell and (C) number of trypomastigote forms in the culture medium after treatment with the IC_{25} of the control (filled circles), Bz (filled squares), compound 6 (filled triangles). The results are the mean

values of three separate experiments and the error bars represent the mean +/- standard deviation.

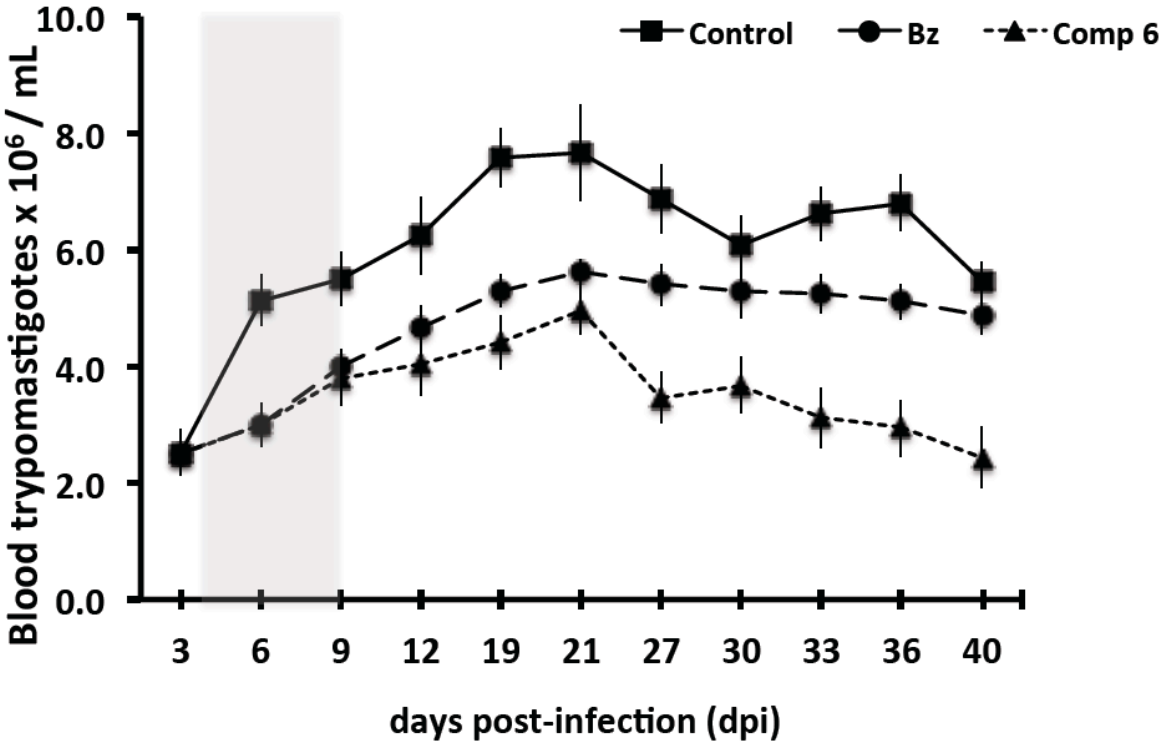

Figure 3. Parasitemia in the murine model of acute Chagas disease. Control (filled squares) and doses of 15 mg/kg body mass of Bz (filled circles), compound 6 (filled triangles). The grey shading represents the days of treatment. Values are the means of six separate mice and error bars represent the mean +/- standard deviation.

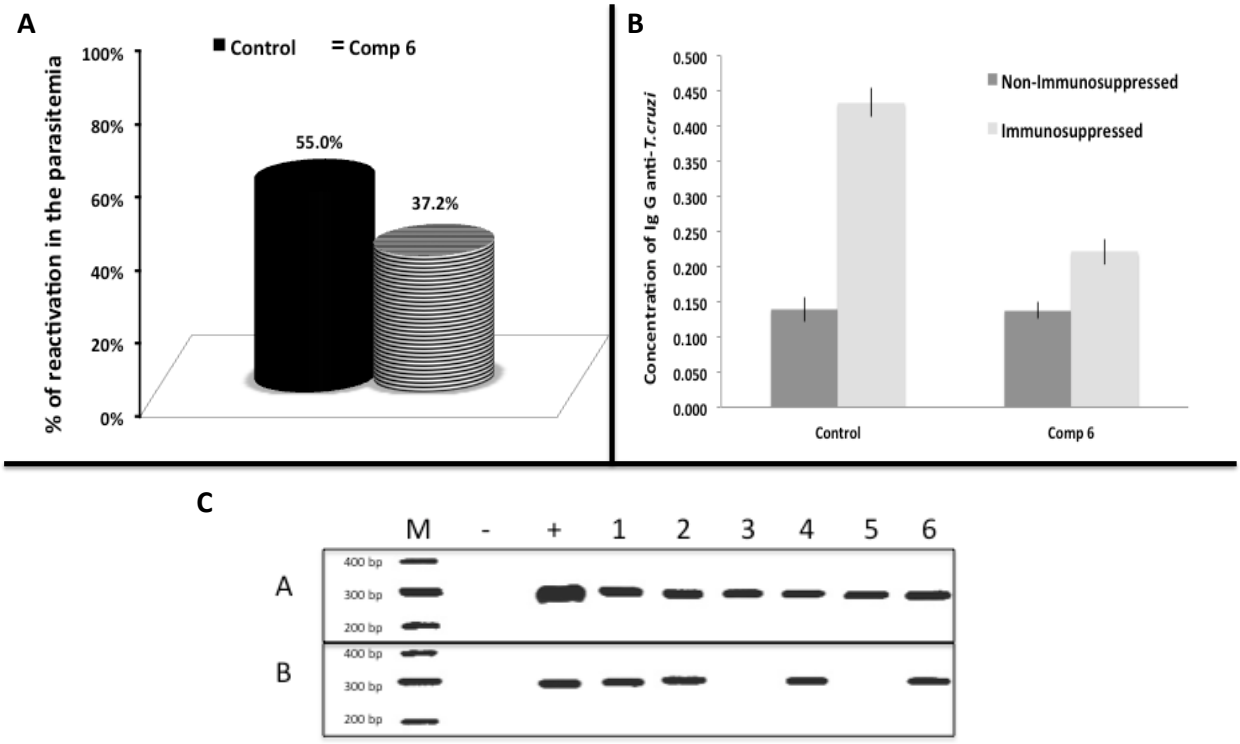

Figure 4. Post-mortem analyses on the final day after necropsy. (A) Percentage of reactivation of blood parasitemia after immunosuppression, (B) total Ig-G levels of anti-*T. cruzi,* where the error bars represent the mean +/- standard deviation of three mice and (C) PCR of mouse heart tissue. M, marker; -, PCR negative control; +, PCR positive control; lanes 1–3 represent the non-IS mice in the final days of the experiment; lanes 4–6 represent the IS mice; A, infection control group; B, infected and treated with compound 6.

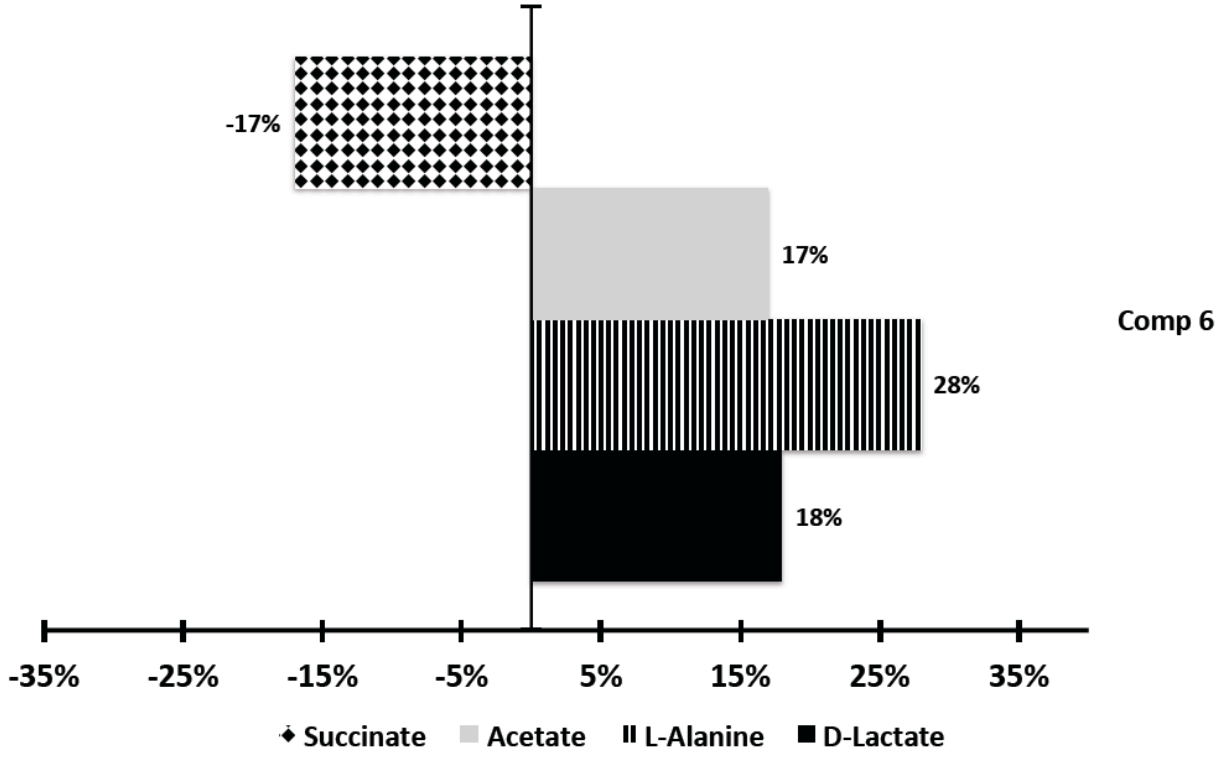

Figure 5. Variation percentages in the height of the peaks corresponding to metabolites excreted by *T. cruzi* epimastigote forms in the presence of comp 6 at its IC_{25} compared to a control sample.

Table 1. In Vitro Activity, Toxicity and Selectivity Index found for polyaminic compounds complex on extracellular and intracellular forms of *Trypanosoma cruzi.*

Compounds	IC_{50} (µM)[a]			Toxicity IC_{50} Vero Cell (µM)[b]	SI[c]		
	Epimastigote forms	Amastigote forms	Trypomastigotes forms		Epimastigote forms	Amastigote forms	Trypomastigotes forms
Bz	15.8±1.1	23.3±4.6	22.4±1.9	13.6±0.9	0.8	0.6	0.6
Comp 1	40.7±2.1	20.8±1.7	19.0±0.8	17.9±3.1	0.4 (1)	0.9 (1)	0.9 (2)
Comp 2	69.9±5.7	19.5±2.2	16.3±0.8	95.6±4.6	1.4 (2)	4.9 (8)	5.9 (10)
Comp 3	52.5±8.5	21.5±2.6	30.0±1.4	80.0±7.7	1.44 (2)	3.7 (6)	2.66 (4)
Comp 4	180.0±8.7	49.5±3.4	44.8±3.7	55.1±2.4	0.3 (1)	1.1 (2)	1.2 (2)
Comp 5	93.8±9.1	34.9±2.3	23.8±0.5	80.2±9.0	0.8 (1)	2.3 (4)	3.4 (6)
Comp 6	7.3±0.8	7.0±0.9	5.7±0.8	71.6±6.3	9.8 (13)	10.2 (17)	12.6 (21)

Results are averages of four separate determinations. [a]IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at concentrations employed (1, 10, 25, 50 and 100 µM). [b]Towards Cell Vero after 72 h of culture. [c]Selectivity index =IC_{50} Cell Vero/IC_{50} extracellularand intracellular form of parasite. In brackets: number of times that compound exceeds the reference drug SI (on extracellular and intracellular forms of *T. cruzi*).