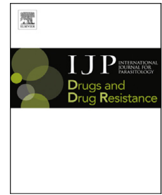
International Journal for Parasitology: Drugs and Drug Resistance 5 (2015) 110–116

Contents lists available at ScienceDirect

International Journal for Parasitology: Drugs and Drug Resistance

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



An *in vitro* iron superoxide dismutase inhibitor decreases the parasitemia levels of *Trypanosoma cruzi* in BALB/c mouse model during acute phase

Francisco Olmo, Kristína Urbanová, Maria Jose Rosales, Ruben Martín-Escolano, Manuel Sánchez-Moreno*, Clotilde Marín

Departamento de Parasitología, Instituto de Investigación Biosanitaria (ibs.GRANADA), Hospitales Universitarios De Granada/Universidad de Granada, Granada, Spain

ARTICLE INFO

Article history:
Received 9 February 2015
Received in revised form
5 May 2015
Accepted 6 May 2015
Available online 20 June 2015

Keywords:
Anti-chagasic
Chemotherapy
Trypanosomiasis
Hydroxyphthalazine derivatives

ABSTRACT

In order to identify new compounds to treat Chagas disease during the acute phase with higher activity and lower toxicity than the reference drug benznidazole (Bz), two hydroxyphthalazine derivative compounds were prepared and their trypanocidal effects against *Trypanosoma cruzi* were evaluated by light microscopy through the determination of IC_{50} values. Cytotoxicity was determined by flow cytometry assays against Vero cells. *In vivo* assays were performed in BALB/c mice, in which the parasitemia levels were quantified by fresh blood examination; the assignment of a cure was determined by reactivation of blood parasitemia levels after immunosuppression. The mechanism of action was elucidated at metabolic and ultra-structural levels, by ^{1}H NMR and TEM studies. Finally, as these compounds are potentially capable of causing oxidative damage in the parasites, the study was completed, by assessing their activity as potential iron superoxide dismutase (Fe-SOD) inhibitors. High-selectivity indices observed *in vitro* were the basis of promoting one of the tested compounds to *in vivo* assays. The tests on the murine model for the acute phase of Chagas disease showed better parasitemia inhibition values than those found for Bz. Compound **2** induced a remarkable decrease in the reactivation of parasitemia after immunosuppression. Compound **2** turned out to be a great inhibitor of Fe-SOD. The high antiparasitic activity and low toxicity together with the modest costs for the starting materials render this compound an appropriate molecule for the development of an affordable anti-Chagas agent.
© 2015 The Authors. Published by Elsevier Ltd on behalf of Australian Society for Parasitology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

American trypanosomiasis is a potentially life-threatening parasitic disease caused by *Trypanosoma cruzi*. There are more than 10–20 million people infected worldwide, mostly in Latin America. Although not a uniform death sentence, *T. cruzi* infection is far from innocuous, as an estimated 30–40% of infected individuals develop debilitating and chronic disease, and this infection accounts for 20,000–50,000 deaths per year (Tarleton and Curran, 2012). Currently, the available drugs used for the treatment of this infection, Benznidazole (Bz) or nifurtimox, show limited therapeutic potential and are associated with serious side effects, such as skin rashes, leucopoenia, neurotoxicity, fever, articular and muscular pain, peripheral neuropathy, lymphadenopathy, agranulocytosis, and thrombocytopenic purpura (Wilkinson et al., 2008; Urbina, 2010). Thus, there is an urgent need for the development of new anti-trypanosomal agents with lower toxicity and greater activity, especially for the chronic phase of the disease. To date, no vaccine has been developed against *T. cruzi* (Dumonteil, 2009). Therefore, the search for new targets for chemotherapy and vaccines is a major challenge. Among the targets, the parasite antioxidant system has attracted attention due to its uniqueness in the trypanosomatids.

Taking into account this need for new drugs to combat *T. cruzi* parasites and considering the previous experience that our group has with this type of chemical structures, the imidazole-based alkylaminophthalazine derivatives (Sánchez-Moreno et al., 2012). In that occasion, we found that the monoalkylaminophthalazine were the more active structures against the acute phase of Chagas disease and we also found out that they were good inhibitors of the

* Corresponding author.
E-mail address: msanchem@ugr.es (M. Sánchez-Moreno).

http://dx.doi.org/10.1016/j.ijpddr.2015.05.002
2211-3207/© 2015 The Authors. Published by Elsevier Ltd on behalf of Australian Society for Parasitology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

parasite specific enzyme Fe-SOD. So, in the present work we considered studying the activity of two hydroxyphthalazine derivatives obtained from the previous selected compounds. In this particular, they were chemically modify to include a hydroxyl group in the main ring, this modification functionalized the molecule increasing its solubility and also giving the molecule an easier ability to future modifications and introduction of new groups. These compounds are quite interesting since their synthesis starts form cheap substrates and the procedures are not very complicated in most of the cases. In this work, their antiproliferative activity and unspecific mammalian cytotoxicity in the species considered were evaluated *in vitro*, and these measures were complemented by infectivity assays on Vero cells. Furthermore, those in whom *in vitro* activity showed remarkable effects were tested *in vivo*. Finally, the parasites were submitted to a thorough study of the possible mechanisms of action of the compounds assayed, as follows: (i) an ^{1}H NMR study concerning the nature and percentage of metabolite excretion was performed in order to obtain information on the inhibitory effect of the compounds on the glycolytic pathway, since it represents the primary source of energy for the parasite, (ii) alterations caused in the cell ultrastructure of the parasites were recorded using transmission electronic microscopy (TEM), and (iii) an enzymatic study of inhibition over the iron superoxide dismutase (Fe-SOD), which represent one of the many mechanisms of antioxidant defence in trypanosomatids.

2. Materials and methods

2.1. Chemistry

Samples of the tested hydroxyphthalazine derivatives **1** and **2** have been donated by the Heterocyclic Synthetic Receptors research group of the Organic Chemistry Department at the Universidad Complutense of Madrid (Sánchez-Moreno et al., 2012, Fig. 1). They are part of a series of compounds that are being currently designed by that group, and their synthesis, characterization and further antiparasitic properties will be described elsewhere.

2.2. Parasite strain culture

T. cruzi SN3 strain of IRHOD/CO/2008/SN3 was isolated from domestic *Rhodnius prolixus*; biological origin is Guajira (Colombia) (Tellez-Meneses et al., 2008). Epimastigote forms were grown in axenic Grace's insect medium (Gibco) supplemented with 10% inactivated foetal bovine serum (FBS) at 28 °C in tissue-culture flasks, Roux flasks (Corning, USA) with a surface area of 75 cm^2, as described by González et al. (2005).

2.3. Transformation of epimastigotes to metacyclic forms

Metacyclogenesis was induced by culturing a 5-day-old culture of epimastigote forms of *T. cruzi* that was harvested by centrifugation at 7000 g for 10 min at 10 °C according to (Cardoso and Soares, 2010).

2.4. Cell culture and cytotoxicity tests

Vero cells (Flow) were grown in RPMI and MEM (Gibco), supplemented with 10% iFBS and the procedure followed was as in Magán et al. (2005).

2.5. In vitro activity assays, extracellular forms

2.5.1. Epimastigotes assay

T. cruzi epimastigotes were collected in the exponential growth phase and distributed in culture trays (with 24 wells) at a final concentration of 5×10^4 parasites/well. The effects on the parasite growth were tested according to Olmo et al. (2013).

2.5.2. Blood trypomastigote forms assay

Compounds 1 and 2 were also evaluated in blood trypomastigotes 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 product. 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.

2.6. In vitro activity assays, intracellular forms: amastigotes assay

Vero cells were cultured in RPMI medium supplemented with 10% iFBS, in a humidified 95% air and 5% CO_2 atmosphere at 37 °C. Then the cells were infected and treated as in González et al. (2005).

2.7. Infectivity assay

Vero cells were cultured in RPMI medium supplemented with 10% iFBS as described above. Afterward, the cells were infected

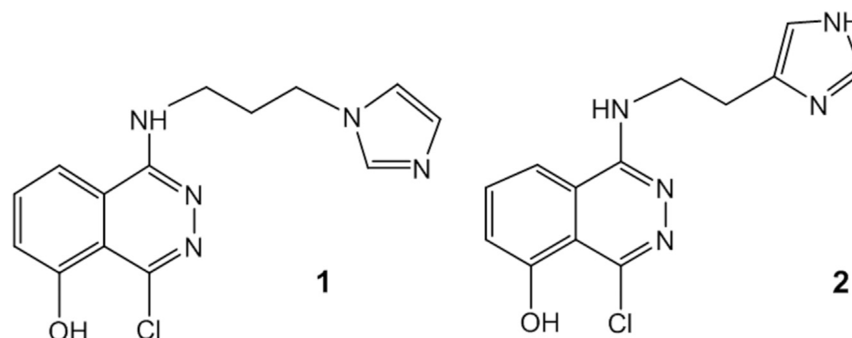


Fig. 1. Chemical structure of the compounds assayed.

in vitro with metacyclic trypomastigote forms of *T. cruzi* at a ratio of 10:1. The assay was performed as in González et al. (2005).

2.8. In vivo trypanosomicidal activity assay

2.8.1. Mice infection and treatment

This experiment was performed with the approval of the Ethical Committee of the University of Granada, Spain. Two groups of six BALB/c albino female mice (6–8 weeks old, 25–30 g weight) were maintained with water and standard chow *ad libitum*, under a 12-h dark/light cycle and 22 ± 3 °C temperature. Each mouse was inoculated by intraperitoneal route with 5×10^5 blood trypomastigotes of *T. cruzi* obtained from previously infected mice blood. Group I was positive control group (mice infected but not treated) and group II was study group (mice infected and treated with the compounds under study). On seventh day of infestation was begun the administration of the selected compounds at doses of 25 mg/kg body mass per day for 5 consecutive days (7–12 days post-infection). Parasitemia levels were evaluated from peripheral blood obtained from the mandibular vein of each mouse (5 μL samples) and dissolved in 495 μL of a PBS solution at a dilution of 1:100. The circulating parasite numbers were quantified with a Neubauer's chamber for counting blood cells and the number of bloodstream forms was expressed as parasites/mL. This counting was performed every 3 days during a 40 day period (acute phase) (Olmo et al., 2014).

2.8.2. Cyclophosphamide-induced immune suppression and assessment of cure

After day 60, parasitaemia showed progressively decreasing levels and it is established that the animals entered the chronic phase of the experiment. Therefore, on day 120, parasitaemia was shown to be undetectable by fresh blood microscopic examination, and the mice received 4 intraperitoneal injections of 200 mg/kg of body mass of cyclophosphamide monohydrate (CP) (ISOPAC®) on alternate days, as previously described (Cencig et al., 2011). Seven days after of the last CP injection, parasitaemia was evaluated according to the procedure described for acute phase to quantify the presence of blood trypomastigote forms as reactivation rate. Finally, mice were bled out, under gaseous anaesthesia (CO_2), via heart puncture and blood was collected. To obtain the serum, blood was incubated for 2 h/37 °C and then overnight/4 °C, followed by centrifuging the supernatant twice at 1000 and 2700 g, consecutively. The serum was aliquoted and used for ELISA and biochemical analysis, as explained below. Hearts were harvested and immediately flushed free of blood by gentle infusion of 10 ml of pre-warmed PBS through the left ventricle (Ye et al., 2008) in order to avoid contamination of the collected tissue with blood parasites. After this, samples were frozen at −80 °C and stored until used for DNA extraction (Olmo et al., 2014).

2.8.3. ELISA tests

Fe-SOD excreted from the parasite, cultured and processed as described in Lopez-Cespedes et al. (2012), was used as the antigen fraction. The ELISA test to measure the antibodies against *T. cruzi* used was performed as in Olmo et al. (2014).

2.8.4. Toxicity tests by clinical chemistry measurements

A fraction of the serum obtained as it was shown above was send to the Biochemical service in the University of Granada where a series of parameters were measured according to their commercial kits acquired from Cromakit® by BS-200 Chemistry Analyzer Shenzhen Mindray (Bio-medical 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% ($100 \times (1-\alpha) = 100 \times (1-0.05)$%). The ranges obtained are shown in Table 2, which allows comparison and analysis of the sera studied in this work.

2.9. Assays to figure out the mechanism of action

2.9.1. Metabolite excretion

Cultures of *T. cruzi* epimastigote forms (initial concentration of 5×10^5 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 ^1H NMR, and the chemical shifts were expressed in parts per million (ppm), using dimethyl sulphoxide (DMSO) as the reference signal. One-dimensional ^1H 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 (Fernandez-Becerra et al., 1997). In addition, the human metabolome database (http://www.hmdb.ca/) was also used for this purpose. The spectral region of 1.0–5.5 ppm was bucketed into a frequency window of 0.1 ppm. The region corresponding to water (4.5–5.5 ppm) was excluded during binning to avoid artefacts due to pre-saturation of water, and the region corresponding to glucose (3.4–3.8 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 (1.0–3.4) 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.

2.9.2. Ultrastructural alterations

The parasites were cultured at a density of 5×10^5 cells/mL in each corresponding medium containing the compounds tested at the concentration of IC_{25}. After 96 h, these cultures were centrifuged at 400 g for 10 min and the pellets produced were washed in PBS before being mixed with 2% (v/v) paraformaldehyde/glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 24 h at 4 °C. Following this, the pellets were prepared for transmission electron microscopy study using a technique described by our group (González et al., 2005).

2.9.3. Superoxide dismutase inhibition assay

The parasites cultured as described above were centrifuged. 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 disrupted by three cycles of sonic disintegration, 30 s each at 60 W. The sonicated homogenate was centrifuged at 1500 g for 5 min at 4 °C, and the pellet was washed three times in ice-cold STE buffer to continue with the supernatant. This fraction was centrifuged (2500 g for 10 min at 4 °C) and the supernatant was collected. The protein concentrations were determined using the Bradford method (Bradford, 1972). Iron and copper–zinc superoxide dismutases (Fe-SOD and CuZn-SOD) activities were determined using the method described by Beyer and Fridovich (Beyer and Fridovich, 1987).

Table 1
In vitro activity, toxicity and selectivity index found for the compounds and the reference drug 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	39.2 ± 2.1	20.8 ± 1.7	29.5 ± 0.8	148.8 ± 3.1	4 (5)	7 (12)	5 (8)
Comp 2	8.5 ± 5.7	7.4 ± 2.2	10.8 ± 0.8	214.7 ± 4.6	25 (31)	29 (48)	20 (33)

In brackets: number of times that compound SI exceeds the reference drug SI. Results are averages of three 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} extracellular and intracellular form of parasite.

Table 2
Summarizes the Biochemical clinical parameters tested in different groups of BALB/c Mice infected with *Trypanosoma cruzi* at different experimental situations.

	Kidney markers profile		Liver markers profile			
	Urea (mg/dL)	Uric acid (mg/dL)	AST/GOT (U/L)	ALT/GPT (U/L)	Total bilirubin (mg/dL)	Phosphatase alkalyne (U/L)
Uninfected mice (n = 15)	39 [36–43]	5 [4.3–5.5]	126 [103–148]	46 [37–54]	0.23 [0.17–0.28]	133 [104–161]
Infected mice-acute phase (n = 15)	49 [39–60]	4.5 [3.7–5.5]	129 [100–157]	48 [38–58]	0.15 [0.12–0.18]	231 [161–300]
Infected mice-chronic phase (n = 15)	49	4.3	148	53	0.12	186
120 days post infection mice and Comp 2125 mg/kg·w treated (n = 6)	=	=	+	+	+	=

Key: =, variation no larger than 10%; +, up to 10% of increasing over the range; ++, up to 30% of increasing over the range; +++, up to 40% of increasing over the range; ++++, more than 50% of increasing over de range; -, up to 10% of decreasing over the range; - -, up to 30% of decreasing over the range; - - -, up to 40% of decreasing over the range; - - - -, more than 50% of decreasing over de range.

3. Results and discussion

3.1. *In vitro trypanosomicidal evaluation*

In order to get preliminary information, *in vitro* activities of **1** and **2** were evaluated against epimastigote, amastigote and trypomastigote forms of *T. cruzi*, as shown in Table 1. It was found that the more sensitive forms to all different compounds tested were the intracellular forms (amastigotas) reaching an effectiveness of 48 times higher than the reference drug in the case of **2**. The compound **1** also showed trypanosomicidal activity against the different forms of the parasite and the effect was always better than that of the reference drug; however, against all forms it showed less than 15 times effectiveness compared with Bz. As a result, compound **2** was chosen to undergo an extra *in vitro* assay and deeper insight of the activity of this compound was found in the infectivity assay, where the process that takes place in the host of the lifecycle of *T. cruzi* in the presence of the drugs was reproduced *in vitro*. Once again, **2** was found to be the more active than the reference drug, decreasing the rate of infection in cells by 80% on the last day of the experiment, as shown in Fig. 2A. It also decreased the average number of amastigote forms found per infected cell; these decreases reached the 57% for cells treated with **2** being more efficient than Bz, which effectively only decreased the number by 15% compared to the control (Fig. 2B). The last data obtained from this assay were regarding the number of trypomastigote forms released by the infected cells, as can be seen in Fig. 2C, where the effect showed a significant decrease of 43% compared to the control assay.

3.2. *In vivo trypanosomicidal evaluation*

Since compound **2** showed remarkable selectivity index (SI, mammalian cells IC_{50}/parasite IC_{50}) values with respect to Bz in the *in vitro* experiments, it was selected for being close to the criteria established by Nwaka et al., 2011 for further *in vivo* studies in the chosen murine model. Its trypanozidal activity during the acute phase of Chagas disease [until 40 days post-infection (pi)] was first investigated. None of the animals treated with either the control or compound **2** died during the treatment. As shown in Fig. 3, the reduction of parasitaemia in mice treated with the compound was evident from the very beginning of the treatment and was maintained until the end, resulting in parasitaemia reduction with respect to the control experiment for **Bz** and **2**.

The next step was to evaluate the behaviour of the compound until the chronic phase. Therefore, the mice treated with the compound under study during the acute phase as described in the material and methods section, were taken up to day 120 pi (advanced chronic phase). In order to evaluate the immune status and the disease extent of the mice at the chronic phase the mice were immunosuppressed as it has been explained in section 2.8.2; then, blood samples were extracted for determining parasitaemia and immunoglobulin G (Ig-G) levels in comparison with the corresponding non-immunosuppressed (control) subgroup of mice (Fig. 4). Concerning the parasitaemia reactivation, Fig. 4A shows a graph indicating the percentage of parasitaemia reactivation for **2** in comparison with the control mice; lower percentages were obtained in the treated case with 54.1% of reactivation, whereas a reactivation of 80.3% was found in the control mice.

The enzyme-linked immunosorbent assay (ELISA) was used for the detection of total Ig-G levels and the antigen source was the Fe-SOD enzyme isolated in our laboratory. The detection of total Ig G allowed evaluation of the immune status of the mice (el Bouhdidi et al., 1994), since that indicates the level of protection that should be attributed to the tested compounds, combined with the innate protection that mice have naturally (Kayama and Takeda, 2010). Results obtained from the ELISA experiments were confirmed by the parasitaemia assay performed as indicated above. In accordance with the ELISA test, the group of mice treated with compound **2** almost maintained levels of total Ig G (Fig. 4B), as did chronic infected mice, lower than that of the control group after being immunosuppressed, while the control group levels were increased as a consequence of the reactivation of parasitaemia.

Clinical chemistry measurements are provided in Table 2. The changes in the transaminases and the total bilirubin, that were observed for the group of mice treated with **2**, were the only differences found when compared to the control group, but these

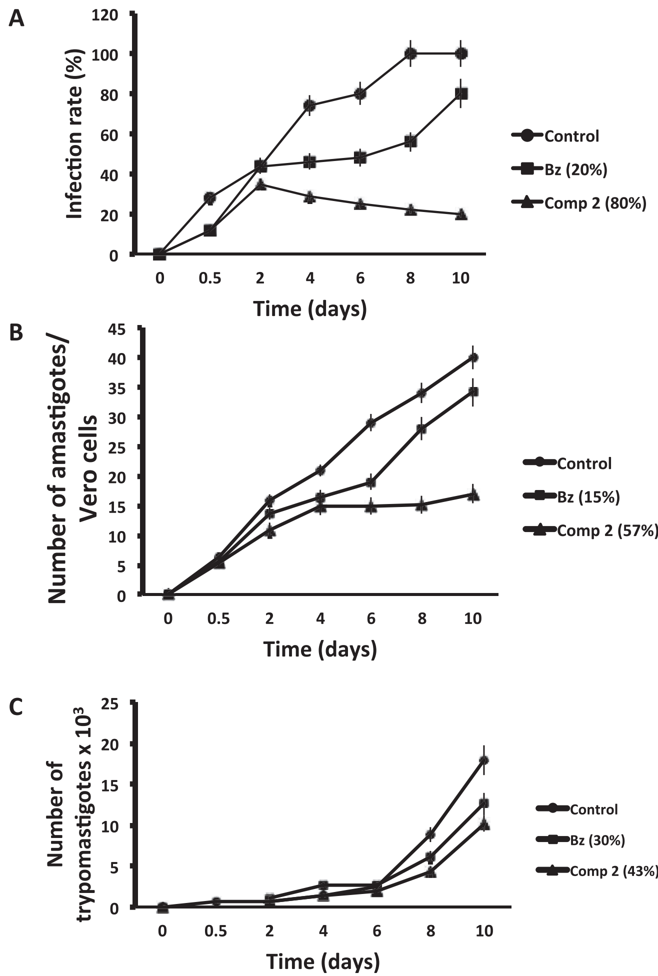


Fig. 2. Reduction of the infectivity of *T. cruzi* in Vero cells treated with **2** and Bz. (A) Rate of infection, (B) mean number of amastigotes per infected Vero cell and (C) number of trypomastigotes in the culture medium. Concentration used was the IC_{25}: 6 μM for **2** and 8.5 μM for Bz Values are the means of three separate experiments.

changes were insignificant since they only reached the 10%. This confirms the evidence that the compounds tested are not toxic in the murine model; in fact, this lack of toxicity added to the better efficacy of **2**, leading us consider this compound as a promising

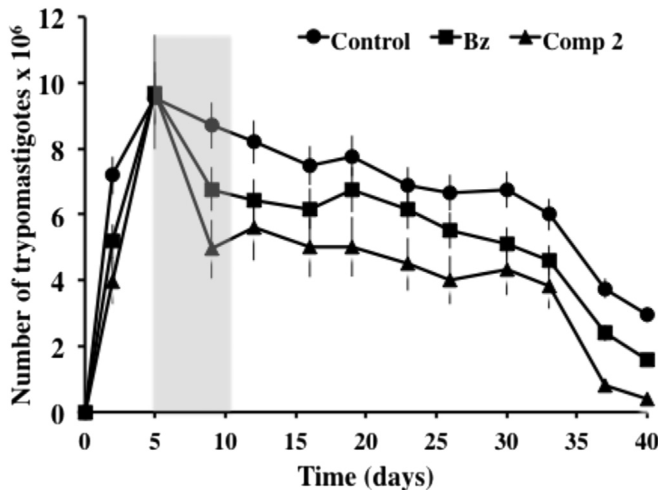


Fig. 3. Parasitemia in the murine model of acute Chagas disease. Grey shade represents the treatment days.

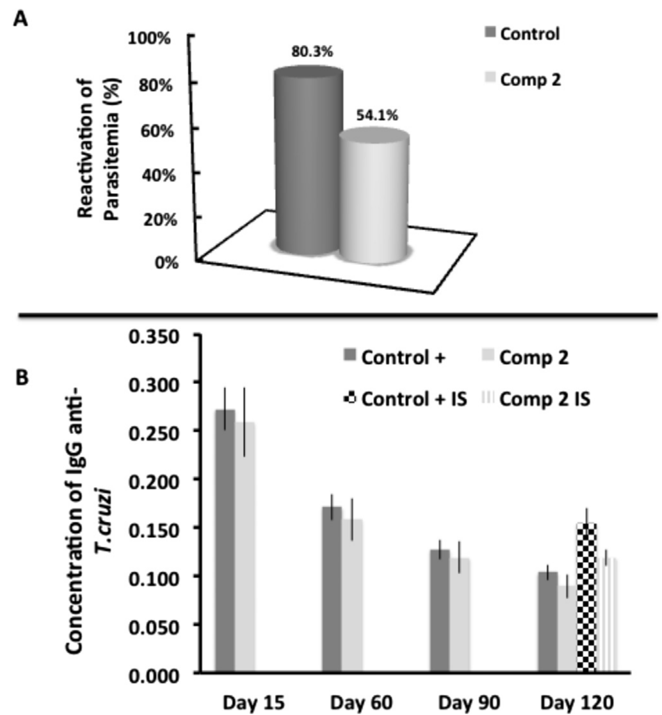


Fig. 4. Immunosuppression *in vivo* assay for mice untreated and treated with 125 mg/kg of body mass of **2**. (A) Shows the reactivation of blood parasitemia after the immunosupression cycles by fresh blood counting comparing to the peak level of parasitemia reached by each group during acute phase. (B) Shows differences in the Ig G levels measured by ELISA at different day post infection for immunosuppressed and non-immunosuppressed group of mice.

candidate for the treatment of Chagas disease. Therefore, the compound should be followed-up in future clinical experiments.

3.3. Possible mechanism of action

Trypanosomatids are unable to completely degrade glucose to CO_2, so they excrete part of the hexose skeleton into the medium as partially oxidised fragments, the nature and percentage of which depend on the pathway used for glucose metabolism (Ginger, 2005). The catabolism products in *T. cruzi* are mainly succinate, acetate, D-lactate and L-alanine (Cazzulo, 1992). In order to obtain some information about the effects of **2** on the glucose metabolism of the parasite, we registered the ^{1}H NMR spectra of *T. cruzi* epimastigotes treated with the test compounds (spectra not shown); the final excretion products were identified qualitatively and quantitatively, and the results obtained were compared with those found for untreated control epimastigotes. Fig. 5 shows the differences found in every case with respect to the control.

Excretion of almost all metabolites was disturbed in the treatment with **2**, with succinate being the most affected, showing an increase of 90%, followed by acetate and D-lactate with decreases of 31% and increases of 12%, respectively. All of these data could be interpreted on the basis of a change in the succinate, D-lactate, pathways occurring in the presence of the compound under investigation. It is well known that D-lactate is formed mainly by the methylglyoxal bypass and L-alanine originate from the transformation of pyruvate in the presence of alanine aminotransferase (Wyllie and Fairlamb, 2011; Bringaud et al., 2006). On the other hand, it is interesting to note that the increase in succinate indicate catabolic changes that could be related to a malfunction of the mitochondria, due to the redox stress produced by inhibition of the mitochondrion-resident Fe-SOD enzyme (Kirkinezos and Moraes,

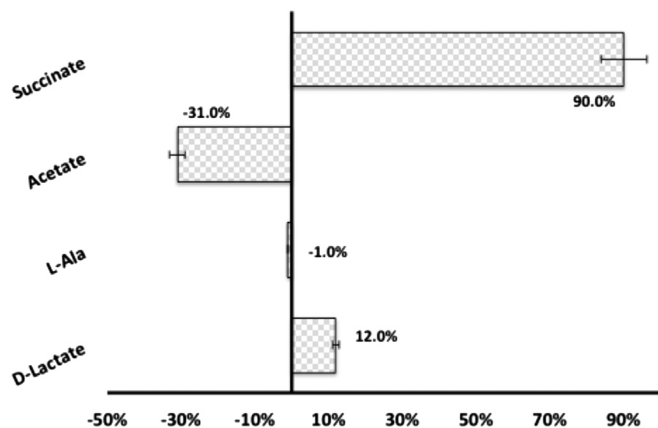


Fig. 5. Variation percentages in the height of the peaks corresponding to catabolites excreted by *T. cruzi* epimastigotes in the presence of **2** at IC_{25}, compared to a control sample after 96 h of incubation.

2001), which should result in decreased pyruvate metabolism and a consequent decrease of the succinate produced in mitochondria. Overall, these data should confirm that the modifications generated in organelles like glycosomes or mitochondria by the compounds assayed are the ultimate reason for the alterations observed in the excreted products of *T. cruzi*.

Modifications at the ultrastructural level caused after incubation of epimastigote forms of *T. cruzi* with the compound under study in the current work, **2**, have been studied. This compound induced strong disturbances in the parasites' morphology and consequently death in some cases. At this level, **2** was found to be the more effective compound, causing more mortality in the parasite cultures compared to the control when the IC_{25} was administered. The parasites were clearly morphologically disturbed when compared with the untreated assay, as shown in Fig. 6. Among the more common disturbances when **2** was added to the medium, as shown in Fig. 6, include the finding that mitochondria were swollen, without cristae and almost unrecognisable; the cytoplasm was also full of small vacuoles, there was a lack of ribosomes and low electron density was appreciated.

These *in vitro* and *in vivo* results prompted us to evaluate the inhibitory effect of compound **2** on SOD activity to test their potential as enzyme inhibitors. The results obtained are shown in Fig. 7, with the corresponding IC_{50} values that were calculated.

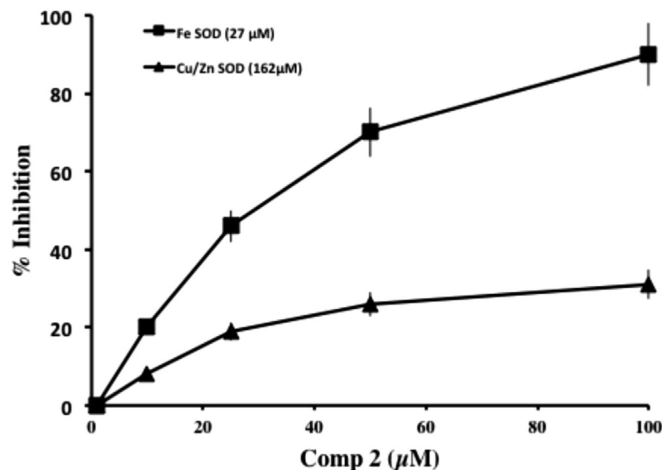


Fig. 7. *In vitro* inhibition (%) of CuZn-SOD from human erythrocytes and Fe-SOD from *T. cruzi* epimastigotes for compound **2** (Cu/Zn-SOD activity 23.36 ± 4.21 U/mg) (Fe-SOD activity 20.77 ± 3.18 U/mg). Differences between the activities of the control homogenate and those ones incubated with compounds were obtained according to the Newman–Keuls test. IC_{50} values are shown in brackets. Values are the average of three separate determinations.

Significant inhibitory values of Fe-SOD activity were found for the tested compound. Compound **2** showed values close to 100% inhibition at 100 μM, with IC_{50} values around 27 μM. The design of an effective drug that is able to inhibit parasite Fe-SOD without inhibiting human Cu/Zn-SOD is an interesting goal. Therefore, we also assayed the effect of the compound **2** on Cu/Zn-SOD in human erythrocytes. The results obtained showed that the inhibition percentages for human Cu/Zn-SOD were lower than for Fe-SOD. Therefore, 162 μM of the studied compound was needed to give a 50% of inhibition.

In conclusion, the trypanocidal properties of compounds **1** and **2** have been examined both *in vivo* and *in vitro*. The two compounds of the present study were chemical modifications of the most efficient compounds previously tested by our group (Sánchez-Moreno et al., 2012). As in the field of medicinal chemistry, any modifications within the structure of the compounds makes that it had to restart the assays from the very first phase. The purpose of this change was the inclusion of a hydroxy group in one of the rings of the pthalazine, this group besides being a new structure with higher polar properties allows for future chemical conjugation to

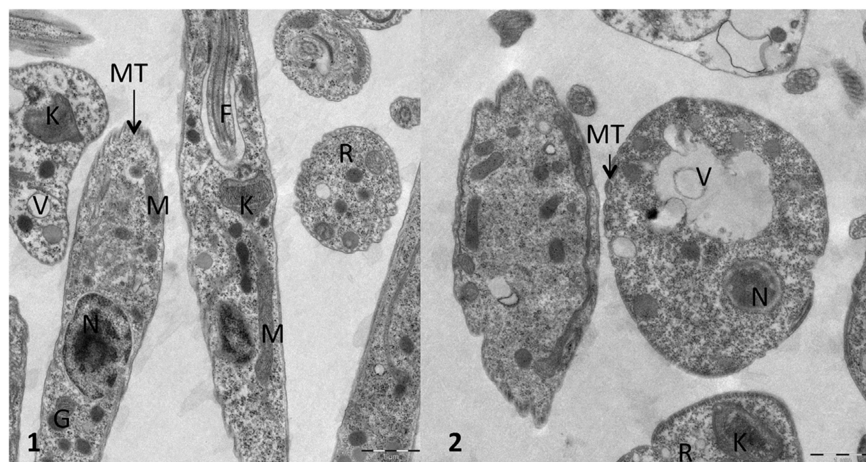


Fig. 6. Ultrastructural alterations shown by TEM in epimastigotes of *T. cruzi* treated with **2** at IC_{25} concentration. (1) Control parasite showing typical organelles such as nucleus (N), mitochondria (M), glycosomes (G), microtubules (MT), vacuoles (V), reservosomes (R), kinetoplast (K) and flagellum (F). (1) Untreated. (2) Treated with **2**. Scale bar = 1 μm.

add new groups and radicals. Although the compounds did not improve the tripanozidal activity *in vitro*, it was found that *in vivo* the toxicity was greatly reduced and therefore permit the administration of higher doses. Also, once again revealed that these chemical structures highly inhibit the parasite enzyme Fe-SOD, as it was anticipated in molecular modelling studies previously performed, (Sánchez-Moreno et al., 2012) confirming once again to be the target for these compounds.

The experiments allowed us to select compound 2, which displayed improved efficiency and lower toxicity than the reference drug. Parallel studies have been carried to establish the mechanisms of action. Compound **2** selectively inhibits the Fe-SOD enzyme of the parasite. Moreover, compound 2 showed an *in vivo* failure of reactivation in parasitaemia after immunosuppression at a dosage of 125 mg/kg body mass. Finally, owing to their promising activity, a further high-level study should be considered to obtain an improved efficiency.

Acknowledgements

We acknowledge financial support from the Spanish Ministry of Economy and Competitiveness (MINECO), CONSOLIDER-INGENIO 2010 CSD2010-00065. F.O. is grateful for a FPU Grant (AP2010-3562) from the Ministry of Education of Spain.

References

Beyer, W.F., Fridovich, I., 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal. Biochem. 161, 559–566.

Bradford, M.M., 1972. A refined and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bringaud, F., Riviere, L., Coustou, V., 2006. Energy metabolism of Trypanosomatids: adaptation to available carbon sources. Mol. Biochem. Parasitol. 149, 1–9.

Cardoso, J., Soares, M.J., 2010. *In vitro* effects of citral on *Trypanosoma cruzi* metacyclogenesis. Mem. Inst. Oswaldo Cruz 5, 1026–1032.

Cazzulo, J.J., 1992. Aerobic fermentation of glucose by trypanosomatids. FASEB J. 6, 3153–3161.

Cencig, S., Coltel, N., Truyens, C., Carlier, Y., 2011. Parasitic loads in tissues of mice infected with *Trypanosoma cruzi* and treated with AmBisome. PLoS Negl. Trop. Dis. 5, e1216.

Dumonteil, E., 2009. Vaccine development against *Trypanosoma cruzi* and *Leishmania species* in the post-genomic era. Infect. Genet. Evol. 9, 1075–1082.

el Bouhdidi, A., Truyens, C., Rivera, M.T., Bazin, H., Carlier, Y., 1994. *Trypanosoma cruzi* infection in mice induces a polyisotypic hypergammaglobulinaemia and parasite-specific response involving high IgG2a concentrations and highly avid IgG1 antibodies. Parasite Immunol. 16, 69–76.

Fernandez-Becerra, C., Sanchez-Moreno, M., Osuna, A., Opperdoes, F.R., 1997. Comparative aspects of energy metabolism in plant trypanosomatids. J. Eukaryot. Microbiol. 44, 523–529.

Ginger, M.L., 2005. Trypanosomatid biology and euglenozoan evolution: new insights and shifting paradigms revealed through genome sequencing. Protist 156, 377–392.

González, P., Marín, C., Rodríguez-González, I., Hitos, A.B., Rosales, M.J., Reina, M., Díaz, J.G., González-Coloma, A., Sánchez-Moreno, M., 2005. *In vitro* activity of C20-diterpenoid alkaloid derivatives in promastigotes and intracellular amastigotes of *Leishmania infantum*. Int. J. Antimicrob. Agents 25, 136–141.

Kayama, H., Takeda, K., 2010. The innate immuneresponse to *Trypanosoma cruzi* infection. Microbes Infect. 12, 511–517.

Kirkinezos, I.G., Moraes, C.T., 2001. Reactive oxygen species and mitochondrial diseases. Cell Dev. Biol. 12, 449–457.

Lopez-Cespedes, A., Villagran, E., Briceño-Alvarez, K., de Diego, J.A., Hernández-Montiel, H.L., Saldaña, C., Sánchez-Moreno, M., Marín, C., 2012. *Trypanosoma cruzi*: seroprevalence detection in suburban population of Santiago de Queretaro (Mexico). Sci. World J. http://dx.doi.org/10.1100/2012/914129.

Magán, R., Marín, C., Rosales, M.J., Salas, J.M., Sánchez-Moreno, M., 2005. Therapeutic potential of new Pt(II) and Ru(III) triazole-pyrimidine complexes against *Leishmania donovani*. Pharmacology 73, 41–48.

Nwaka, S., Besson, D., Ramirez, B., Maes, L., Matheeussen, A., Bickle, Q., Mansour, N.R., Yousif, F., Townson, S., Gokool, S., Cho-Ngwa, F., Samje, M., Misra-Bhattacharya, S., Murthy, P.K., Fakorede, F., Paris, J.M., Yeates, C., Ridley, R., Van Voorhis, W.C., Geary, T., 2011. Integrated dataset of screening hits against multiple neglected disease pathogens. PLoS Negl. Trop. Dis. 5, e1412.

Olmo, F., Marín, C., Clares, M.P., Blasco, S., Albelda, M.T., Soriano, C., Gutiérrez-Sánchez, R., Arrebola-Vargas, F., García-España, E., Sánchez-Moreno, M., 2013. Scorpiand-like azamacrocycles prevent the chronic establishment of *Trypanosoma cruzi* in a murine model. Eur. J. Med. Chem. 70, 189–198.

Olmo, F., Rotger, C., Ramírez-Macías, I., Martínez, L., Marín, C., Carreras, L., Urbanová, K., Vega, M., Chaves-Lemaur, G., Sampedro, A., Rosales, M.J., Sánchez-Moreno, M., Costa, A., 2014. Synthesis and biological evaluation of N,N'-Squaramides with high in vivo efficacy and low toxicity: toward a low-cost drug against chagas disease. J. Med. Chem. 57, 987–999.

Sánchez-Moreno, M., Gómez-Contreras, F., Navarro, P., Marín, C., Olmo, F., Yunta, M.J., Sanz, A.M., Rosales, M.J., Cano, C., Campayo, L., 2012. Phthalazine derivatives containing imidazole rings behave as Fe-SOD inhibitors and show remarkable anti-*T. cruzi* activity in immunodeficient-mouse mode of infection. J. Med. Chem. 26, 9900–9913.

Tarleton, R.L., Curran, J.W., 2012. Is Chagas disease really the “new HIV/AIDS of the Americas”? PLoS Negl. Trop. Dis. 6, e1861.

Tellez-Meneses, J., Mejía-Jaramillo, A.M., Triana-Chavez, O., 2008. Biological characterization of *Trypanosoma cruzi* stocks from domestic and sylvatic vectors in Sierra Nevada of Santa Marta, Colombia. Acta Trop. 108, 26–34.

Urbina, J.A., 2010. Specific chemotherapy of chagas disease: relevance, current limitations and new approaches. Acta Trop. 115, 55–68.

Wilkinson, S.R., Taylor, M.C., Horn, D., Kelly, J.M., Cheeseman, I., 2008. A mechanism for cross-resistance to nifurtimox and Bz in trypanosomes. Proc. Natl. Acad. Sci. 105, 5022–5027.

Wyllie, S., Fairlamb, A.H., 2011. Methylglyoxal metabolism in trypanosomes and leishmania. Semin. Cell Dev. Biol. 22, 271–277.

Ye, X., Ding, J., Zhou, X., Chen, G., Liu, S.F., 2008. Divergent roles of endotelial NF-kappaB in multiple organ injury and bacterial clearance in mouse models of sepsis. J. Exp. Med. 205, 1303–1315.