Simple dialkyl pyrazole-3,5-dicarboxylates show *in vitro* and *in vivo* activity against disease-causing trypanosomatids

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SUMMARY

The synthesis and antiprotozoal activity of some simple dialkyl pyrazole-3,5-dicarboxylates (*2–6*) and their sodium salts (pyrazolates) (*7–9***)** against *Trypanosoma cruzi*, *Leishmania infantum* and *Leishmania braziliensis* are reported. In most cases the studied compounds showed, especially against the clinically significant amastigote forms, *in vitro* activities higher than those of the reference drugs (benznidazole for *T. cruzi* and glucantime for *Leishmania* spp.); furthermore, the low unspecific cytotoxicities against Vero cells and macrophages shown by these compounds led to good selectivity indexes (SI), which are 8−72 times higher for *T. cruzi* amastigotes and 15−113 times higher for *Leishmania* spp. amastigotes than those of the respective reference drugs. The high efficiency of diethyl ester *3* and its sodium salt *8* against the mentioned protozoa was confirmed by further *in vitro* assays on infection rates and by an additional *in vivo* study in a murine model of acute and chronic Chagas disease. The inhibitory capacity of compounds *3* and *8* on the essential iron superoxide dismutase (Fe-SOD) of the aforementioned parasites may be related to the observed antitrypanosomatid activity. The low acute toxicity of compounds *3* and *8* in mice is also reported in this article.

**Key words**: pyrazole,*Trypanosoma*, *Leishmania*, antichagasic activity, leishmanicidal activity, cytotoxicity

KEY FINDINGS

Dialkyl pyrazole-3,5-dicarboxylates display high antitrypanosomatid activity – They are active against different forms of *T. cruzi, L. infantum and L. braziliensis* – Esters display low unspecific cytotoxicity and high selectivity indexes– It is proposed that esters act as inhibitors of superoxide dismutase of parasites

INTRODUCTION

Trypanosomatid protozoa are the etiological agents of several major insect transmitted parasitic illnesses such as Chagas disease (*Trypanosoma cruzi*), leishmaniasis (*Leishmania* spp.) and sleeping sickness (*Trypanosoma brucei* subsp. *rhodesiense* and subsp. *gambiense*). These infections are concentrated in the poorest areas of the planet and are considered the three “Neglected Tropical Diseases” (NTDs) with the highest rates of death (Cavalli and Bolognesi, 2009; Espuelas *et al.*, 2012).

Existing drug treatments for these diseases are far from satisfactory and the development of a vaccine is an unachieved goal. Current therapies are not adequate due essentially to several factors such as the low therapeutic indexes leading to high toxicities and unacceptable side-effects, the emergence of resistant parasites, the difficulty of treatment compliance due to complex protocols, high prices that are unaffordable for the affected countries, etc. These drawbacks of the current therapy make the search for new drugs of urgent need. Nevertheless, owing to the low income of affected population, the investment in the development of new drugs against these diseases has not been financially attractive for pharmaceutical companies, and the interest of academic institutions is rather limited. This bleak picture is changing in recent years thanks to the financial backing from not-for-profit organizations and the involvement of public-private partnerships (Dujardin *et al.*, 2010). In this article we have focused on developing new compounds against the etiological agents of two of the mentioned diseases caused by trypanosomatids, *T. cruzi* and *Leishmania* spp. Several excellent articles covering different aspects and providing a current overview of chemotherapy of Chagas disease (Soeiro and de Castro, 2009; Cerecetto and González, 2010; Sánchez-Sancho *et al.*, 2010; Urbina, 2010; Guedes *et al.*, 2011; Bermudez *et al.*, 2016) and leishmaniasis (Mishra *et al.*, 2007; Santos *et al.*, 2008; Singh *et al.*, 2012; Rajasekaran and Chen, 2015) have been published recently.

In this context, the antichagasic and leishmanicidal properties of some pyrazole-containing benzo[*g*]phthalazines (Sánchez-Moreno *et al.*, 2011; Sánchez-Moreno *et al.*, 2012b) and pyrazole-derived macrocyclic polyamines (Sánchez-Moreno *et al.*, 2012c; Navarro *et al.*, 2014) have been reported in the last years. It has been proposed that these compounds act as inhibitors of iron superoxide dismutase (Fe-SOD), essential for the parasites survival. In all cases, the complexing ability of these compounds has been related to antitrypanosomatid activity. In fact, it has been pointed out that complexation of iron atom may modify the enzyme active site by dissociation of the metal ion, by changes in the coordination geometry or reducing the interaction between the two monomers of the enzyme.

On the other hand, some pyrazole-3-carboxylic acid derivatives have shown to be weak inhibitors of *T. cruzi* proline racemase, which has been validated as a target for the design of new chemotherapeutic agents against Chagas disease (Berneman *et al.*, 2013).

On these bases, taking in mind the important role of azole unit in the complexation of metals by pyrazole-containing macrocycles (Sharma *et al.*, 1994; Lamarque *et al.*, 2001; Escartí *et al.*, 2002; Miranda *et al.*, 2005), we decided to study the activity against *T. cruzi*, *L. infantum* and *L. braziliensis* of some simple dialkyl pyrazole-3,5-dicarboxylates; in order to improve solubility in water, the corresponding sodium 3,5-bis(alkoxycarbonyl)pyrazolates were also tested. Since initial *in vitro* assays showed interesting activity against different morphological forms of the mentioned protozoa, further studies on infection rates and *in vivo* antichagasic activity were also carried out for the more interesting pyrazole derivatives. Complementary analyses of inhibition of Fe-SDS of parasites and acute toxicity of the most active compounds were also conducted.

MATERIALS AND METHODS

*Preparation of dialkyl 1*H*-pyrazole-3,5-dicarboxylates* 2*–*6 and *sodium 3,5-bis(alkoxycarbonyl)pyrazolates* 7–9

Diesters *2–6* were prepared by acid-catalyzed (HCl) esterification of the commercially available dicarboxylic acid *1* with the corresponding alcohols, following a procedure closely related to those previously reported for dimethyl (*2*) (Askew *et al.*, 1997) and diethyl (*3*) (Schenck *et al.*, 1985) pyrazole-3,5-dicarboxylates (Fig. 1). Sodium pyrazolates *7*–*9* were prepared by treatment of esters *2*–*4* with sodium hydroxide, following the procedure reported for the corresponding bis(ethoxycarbonyl) derivative *8* (Reviriego *et al.*, 2006) (Fig. 1). Analytical and spectral data of the obtained compounds are in agreement with their respective structures. Detailed experimental procedures are given as supplementary material.

Fig. 1

*Parasites strains cultures*

Epimastigotes of *T. cruzi* SN3 strain (IRHOD/CO/2008/SN3) isolated from domestic *Rhodnius prolixus* from Colombia (Téllez-Meneses *et al.*, 2008), and promastigotes of *L. infantum* (MCAN/ES/2001/UCM-10) and *L. braziliensis* (MHOM/BR/1975/M2904) were cultivated *in vitro* in medium trypanosomes liquid (MTL) with 10% inactivated foetal bovine serum and were kept in an air atmosphere at 28 °C in Roux flasks (Corning, USA) with a surface area of 75 cm2, as reported is previous articles (González *et al.*, 2005; Marín *et al.*, 2013; Olmo *et al.*, 2014c).

*Cells culture and cytotoxicity tests*

Vero cells (ECACC number 84113001) were grown in RPMI (Gibco), supplemented with 10% inactivated foetal bovine serum in a humidified 95% air, 5% CO2 atmosphere at 37 ºC for two days. The cytotoxicity test for Vero cells was performed according to a previously described methodology (Marín *et al.*, 2011). In summary, Vero cells cultures were treated with the compounds to be tested at concentrations 100, 50, 25, 10 and 1 μM. After 72 h cell viability was determined by flow cytometry (FACSVantage flow cytometer, Becton Dickinson) using propidium iodide and fluorescein diacetate to detect cells with plasma membrane damaged or intact, respectively. The viability percentage was calculated in comparison with the control culture. The IC50, i.e. the concentration required to give 50% of growth inhibition, was calculated using linear regression analysis from the *K*c values corresponding to the concentrations employed. Values included in Table 1 are the means of three separate determinations.

On the other hand, J774.2 macrophages (ECACC number 85011428) were grown in MEM medium, supplemented with 20% inactivated foetal bovine serum in a humidified 95% air and 5% CO2 atmosphere at 37 ºC for two days. The cytotoxicity test for macrophages was performed according to the procedure described for Vero cells (Marín *et al.*, 2011). The obtained values are gathered in Table 2.

In vitro *activity against extracellular forms of* T. cruzi, L. infantum *and* L. braziliensis*.*

The assayed compounds and the reference drugs (benznidazole or glucantime) were dissolved in dimethyl sulfoxide, which at a final concentration of 0.01% was shown to be nontoxic and without inhibitory effects on parasite growth. The compounds to be evaluated were added to the culture medium of epimastigotes (*T. cruzi*) or promastigotes (*Leishmania* spp.) at concentrations 100, 50, 25, 10 and 1 μM, and the effect of each compound was tested at 72 h using a Neubauer haemocytometric chamber according to a reported procedure (González *et al.*, 2005; Ramírez-Macías *et al.*, 2012). The antiparasitic effect is expressed as the IC50, calculated by linear regression analysis from the *K*c values corresponding to the concentrations employed. Results gathered in Tables 1 and 2 are the average values of four separate experiments.

In vitro *activity against intracellular forms of* T. cruzi*,* L. infantumandL. braziliensis*.*

According to previously developed experimental procedures (Ramírez-Macías *et al.*, 2012), Vero cells (for *T. cruzi*) or macrophages (for *Leishmania* spp.) were grown as commented in the section “*Cells culture and cytotoxicity tests”*. Cells were seeded at a density of 1 × 104 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultured for 2 days. Afterwards the cells were infected *in vitro* with metacyclic forms of *T. cruzi* or promastigotes of *L. infantum* or *L. braziliensis* during 24 h. The non-phagocytosed parasites were removed by washing, and then the drugs were added at concentrations 100, 50, 25, 10 and 1 μM. Cells with the drugs were incubated for 72 h at 37 °C in 5% CO2. Drug activity was determined on the basis of number of amastigotes in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The number of amastigotes was determined by analysing 200 host cells distributed in randomly chosen microscopic fields. The antitrypanosomatid effect is expressed as the IC50 values. Results given in Tables 1 and 2 are averages of four separate experiments.

*Infectivity assays with* T. cruzi, L. infantum *and* L. braziliensis*.*

According to a previously described method (Marín *et al.*, 2011; Ramírez-Macías *et al.*, 2012), Vero cells (for *T. cruzi* infection) or macrophages (for *Leishmania* spp. infection) were grown under the same conditions expressed in the previously described intracellular forms assay during two days. The cells were then infected *in vitro* with metacyclic forms of *T. cruzi* or promastigotes of *L. infantum* or *L. braziliensis*. The tested drugs (IC25 concentrations) were added immediately after infection and the cultures incubated for 12 h at 37 °C in 5% CO2. After removal of non-phagocytosed parasites and drugs by washing, the infected cultures were grown for 10 days in fresh medium. Activity of the different compounds was determined from the percentage of infected cells and the number of amastigotes per infected cell in treated and untreated cultures, in methanol-fixed and Giemsa-stained preparations; in the case of infection with *T. cruzi*, number of trypomastigotes in the culture medium was also determined. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analyzing 200 host cells distributed in randomly chosen microscopic fields. Values represented in Fig. 2, 5 and 6 are the mean values of four separate experiments.

In vivo *trypanocidal activity*

According to previous reports (Marín *et al.*, 2011; Olmo *et al.*, 2014c), groups of 6 BALB/c female mice (6 to 8 weeks old, 20–25 g) were infected with 1 × 105 *T. cruzi* metacyclic forms by the intraperitoneal route. The animals were divided into the following groups: (I) group 1: untreated (infected with *T. cruzi* but not treated; study of acute and chronic phases of disease); (II) group 2: treated [infected and treated for five consecutive days (5 to 10 days post-infection) with the tested compounds (*3* and *8*; 50 mg/kg/day doses)]. This animal experiment was performed with the approval of the Bioethical Committee of the University of Granada.

A blood sample (5 μL) drawn from the mandibular vein of each treated mouse was taken and diluted 1:15 (50 μL of citrate buffer: 0.1 M citric acid, 0.1 M sodium citrate and 20 μL of lysis buffer at pH 7.2: 2 M Tris–Cl, MgCl2). The parasites were counted by the Neubauer chamber. The number of bloodstream *T. cruzi* forms was recorded every 2 days from 5 to 30 days post-infection. The number of trypomastigotes was expressed as parasites/mL. The obtained mean values are gathered in Fig. 3.

After day 60, the animals reach the chronic phase and parasitemia decreases independently of the treatment; in fact, day 120 post-infection blood parasites are undetectable by microscopic examination. This day an immunosuppression treatment with cyclophosphamide is applied to subgroups of animals untreated and treated with compounds *3* and *8* as reported (Cencig *et al.*, 2011; Olmo *et al.*, 2015), and parasitemia reactivation is calculated as before by counting blood trypomastigotes (Fig. 4A).

Additionally, quantitative evaluation of circulating anti-*T. cruzi* antibodies was performed at days 15, 60, 90 and 120 post-infection using an enzyme-linked immunoassay (ELISA). The sera, diluted 1:100 in PBS, were reacted with an antigen composed of a soluble Fe-SOD of *T. cruzi* epimastigotes isolated in our laboratory (Longoni *et al.*, 2011; Sánchez-Moreno *et al.*, 2012a). The results are expressed as the ratio of the absorbance (Abs) of each sample at 490 nm to the cutoff value. The cutoff for each reaction was the mean of the values determined for the negative controls plus three times the standard deviation (Fig. 4B).

On the other hand, non-immunosuppressed subgroups of mice, untreated and treated with compounds *3* and *8*, are sacrificed (day 120) and the hearts extracted for detecting the presence of the parasite by PCR using specific primers for the gene of *T. cruzi* Fe-SOD (sod-b) as reported (Olmo *et al.*, 2014b, 2015) (Fig. 4C).

*Acute toxicity study*

This study was carried out using albino mice of BALB/c strain. Doses of 100 mg/kg of compounds *3* and *8* were initially administered on alternative days in order to determine the median lethal dose (LD50). Since after administration of 500 mg/kg neither apparent toxic effects nor deaths were observed, doses of 500 mg/kg of each compound were given on alternative days to reach 6 g/kg. At this point it was decided to cease the experiment considering that these levels of administration of the compound evidenced their very low toxicity.

*SOD inhibition assays*

Iron superoxide dismutase (Fe-SOD) activity was measured in homogenates obtained from parasites cultured as described in the section *“Parasites strains cultures”* and disrupted by sonic disintegration. CuZn-SOD was obtained from Sigma Chemical Co. The studied compounds were added at concentrations 100, 50, 25, 10, 5 and 1 μM, and SOD activities were determined spectrophotometrically following a reported procedure based on the reduction of nitroblue tetrazolium (NBT) by superoxide ions (Beyer Jr. and Fridovich, 1987; Olmo *et al.*, 2014a). Differences between activities of the control homogenate and those incubated with compounds *3* and *8* were obtained according to the Newman-Keuls test. Values of SOD inhibition included in Fig. 7 are the averages of three separate determinations; IC50 were calculated by lineal-regression analysis from the inhibition values at the concentrations employed.

RESULTS AND DISCUSSION

In vitro *anti-*T. cruzi *evaluation*

Epimastigotes of *T. cruzi* SN3 strain were used for the initial *in vitro* evaluation of compounds *2–9*; this extracellular insect vector stage is easily handled in the laboratory and is very appropriate for preliminary studies of antichagasic activity. Further studies were carried out on amastigotes, intracellular mammalian host cells forms, and consequently, more interesting from the point of view of human disease; with this aim, Vero cells were infected with metacyclic forms of the parasite, which transformed quickly into amastigotes. Vero cells were also used as a model for the study of unspecific cytotoxicity of pyrazole derivatives against mammalian cells. Compounds *2–9* as well as the standard drug benznidazole were assayed at concentrations of 1–100 μM; *in vitro* activities (IC50 values) of these compounds on epimastigotes and amastigotes of *T. cruzi*, the unspecific cytotoxicity against Vero cells (IC50 values) and the corresponding selectivity indexes (SI) are gathered in Table 1.

Table 1

All compounds showed good activities against the two stages of the parasite (IC50 = 9.3*–*40.5 μM), of the same order or slightly smaller or larger than those of the reference drug. The most active compounds, especially against amastigotes, were diethyl ester *3* (IC50 = 10.8 μM) and the corresponding sodium pyrazolate *8* (IC50 = 9.3 μM). The major differences among the assayed compounds were related to unspecific cytotoxicities against Vero cells, reflected in the obtained SI indexes. Considering all the data included in Table 1, compounds *3* and *8*, with SI values above 25−72 times those of benznidazole depending on the stage of the parasite, showed to be the best compounds.

Fig. 2

Following the *in vitro* tests, a study of *T. cruzi* propagation in Vero cells with and without addition of the products showing highest activity in the previously described assays, compounds *3* and *8*, was carried out (Fig. 2). During a 10-days period, different parameters such as the percentage of infection, the average number of amastigotes per infected Vero cell and the number of trypomastigotes present in the culture medium were studied. Following the infection of Vero cells with metacyclic forms, the parasites invaded the former and underwent morphological conversion to amastigotes within 1 day. In the absence of drugs, the rate of host-cell infection reached its maximum, ca. 92% of invasion, on day 10 (Fig. 2A). Then the assay was carried out in the presence of compounds *3* and *8* at their IC25 concentrations in order to see their effects but minimising the number of dead parasite cells; in these cases, at the end of the experiment, the rate of infection decreased 54% and 48% for *3* and *8*, respectively, in relation to the untreated control; taking in mind that typical values for benznidazole in this test are around 23% of reduction (Sánchez-Moreno *et al.*, 2012c), compounds *3* and *8* were shown to be much more efficient than the reference drug.

On the other hand, in the absence of drugs, the number of amastigotes per Vero cell gradually increased during the 10-days period to a value of ca. 57 (Fig. 2B). Treatment with compounds ***3*** and ***8*** significantly reduced the number of amastigotes per cell in 86% and 56%, respectively, while benznidazole showed typically in this test only a reduction of 13% in relation to the control (Sánchez-Moreno *et al.*, 2012c).

Finally, trypomastigotes in the culture medium are coming from the rupture of Vero cells infected by amastigotes; the number of trypomastigotes/mL found in the untreated culture medium (Fig. 2C) reached a maximum (ca. 9.5 × 103) on day 10. Once again this value was substantially reduced by compounds *3* and *8* (54% and 49%, respectively), values slightly better than those typically found for benznidazole in this assay (ca. 46% of decrease) (Sánchez-Moreno *et al.*, 2012c). Summarizing, the results of propagation of parasite in Vero cells agree with those of trypanocidal activity gathered in Table 1 for the different morphological stages of *T. cruzi*.

Discrepancies sometimes observed in all these experiments between the values found for esters and for their respective sodium salts may be due to pharmacokinetic issues, mainly the low solubility of the former in water.

In vivo *anti-*T. cruzi *evaluation*

Considering the promising results obtained in the previously mentioned *in vitro* assays, compounds *3* and *8* were selected for the study of *in vivo* activity on infected female BALB/c mice. Both significant stages of Chagas disease, i.e., the acute and the chronic phases, were taken into account. For the acute phase experiments, the first 30 days after infection were considered. Mice were inoculated with *T. cruzi* metacyclic forms and treatment with compounds *3* and *8* (50 mg/kg/day doses) was initiated intraperitoneally 5 days after infection and continued for 5 days. Different control groups were included in the study as described in “*Materials and Methods*” section. The level of parasitemia, expressed as the number of circulating trypomastigotes/mL of blood vs days since infection, was determined every 2 days (Fig. 3). On the days of maximum parasite load (ca. 15 days after infection) the assayed compounds decreased significantly the number of trypomastigotes. At the end of the acute phase period (day 30), compounds *3* and *8* reduced the level of parasitemia by 88 and 53%, respectively, in relation to the control, values significantly higher than those found typically for the reference drug benznidazole (ca. 25%) (Sánchez-Moreno *et al.*, 2012c). None of the control animals or those treated with compounds *3* and *8* died during the treatment; however, similar assays performed in the presence of benznidazole always led to mice mortality values of about 20% (Sánchez-Moreno *et al.*, 2012c).

Fig. 3

In relation to the last stage of the disease, the mice used for the study of the acute phase were kept up to day 120, after which the advanced chronic phase was reached and blood parasites could not be detected by microscopic examination; this day mice were divided into two subgroups, one that was kept under the same conditions and another that was subjected to immunosuppression induced by cyclophosphamide. This treatment allowed determining the degree of reactivation of parasitemia and therefore the parasitological cure of animals. As shown in Fig. 4A, the percentage of parasitemia reactivation after immunosuppression was 61% for the untreated control, while the values for compounds *3* and *8* were 5% and 24%, respectively. Additionally, variations observed in the concentrations of antibodies against *T. cruzi,* showing the immunological state of the mouse, were also recorded as shown in Fig. 4B; after reaching the chronic phase an immunological balance is achieved in all cases; this equilibrium stage is artificially broken after immunosuppression, which induces in the untreated control a considerable increase in immunoglobulin G (Ig G) levels as a result of the presence of parasite, while in animals treated with compounds *3* and *8*, levels remain fairly constant, indicating lower parasitemia. Finally, results of post-mortem analysis of parasitemia clearance in heart tissue of immunocompetent animals using the PCR technique are gathered in Fig. 4C. We have found that after treatment with compound *3*, the heart tissue of 66% of mice are clean of parasites (lanes 1–3), while the percentage drops to 33% in those treated with compound *8* (lanes 4–6).

Fig. 4

In vitro *leishmanicidal activity evaluation*

*In vitro* activity of compounds *2–9* on promastigote and amastigote forms of *L. infantum* and *L. braziliensis*, representative species causing respectively visceral and cutaneous leishmaniasis, the unspecific cytotoxicity against macrophages and the corresponding selectivity indexes (SI) are gathered in Table 2. Early *in vitro* studies are usually performed on promastigotes since they are more easily handled in the laboratory. Nevertheless, results obtained for this extracellular insect stage are routinely complemented with studies conducted on amastigotes, which are the forms parasitizing the cells of the mammalian host and constitute the real goal of antileishmanial chemotherapy. Assays with amastigotes were performed by infecting macrophages with promastigotes, which were transformed into amastigotes within 1 day after infection. Pyrazoles *2–9* as well as the standard drug glucantime (meglumine antimoniate) were assayed at concentrations of 1–100 μM; the obtained data enabled the calculation of IC50 values gathered in Table 2.

Table 2

Activities of these compounds against *Leishmania* range from c. 11 times to 3/4 of those of the reference drug, depending on the concrete species and the morphological state. Once more, there are great differences in toxicities against macrophages, which are of course reflected in the corresponding selectivity indexes, higher in most cases for amastigotes than for promastigotes (Table 2).

Additionally, as described above for *T. cruzi*, the effect of representative active compounds *3* and *8* on the propagation in macrophages of *L. infantum* and *L. braziliensis* was studied by measuring the rates of infection and the average number of amastigotes per macrophage present during a 10-days experiment. During this period the rate of infection of the host cells gradually increased reaching at the end 73% of invasion for *L. infantum* (Fig. 5) and 81% for *L. braziliensis* (Fig. 6).

For *L. infantum* similar experiments were conducted in the presence of the mentioned pyrazole derivatives at their IC25 concentrations (Fig. 5A). Compounds *3* and *8* were more effective than the reference drug glucantime [typical value: 24% reduction (Marín *et al.*, 2013) in relation to the untreated control], displaying infection rates decreases of 59 and 53%, respectively. In relation to the number of amastigotes per macrophage (Fig. 5B), related results were obtained, showing pyrazole derivatives *3* and *8* decreases of 37 and 58%, respectively, higher than those typically obtained for the reference drug (ca. 30% reduction) (Marín *et al.*, 2013).

Fig. 5

In the case of *L. braziliensis* compounds *3* and *8* were subjected to a similar infection rate study (Fig. 6A), showing that they were more effective (55 and 51% decrease in relation to the control, respectively) than the reference drug for which typical values of ca. 22% decrease have been reported in this kind of assay (Marín *et al.*, 2013). In connection with the average number of amastigotes per macrophage (Fig. 6B), glucantime shows typically a reduction of ca. 37% relative to the control (Marín *et al.*, 2013), while compounds *3* and *8* significantly decrease this value in 50 and 45%, respectively.

In conclusion, the differences among the IC50 values of compounds *3* and *8* gathered in Table 2, lower than those of glucantime for amastigotes, are also reflected in Fig. 6. According to the latter, compounds *3* and *8* are in all cases much more efficient than the reference drug.

*Acute toxicity in mice*

Acute toxicity of compounds *3* and *8* was shown to be very low. After progressive dose increases up to 500 mg/kg of each compound on alternative days to reach 6 g/kg, neither apparent toxic effects nor deaths were observed. A week after cessation of treatment, a routine analysis of biochemical parameters was performed (not shown) and no significant changes were observed. The only finding was that in the 15−20 min immediately after administration of the compounds and after their transfer to the cage, mice displayed some hyperactivity, which ceased after this period and left no sequel or appreciable change in behaviour.

*Possible deciphered of the mechanisms of action: inhibition of Fe-SOD of* T. cruzi*,* L. infantum *and* L. braziliensis *assays*

As mentioned in the Introduction, antitrypanosomatid activity of some pyrazole derivatives has been related to their ability to interact with Fe-SOD of parasites (Sánchez-Moreno *et al.*, 2011; Sánchez-Moreno *et al.*, 2012b; Sánchez-Moreno *et al.*, 2012c; Navarro *et al.*, 2014). Therefore, in order to verify if this mode of action contributes to the activity of pyrazole derivatives treated in the current work, we studied the effect of compounds *3* and *8* on the parasites enzymes. Compounds were assayed at concentrations 1−100 μM, and the inhibition data (IC50 values) obtained for CuZn-SOD from human erythrocytes, and for *T. cruzi*, *L. infantum* and *L. braziliensis* Fe-SOD are shown in Fig. 7. The inhibition of *T. cruzi* enzyme was substantially higher than that of the human origin, 1.7 and 28.7 times for compounds *3* (Fig. 7A) and *8* (Fig. 7B), respectively. Results were especially noteworthy for compound *8*, which showed an IC50 value of ca. 5 μM for *T. cruzi* Fe-SOD and reached 100% inhibition of this enzyme at ca. 10 μM concentration.

For *L. infantum* and *L. braziliensis*, the obtained inhibition data for compounds *3* and *8* are shown in Fig. 7A and 7B, respectively. In this case, inhibition of Fe-SOD from both species of *Leishmania* was also higher (1.6−4.3 times) than that of human erythrocytes CuZn-SOD, but values were not as remarkable as those previously found for *T. cruzi* enzyme. Once more, compound *8* gave the best results, but concentrations near 100 μM were required to achieve 100% inhibition of Fe-SOD of both *Leishmania* species.

Fig. 7

CONCLUSIONS

In the current work we describe the high trypanocidal activity of some simple and very easily available dialkyl pyrazole-3,5-dicarboxylates against *T. cruzi*, *L. infantum* and *L. braziliensis*. The high efficiency of these compounds has been demonstrated by *in vitro* tests against various morphological forms of these parasites, and in the case of *T. cruzi*, by an *in vivo* study in a murine model of Chagas disease.

It is proposed that the activity of these compounds may be due, at least in part, to their ability to inhibit Fe-SOD enzyme, essential for parasites survival. Further studies are required to verify if the inhibition of parasites proline racemase may also be contributing to the activity shown by our pyrazole-3,5-dicarboxylic acid derivatives.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://............

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LEGENDS TO FIGURES

Fig. 1. Synthesis of dialkyl pyrazole-3,5-dicarboxylates *2–6* and the sodium salts (pyrazolates) *7–9*.

Fig. 2. Effect of pyrazole derivatives *3* and *8* (IC25 concentrations) on the infection rate and growth of *T. cruzi* in Vero cells: (A) rate of infection; (B) mean number of amastigotes per infected Vero cell; (C) number of trypomastigotes/mL in the growth medium.

Fig 3. *T. cruzi* parasitemia in the murine model of acute Chagas disease: effect of pyrazole derivatives *3* and *8* (50 mg/kg/day doses during 5 days). The gray shading represents the days of treatment.

Fig. 4. Murine model of chronic Chagas disease: (A) *T. cruzi* parasitemia reactivation percentage following administration of an immunosuppressive treatment for both the control group and the group of mice treated with compounds *3* and *8*; (B) total levels of anti-*T. cruzi* Ig G measured by ELISA, expressed in absorbance units (OD, optical density) for the control and treated groups at different days post-infection; IS: immunosuppressed subgroup; results are expressed as the mean values ± standard deviations; (C) post-mortem PCR analysis of heart tissue of non-immunosuppressed mice at the end of experiment; lanes: (M) base pair marker; (+) PCR positive control; (-) PCR negative control; (1–3) mice treated with compound *3*; (4–6) mice treated with compound *8*.

Fig. 5. Effect of pyrazole derivatives *3* and *8* (IC25 concentrations) on the infection rate and growth of *L. infantum* in macrophages: (A) rate of infection; (B) mean number of amastigotes per infected macrophage.

Fig. 6. Effect of pyrazole derivatives *3* and *8* (IC25 concentrations) on the infection rate and growth of *L. braziliensis* in macrophages: (A) rate of infection; (B) mean number of amastigotes per infected macrophage.

Fig. 7. *In vitro* inhibition (%) of CuZn-SOD of human erythrocytes, and Fe-SOD of *T. cruzi* epimastigotes, and *L. infantum* and *L. braziliensis* promastigotes: (A) for compound *3*; (B) for compound *8*. In brackets, IC50 values for the indicated SOD enzymes from different origin.

Table 1. *In vitro* activity, unspecific cytotoxicity and selectivity index (SI) found for pyrazole derivatives *2–9* and benznidazole (reference drug) on extra- and intracellular forms of *T. cruzi*.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Compounds | IC50 (µM)a | | Vero cells  toxicity  IC50 (µM)a | SIb | |
| Epimasti-gote forms | Intracellu-lar amastigote forms | Epimasti-gote forms | Intracellu-lar amastigote forms |
| Benznidazole | 15.8 ± 1.1 | 23.3 ± 4.6 | 13.6 ± 0.9 | 0.9 | 0.6 |
| *2* | 40.5 ± 6.3 | 16.9 ± 2.8 | 186.5 ± 9.3 | 4.6 (5) | 11.0 (18) |
| *3* | 17.2 ± 2.4 | 10.8 ± 0.7 | 385.4 ± 11.5 | 22.4 (25) | 35.7 (59) |
| *4* | 38.7 ± 7.7 | 23.6 ± 1.4 | 154.5 ± 12.5 | 4.0 (4) | 6.5 (11) |
| *5* | 31.2 ± 3.8 | 30.9 ± 4.7 | 179.4 ± 13.1 | 5.7 (6) | 5.8 (10) |
| *6* | 35.8 ± 4.1 | 23.7 ± 3.3 | 242.1 ± 10.6 | 6.8 (7) | 10.2 (17) |
| *7* | 27.5 ± 3.2 | 27.1 ± 1.1 | 139.1 ± 9.3 | 5.0 (6) | 5.1 (9) |
| *8* | 16.5 ± 1.6 | 9.3 ± 5.8 | 404.1 ± 13.3 | 24.5 (27) | 43.4 (72) |
| *9* | 34.5 ± 2.2 | 34.5 ± 1.7 | 170.2 ± 13.8 | 4.9 (6) | 4.9 (8) |

a IC50 = concentration required to give 50% inhibition, calculated by linear regression analysis from the *K*c values at concentrations employed (1, 10, 25, 50 and 100 µM); *K*c (culture growth constant) corresponds to the slope resulting from plotting the log of the growth measurement versus time for each drug concentration. b Selectivity index (SI) = IC50 Vero cells/IC50 extra- and intracellular forms of parasites. In brackets: number of times that compound SI exceeds the reference drug SI on the different morphological forms of parasites.

Table 2. *In vitro* activity, unspecific cytotoxicity and selectivity index (SI) found for pyrazole derivatives *2–9* and glucantime (reference drug) on extra- and intracellular forms of *L. infantum* and *L. braziliensis*.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Compounds | IC50 (µM)a | | | | Macro-phages toxicity  IC50 (µM)a | SIb | | | |
| *L. infantum* | | *L. braziliensis* | | *L. infantum* | | *L. braziliensis* | |
|  | Promasti-gote forms | Intracellu-lar amastigote forms | Promasti-gote forms | Intracellu-lar amastigote forms | Promasti-gote forms | Intracellu-lar amastigote forms | Promasti-gote forms | Intracellu-lar amastigote  forms |
| Glucantime | 18.0 ± 3.1 | 30.0 ± 2.7 | 25.6 ± 1.6 | 31.1 ± 3.0 | 15.2 ± 1.3 | 0.8 | 0.5 | 0.6 | 0.5 |
| *2* | 23.5 ± 4.2 | 18.4 ± 2.0 | 21.4 ± 1.7 | 18.2 ± 0.9 | 166.2 ± 11.4 | 7.1 (9) | 9.0 (18) | 7.8 (13) | 9.1 (18) |
| *3* | 24.4 ± 1.5 | 16.3 ± 0.9 | 18.8 ± 1.8 | 9.3 ± 0.8 | 233.5 ± 15.6 | 9.6 (12) | 14.3 (29) | 12.4 (21) | 25.1 (50) |
| *4* | 15.6 ± 0.8 | 7.6 ± 0.6 | 16.0 ± 1.4 | 17.9 ± 2.2 | 170.2 ± 13.5 | 10.9 (14) | 22.4 (45) | 10.6 (18) | 9.5 (19) |
| *5* | 19.7 ± 0.8 | 2.8 ± 0.7 | 32.1 ± 2.9 | 21.5 ± 1.5 | 158.1 ± 7.4 | 8.0 (10) | 56.1 (113) | 4.9 (8) | 7.35 (15) |
| *6* | 24.8 ± 2.6 | 19.1 ± 1.1 | 36.2 ± 3.6 | 20.8 ± 1.4 | 271.1 ± 16.1 | 10.9 (14) | 14.2 (28) | 7.5 (13) | 13.0 (26) |
| *7* | 17.5 ± 3.2 | 22.4 ± 1.5 | 15.9 ± 0.8 | 23.2 ± 3.5 | 222.4 ± 21.2 | 12.7 (16) | 9.9 (20) | 14.0 (23) | 9.6 (19) |
| *8* | 5.9 ± 0.2 | 6.7 ± 1.0 | 23.8 ± 3.2 | 6.8 ± 0.7 | 288.6 ± 23.1 | 48.9 (61) | 43.1 (86) | 12.1 (20) | 42.4 (85) |
| *9* | 17.7 ± 2.5 | 21.3 ± 1.0 | 16.6 ± 1.4 | 14.7 ± 0.5 | 264. 3 ± 16.9 | 14.9 (19) | 12.4 (25) | 15.9 (26) | 18.0 (36) |

a IC50 = concentration required to give 50% inhibition, calculated by linear regression analysis from the *K*c values at concentrations employed (1, 10, 25, 50 and 100 µM); *K*c (culture growth constant) corresponds to the slope resulting from plotting the log of the growth measurement versus time for each drug concentration. b Selectivity index (SI) = IC50 macrophages/IC50 extra- and intracellular forms of parasites. In brackets: number of times that compound SI exceeds the reference drug SI on the different morphological forms of parasites.