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In vitro assessment of 3-alkoxy-5-nitroindazole-derived ethylamines and related compounds as potential antileishmanial drugs

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Authors would like to dedicate this work to their colleague Dr. Pilar Navarro (IQM, CSIC, Madrid), who recently retired.

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ABSTRACT

Leishmaniasis is a widespread neglected tropical disease complex that is responsible of one million new cases per year. Current treatments are outdated and pose many problems that new drugs need to overcome. With the goal of developing new, safe, and affordable drugs, we have studied the *in vitro* activity of 12 different 5-nitroindazole derivatives that showed previous activity against different strains of *Trypanosoma cruzi* in a previous work. *T. cruzi* belongs to the same family as *Leishmania* spp., and treatments for the disease it produces also needs renewal. Among the derivatives tested, compounds **1**, **2**, **9**, **10**, **11**, and **12** showed low J774.2 macrophage toxicity, while their effect against both intracellular and extracellular forms of the studied parasites was higher than the ones found for the reference drug Meglumine Antimoniate (Glucantime®). In addition, their Fe-SOD inhibitory effect, the infection rates, metabolite alteration, and mitochondrial membrane potential of the parasites treated with the selected drugs were studied in order to gain insights into the action mechanism, and the results of these tests were more promising than those found with glucantime, as the leishmanicidal effect of these new drug candidates was higher. The promising results are encouraging to test these derivatives in more complex studies, such as in vivo studies and other experiments that could find out the exact mechanism of action.

1. Introduction

Leishmaniasis is a widespread neglected tropical disease complex that is endemic in 98 countries and causes about 1 million of new cases and 20,000–30,000 deaths per year. It has three main clinical forms, which range from: a form that causes an ulcer or ulcers or disseminated lesions which leave lifelong scarring when healed (cutaneous leishmaniasis or CL); another form that causes facial deformations and destruction of connective tissue in the mouth, nose, and throat (mucocutaneous leishmaniasis or ML); and the most dangerous form, visceral leishmaniasis or VL, which causes enlargement of the spleen and liver, anemia, and irregular fever. This form is fatal if untreated in a 95% of the cases [1].

CL is found in the Americas, the Mediterranean basin, Africa, and central Asia, while ML is present only in South America (Brazil, Bolivia, and Peru). VL is located especially in India and East Africa, but recently cases have been reported in Brazil. In Madrid (Spain) 560 cases have been reported since 2009, 70% of these cases occurring in immunocompetent patients [2]. These cases occur due to the recent overpopulation of non-depredated lagomorphs (*Oryctolagus cuniculus* and *Lepus granatensis*) in natural areas of Madrid and the ecological conditions that allow the vector species, the sandfly *Phlebotomus perniciosus*, to exist [3]. The coexistence of a new wild reservoir and the aforementioned vector constitute a new life cycle for the parasite in Spain [4].

Paraguay was thought to be free of leishmaniasis, but 19 cases of VL were reported in 2018. This is quite remarkable because the clinical form that was thought to be found there was the mucocutaneous form. This finding, together with 87 cases diagnosed in the Madrid community, rank it as the location with most cases in Spain, forcing the authorities to allow the hunting of wild animals as a control measure [5] to curb the expansion of the disease.

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The parasite currently found in the Iberian Peninsula is *L. infantum*, which causes the most life-threatening clinical form, VL and also CL. An estimated 40% of the cases in Spain are VL [6].

Current treatments are based mainly on pentavalent antimonials such as sodium stibogluconate and meglumine antimoniate [7], which are outdated drugs that present many negative effects such as hepatotoxicity and nephrotoxicity. The lack of vaccines and effective treatments cause an urgent need to search for new drugs that can overcome the problems that current drugs have, such as aforementioned toxicity, long administration periods and overprice. For this reason, understanding the biochemistry of the parasite (such as enzymatic reactions or metabolic pathways and other inner processes) is critical in order to design new, effective drugs, because current drugs (such as pentavalent antimonials) date from the 1960s and the parasites have developed resistance.

One of the *Leishmania* targets is a kinetoplastid exclusive protein known as iron superoxide dismutase (Fe-SOD), which acts as a protector against the host oxidative burst defense. This enzyme differs from its vertebrate counterpart in the iron atom that is associated with it; vertebrates possess Cu/Zn-SOD, thus making Fe-SOD a perfect therapeutic target due to the parasite dependence on its protection in order to survive and divide [8,9]. Another possible target is the parasite glycolysis, which takes place in the mitochondria, as sometimes drugs can interfere with the metabolic pathway by altering enzymatic function or the mitochondrial membrane potential, ending in a lack of energy that could compromise parasite survival.

In recent years, we have studied the synthesis and antiprotozoal properties of many 5-nitroindazole derivatives. In connection with the current work, the antileishmanial activity of some 5-(indazol-1-yl) pentyl- and 3-oxapentylamines has been reported [10,11]. Additionally, we have recently described the antichagasic activity of some primary, secondary, and tertiary 2-(5-nitroindazol-1-yl)ethylamines [12].

In the present work, we have studied the effects of the above-mentioned 2-(indazolyl)ethylamines (**1**–**8**), as well as some related reaction intermediates (**9**, **10**) and byproducts (**11**, **12**) (Fig. 1), against both forms of three *Leishmania* species, each causing one of the aforementioned clinical forms (*L. infantum* for cutaneous leishmaniasis, *L. braziliensis* for mucocutaneous leishmaniasis, and *L. donovani* for visceral leishmaniasis), their metabolite alteration, their SOD activity, and their mitochondrial membrane potential.

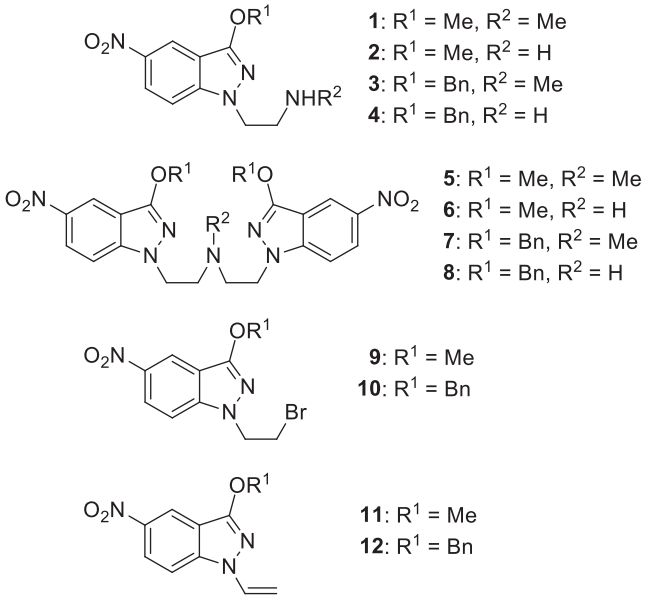

Fig. 1. 5-Nitroindazole derivatives studied in the current work.

2. Materials and methods

2.1. Chemistry

The amines **1**–**8** (Fig. 1) were prepared from 3-methoxy- or 3-benzyloxy-5-nitroindazole according to previously reported procedures. Alkylation of the aforementioned 3-alkoxy derivatives with 1,2-dibromoethane afforded the corresponding 3-alkoxy-1-(2-bromoethyl) indazoles **9**, **10**, and 3-alkoxy-1-vinylindazoles **11**, **12** were obtained as byproducts in this process. Finally, the reaction of 2-bromoethyl derivatives **9** and **10** with ammonia or the required amines afforded the final compounds **1**–**8** [12].

2.2. Parasite strain and culture

Extracellular forms (promastigotes) of the three *Leishmania* species were harvested in Roux flasks (Corning, USA) with 75 cm^2 surface with Medium Trypanosomes Liquid (MTL) supplemented with 10% inactivated fetal calf serum and kept at 27 °C in an air atmosphere. The strains used were MHOM/BR/1975/M2904 for *L. braziliensis*, MCAN/ES/2001/UCM-10 for *L. infantum* and LCR-L 133 LRC, Jerusalem, Israel for *L. donovani*, following a previously described methodology [13].

2.3. In vitro activity assays

The compounds tested were dissolved in dimethyl sulfoxide (DMSO, Panreac, Barcelona, Spain) and added to the cultures of mammalian cells or extracellular and intracellular forms of the parasite in order to give the required final concentrations as described [13,14]; the final concentration of DMSO in cultures was 0.01% (v/v).

For the amastigote inhibitory effect, J774.2 macrophages were harvested in 24-well sterile microplates (Nunc®) with a round coverslip in the bottom of each well at a concentration of 10^4 cells per well. After the cells were fixed to the coverslip, they were infected with promastigotes (10 promastigotes per J774.2 macrophage) of each species studied. At the same time, the drugs were added in order to reach final concentrations of 12.5, 25, 50, and 100 μM. Promastigotes underwent conversion to amastigotes one day after infection. Those microplates were maintained for 2 days, and then coverslips were collected, fixed to a microscope slide, and stained with Giemsa for subsequent examination under an optical microscope.

Results are the median of three separated experiments.

2.4. Effects on infected J774.2 macrophages and infection rates

The compounds that showed a selectivity index (SI) 20 times or more higher than that of the reference drug and an IC_{50} value close to (or lower than) 10 μM were chosen for further studies because they were more selective towards parasite cells [15]. The first of those studies concerned the effect of the drug on the parasite's ability to infect and divide inside a host cell. We cultured macrophage cells of the J774.2 cell line and then infected them with promastigotes during the stationary phase. One day later, promastigotes underwent morphological conversion to amastigotes, and 10 days after infection they reached their peak. We used IC_{25} as the test dosage following the method described in [16].

2.5. Superoxide dismutase (SOD) inhibition studies

As SOD is a key enzyme for fighting oxidative stress, many life forms rely on it to avoid damage caused by reactive oxygen species (ROS) generated by cell oxidative burst defense mechanism. Kinetoplastids evolved to have a version of this enzyme linked to an iron atom, thus making it different from the vertebrate counterpart that appears linked to a copper or zinc atom (Cu/Zn-SOD). Because of the parasite dependence on this molecule and its difference from the human host

homologue, this enzyme constitutes a suitable therapeutic target. For these reasons, we studied the effect of the chosen drugs against the activity of both parasite and human erythrocytes SOD, which could partially explain their action mechanism.

We performed this experiment extracting Fe-SOD from the parasites as described [16]; commercial Cu/Zn-SOD extracted from human erythrocytes was purchased from Sigma-Aldrich®. We measured the protein quantity by the Sigma Bradford test [17] and analyzed the SOD activity following the method described by Beyer and Fridovich [18].

2.6. Metabolite excretion

In order to determine whether the drugs have some effect on the main metabolic pathway (glycolysis) of the parasite, we measured the quantity of the most important metabolites by ^{1}H NMR using the method reported by Fernandez-Becerra et al. [19].

2.7. Effects on the mitochondrial membrane potential

Another approach for understanding the possible action mechanism is to determine whether the leishmanicidal effect of the compounds can be caused by an alteration of the mitochondrial membrane potential. To confirm this, we treated cultures with IC_{25} of each drug, in a way similar to the approach followed for the metabolite excretion test, and were incubated for 72 h at 27 °C. Afterwards, the cultures were centrifuged (800xg for 10 min) and the pellets were collected, stained with Rho 123 (Sigma-Aldrich®), and analyzed by flow cytometry. Changes in fluorescence intensity were calculated using the equation VI = (TM-CM)/CM, meaning: VI (variation index, TM (median fluorescence for treated parasites) and CM (median fluorescence for untreated parasites) [20].

3. Results and discussion

3.1. In vitro activity assays

First, the compounds cytotoxicity in macrophages belonging to the J774.2 cell line was determined. We found that most compounds were at least half as toxic as the reference drug glucantime, except compound **4**, which proved to be severely cytotoxic. With those results, we proceeded to test compounds with the promastigote forms, for which the results were not as decisive as for those of the amastigote form, but served as a preliminary filter for the screening process. We gathered the IC_{50} data and used it to calculate the selectivity index, dividing the IC_{50} value for J774.2 macrophages by the value for promastigotes. The compounds that registered a good SI value according to Nwaka criteria [15] were tested again with the amastigote forms, and the same calculations were performed. Compounds **1**, **2**, **9**, **10**, **11**, and **12** showed much better activity than the reference drug; for instance, compound **2** was 134 times more active than glucantime against *L. infantum* and 77 times against *L. braziliensis* amastigotes. On the other hand, compound **10** was the most active against *L. donovani* amastigotes, being 200 times more active than glucantime (Tables 1–3).

For the above-mentioned reasons, we made further studies with compounds **1**, **2**, **9**, **10**, **11**, and **12**.

For the currently studied compounds we have not been able to find a clear relationship between structure and *in vitro* activity. It is clear that bis(indazolylethyl)amines (**5**–**8**) are not good antileishmanial agents owing the their low SI. On the other hand, the structures of the most efficient compounds are heterogeneous, including a primary amine (**2**), a secondary amine (**1**), two 1-(2-bromoethyl)indazoles (**9**, **10**) and two 1-vinyl derivatives (**11**, **12**).

For simple 2-(indazolylethyl)amines, 3-benzyloxy derivatives (**3**, **4**) were much more toxic for macrophages than the corresponding 3-methoxy derivatives (**1**, **2**), leading to the very low values of SI found for the former. However, for 2-bromoethyl (**9**, **10**) and vinyl derivatives (**11**, **12**), the effect of the substituent at position 3 appears to be low. The activity of 5-(indazol-1-y)-3-oxapentylamines and related 3-alkoxy-1-alkylindazoles against *L. infantum* and *L. braziliensis* has been previously studied [11]. In general, activities of these compounds against promastigotes and amastigotes (IC_{50} = 9–56 μM) are similar to those found for the current products; nevertheless, these initially reported indazoles are quite toxic for macrophages and, consequently, their SI are much lower (SI < 22). On the other hand, activities against *Leishmania* spp. of some 3-alkoxy-1-[5-(dialkylamino)pentyl and 3-oxapentyl]indazoles have been published [10]; however, IC_{50} and SI values were not determined in this article, so the provided activity data cannot be compared directly with those obtained in the present work.

3.2. In vitro infectivity

We tested the drug ability to reduce parasite infection and division. We display four charts for each parasite, two showing the infection rate through the days the experiment lasted, and the others showing the number of amastigotes per infected cell through 10 days. Overall, a notable reduction in those parameters was found for each product in every parasite. This reduction was higher than the one caused by the reference drug.

Compound **2** was the most effective at reducing the infection rate in all the species studied. In *L. infantum,* the infection rate was lowered by 86%, by 73% in *L. braziliensis*, and by 49% in the case of *L. donovani*. With respect with the number of amastigotes per infected cell, the results were more varied. That is, compound **10** reached a reduction of 71% in *L. infantum*, compound **9** caused a 72% reduction in *L. braziliensis*, and compound **11** reduced the number of amastigotes 64% in *L. donovani* (see Figs. 2–4).

The reductions in the rate of infection and the number of amastigotes per macrophage observed for the current compounds are similar or slightly lower than those observed for some 5-(indazol-1-y)-3-oxapentylamines previously studied against *L. infantum* and *L. braziliensis* (56–81% and 65–85%, respectively) [11].

3.3. Mechanism of action-SOD inhibition studies

Because Fe-SOD is critical for parasite survival, its activity inhibition caused by the compounds studied was assessed. Previous works have demonstrated that a vast majority of the drugs tested were effective against parasites by inhibiting Fe-SOD activity, while their inhibition of human Cu/Zn-SOD was not significant in comparison. [16,21,22] This was not the case in the present study. The experiment was carried out as usual and the drugs chosen proved to have a quite marginal effect on Fe-SOD, which in this case does not serve to explain the action mechanism. The compounds tested had no effect on *L. infantum* or *L. braziliensis* Fe-SOD. In the case of *L. donovani*, compound **2** had a slight inhibition (59%) as did compound **9** (69%) but these inhibitions occurred only when the parasites were treated with 100 μM of each drug. Compound **10** was the most effective, as it completely inhibited the enzymatic activity when 50 μM concentration was reached; at lower concentrations, inhibition reached 25.4% with 12.5 μM and 77.8% with 25 μM.

For related antichagasic 5-nitroindazole derivatives, different alternative mechanisms, similar to those suggested for other antiprotozoal nitroheterocycles, have been proposed. All these possible mechanisms, discussed in a recent article [23], start with the intracellular reduction of the nitro group, followed by the generation of reactive oxygen species (ROS) and induction of oxidative stress in parasites, or by the production of highly reactive metabolites capable of reacting with essential biomolecules of parasites. In our case, the obtained results on the mode of action of these compounds are somewhat contradictory and we do not have conclusive results. In fact, a study of electrochemical and enzymatic reduction of antichagasic 5-nitroindazolinones suggested that these compounds could induce

Table 1
In vitro activity, toxicity, and selectivity index for the 5-nitroindazole derivatives on extra- and intracellular forms of *L. infantum*. Numbers in brackets show the number of times drugs are more effective than the reference drug.

Compounds	Activity IC_{50} (μM)		J774.2 Macrophage toxicity IC_{50} (μM)	Selectivity index	
	Promastigote forms	Amastigote forms		Promastigote forms	Amastigote forms
Glucantime	18.0 ± 3.1	24.2 ± 2.6	15.2 ± 1.0	0.8	0.6
1	13.2 ± 5.4	16.8 ± 0.8	289.7 ± 32.7	22 (27)	17 (29)
2	3.5 ± 0.01	6.1 ± 0.1	489.2 ± 66.8	140 (1 7 4)	80 (1 3 4)
3	4.3 ± 0.0	nd	32.9 ± 3.6	8 (10)	nd
4	17.7 ± 4.2	nd	0.01 ± 0.0	0 (0)	nd
5	9.9 ± 1.5	nd	79.3 ± 7.1	8 (10)	nd
6	9.9 ± 2.6	nd	29.8 ± 2.1	3 (4)	nd
7	36.2 ± 3.4	nd	103.6 ± 8.6	3 (4)	nd
8	2.5 ± 0.6	nd	30.6 ± 2.4	12 (15)	nd
9	11.5 ± 1.1	21.8 ± 1.0	234.5 ± 18.7	20 (25)	11 (18)
10	13.1 ± 2.2	14.4 ± 0.6	587.8 ± 89.6	45 (56)	41 (68)
11	9.3 ± 0.1	43.7 ± 1.5	667.8 ± 74.5	72 (90)	15 (25)
12	1.7 ± 0.3	18.0 ± 1.6	306.8 ± 18.9	180 (225)	17 (28)

oxidative stress in the parasites, i.e., a mode of action similar to that initially accepted for nifurtimox [24]; however, an additional study using 1,2-disubstituted indazolinones has shown that their action on *T. cruzi* trypomastigotes is due to damage induced at the mitochondrial level, and that oxidative stress is not involved [25].

On the other hand, interference with glycosomal or mitochondrial enzymes involved in the catabolism of *T. cruzi* [24] as well as the inhibition of trypanothione reductase has also been proposed for some antichagasic 5-nitroindazoles [26–28].

3.4. Metabolite production and excretion

Another approach used to gain insight into the mechanism of action of our drugs was to assess their effect on the parasite metabolism. Trypanosomatids are unable to oxidize glucose to carbon dioxide and water as vertebrates do; instead, they excrete part of the carbon skeleton in the form of different organic acids [29]. There is an enzyme responsible of the production of each excreted compound of the metabolic pathway, so we can infer which enzyme has its activity altered because of the drug just by determining which metabolite registers higher or lower production than control.

In the case of *L. infantum*, metabolite production was reduced with respect to the control for every tested drug, except an exiguous increase in D-lactate production for compound **10**. The most pronounced alteration was succinate production when *L. infantum* was treated with compound **1**, which caused an increase of 243.1%. When the *L. braziliensis* metabolite alteration was assessed, we found that all metabolite production was increased, especially pyruvate. For *L. donovani*, the same phenomenon again occurred, but the increase was much less notorious than in the previous case. An exception to this was that compound **1** reduced metabolite production instead of augmenting it, and the reduction was slight with respect to the control. Another minor reduction occurred in succinate production for compound **10**. Apart from this, the most remarkable metabolite alteration was found in *L. braziliensis*, which underwent an extraordinary surge in succinate production triggered by action of compound **11**, values being almost 600% higher than control (see Tables 4–6).

3.5. Mitochondrial membrane potential alterations

The mitochondrial membrane potential of promastigotes treated with an IC_{25} dosage of the compounds was assessed, with the expectation that their antileishmania activity might be explained by altering the potential and thus triggering programmed cell death. However, only one alteration was detected (and a remarkable one) in the case of *L. donovani* when treated with compound **11**. For the other drugs and parasites, no difference was found between control and different treatments. The membrane depolarization can be seen as a shift to the left for the treatment curve with respect to the control curve (see Fig. 5).

This phenomenon is related with the previous metabolite excretion test, because, as stated previously, the most notable effect in *L. donovani* metabolite excretion was the extraordinary increase in succinate excretion when treated also with compound **11**. According to Barisón et al. [30] in their study of a metabolic switch of induced starvation in *T. cruzi* epimastigotes, another metabolic pathway occurs in the

Table 2
In vitro activity, toxicity, and selectivity index for the 5-nitroindazole derivatives on extra- and intracellular forms of *L. braziliensis*. Numbers in brackets show the number of times drugs are more effective than the reference drug.

Compounds	Activity IC_{50} (μM)		J774.2 Macrophage toxicity IC_{50} (μM)	Selectivity index	
	Promastigote forms	Amastigote forms		Promastigote forms	Amastigote forms
Glucantime	25.6 ± 1.7	30.4 ± 2.6	15.2 ± 1.0	0.6	0.6
1	10.6 ± 2.0	27.9 ± 1.2	289.7 ± 32.7	27 (40)	10 (17)
2	8.1 ± 0.8	10.6 ± 0.6	489.2 ± 66.8	60 (1 0 1)	46 (77)
3	7.4 ± 1.6	nd	32.9 ± 3.6	4 (7)	nd
4	1.2 ± 0.2	nd	0.01 ± 0.0	0 (0)	nd
5	51.3 ± 3.6	nd	79.3 ± 7.1	1 (3)	nd
6	27.6 ± 3.5	nd	29.8 ± 2.1	1 (2)	nd
7	22.6 ± 1.9	nd	103.6 ± 8.6	5 (8)	nd
8	6.8 ± 0.7	nd	30.6 ± 2.4	4 (8)	nd
9	5.5 ± 0.6	11.7 ± 1.3	234.5 ± 18.7	43 (71)	20 (33)
10	11.4 ± 1.1	25.5 ± 2.0	587.8 ± 89.6	52 (86)	23 (38)
11	14.1 ± 3.7	19.8 ± 1.4	667.8 ± 74.5	47 (79)	34 (56)
12	23,2 ± 2,8	30.7 ± 2.3	306.7 ± 18.9	13 (22)	10 (17)

Table 3
In vitro activity, toxicity, and selectivity index for the 5-nitroindazole derivatives on extra- and intracellular forms of *L. donovani*. Numbers in brackets show the number of times drugs are more effective than the reference drug.

Compounds	Activity IC_{50} (μM)		J774.2 Macrophage toxicity IC_{50} (μM)	Selectivity index	
	Promastigote forms	Amastigote forms		Promastigote forms	Amastigote forms
Glucantime	26.6 ± 5.4	33.3 ± 1.2	15.2 ± 1.0	0.7	0.6
1	6.6 ± 2.5	18.3 ± 1.1	289.7 ± 32.7	44 (63)	16 (26)
2	7.1 ± 0.9	14.6 ± 0.8	489.2 ± 66.8	69 (98)	33 (56)
3	5.7 ± 0.7	nd	32.9 ± 3.6	6 (8)	nd
4	11.4 ± 2.5	nd	0.01 ± 0.0	0 (0)	nd
5	26.9 ± 3.3	nd	79.3 ± 7.1	3 (4)	nd
6	3.6 ± 0.2	nd	29.8 ± 2.1	8 (12)	nd
7	21.6 ± 1.7	nd	103.6 ± 8.6	5 (7)	nd
8	27.2 ± 1.8	nd	30.6 ± 2.4	1 (2)	nd
9	14.1 ± 2.3	55.2 ± 2.5	234.5 ± 18.7	17 (24)	4 (7)
10	1.6 ± 0.0	4.9 ± 0.6	587.8 ± 89.6	367 (5 2 5)	120 (200)
11	37.7 ± 3.0	36.5 ± 2.6	667.8 ± 74.5	18 (25)	18 (30)
12	21.6 ± 1.6	27.9 ± 1.7	306.7 ± 18.9	14 (20)	11 (18)

glycosomes and not in the mitochondria, for which the final product is succinate. Bearing in mind how close *T. cruzi* and *Leishmania* are phylogenetically, we suggest that they could react in a similar way when these conditions appear.

Considering that mitochondrial membrane potential is nullified by the action of compound **11**, as the graph suggests, we might infer that the mitochondria are not working, which could force the cell to rely in this alternative, less efficient route to gain energy. This could explain the massive production and consequent excretion of this metabolite, making the results of NMR and Rhodamine assay support each other in the case of compound **11** against *L. donovani*. This might lead us to deduce that the promastigotes die of energetic collapse, as the mitochondria and all the metabolic events that occur within it cease to work.

4. Conclusions and future outlooks

As seen in previous studies, the activity of the 2-(5-nitroindazol-1-yl)ethylamines and related compounds was assessed in *T. cruzi* [12], where only two compounds showed noteworthy *in vitro* activity. In the present study, those drugs were found to be more effective against *Leishmania* species, as 6 compounds were active against at least one species (**1**, **9**, **12**), and three were active against all three of the species studied (**2**, **10**, **11**). Those 6 selected compounds proved to be

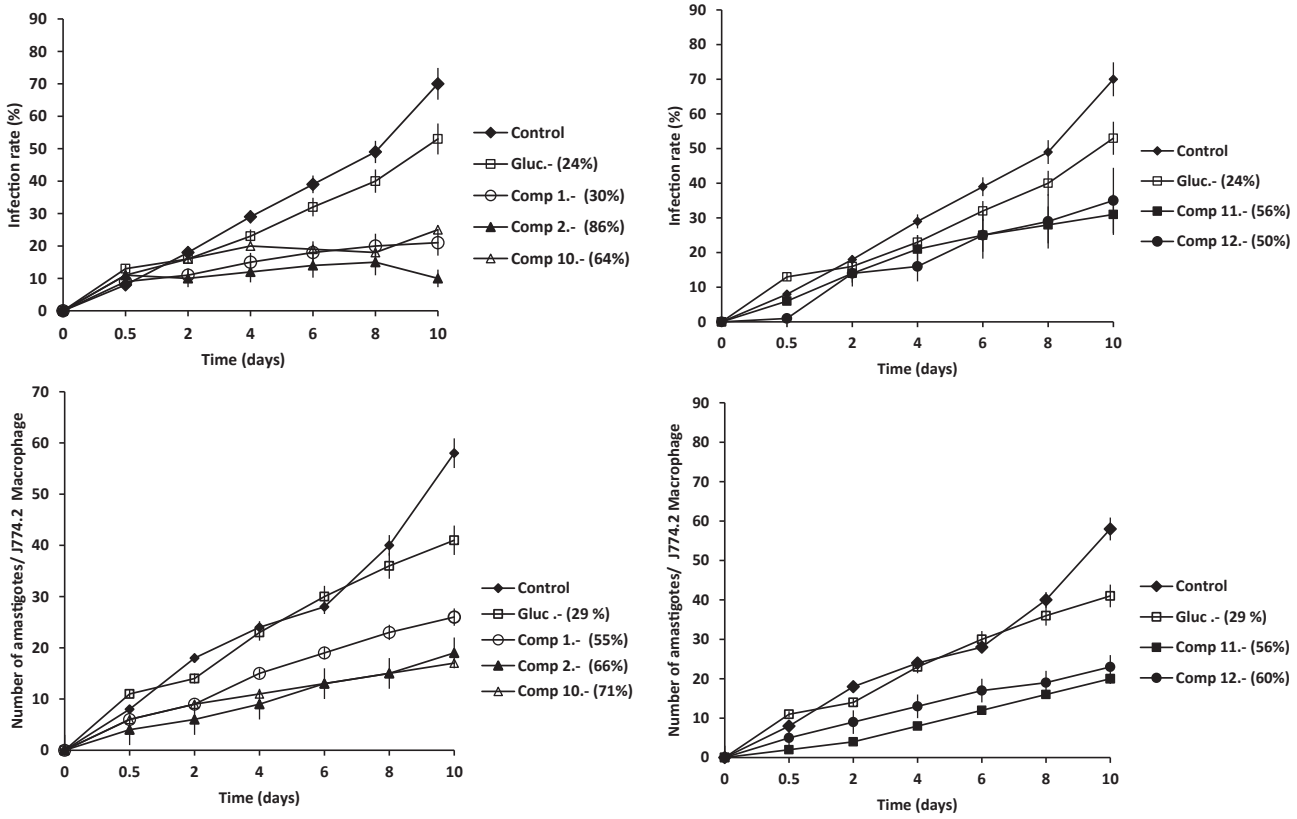

Fig. 2. Infection rates and number of amastigotes per infected cell for *L. infantum*. Effect of 3-alkoxy-5-nitroindazole-derived ethylamine derivatives **1**, **2**, **10**, **11**, and **12** on the infection and growth rates and mean numbers of amastigotes per infected J774.2 macrophage cell (at IC_{25} concentration) of *L. infantum*. The inhibition percentage is indicated in parenthesis. Values are the means of three separate experiments. All compounds are statistically significant against glucantime at a P value < 0.05.

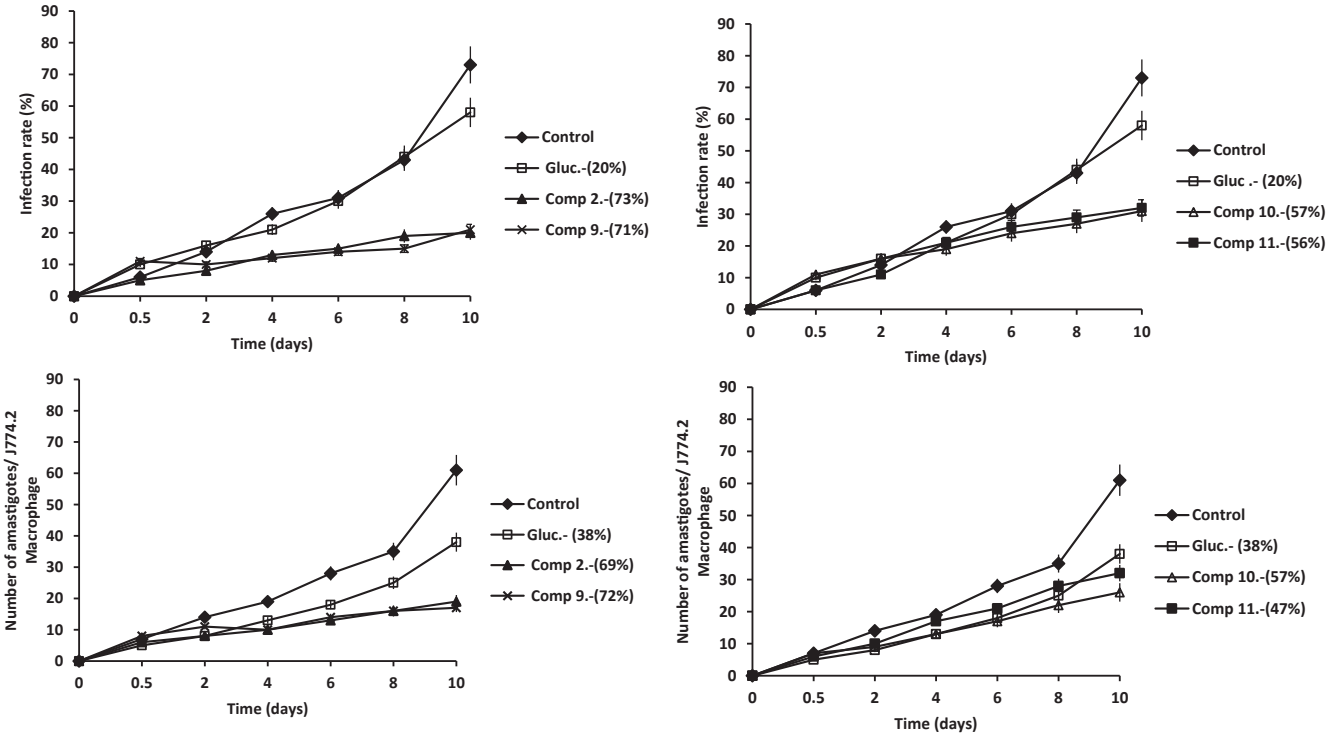

Fig. 3. Effect of 3-alkoxy-5-nitroindazole-derived ethylamines derivatives **2**, **9**, **10**, and **11** on the infection and growth rates and mean numbers of amastigotes per infected J774.2 macrophage cell (at IC_{25} concentration) of *L. braziliensis*. Inhibition percentage is indicated in parenthesis. Values are the means of three separate experiments. All compounds are statistically significant against glucantime at a P value < 0.05.

considerably less toxic than the reference drug and were also effective in lowering the parasite's ability to infect and divide inside the host cell.

As the Fe-SOD is key for parasite survival inside the host cell, our group uses this enzyme to test the drug's ability inhibit this enzymatic activity, which is one of the most usual ways to explain why the drugs are effective [16,21,22]. However, in this present study the drugs which

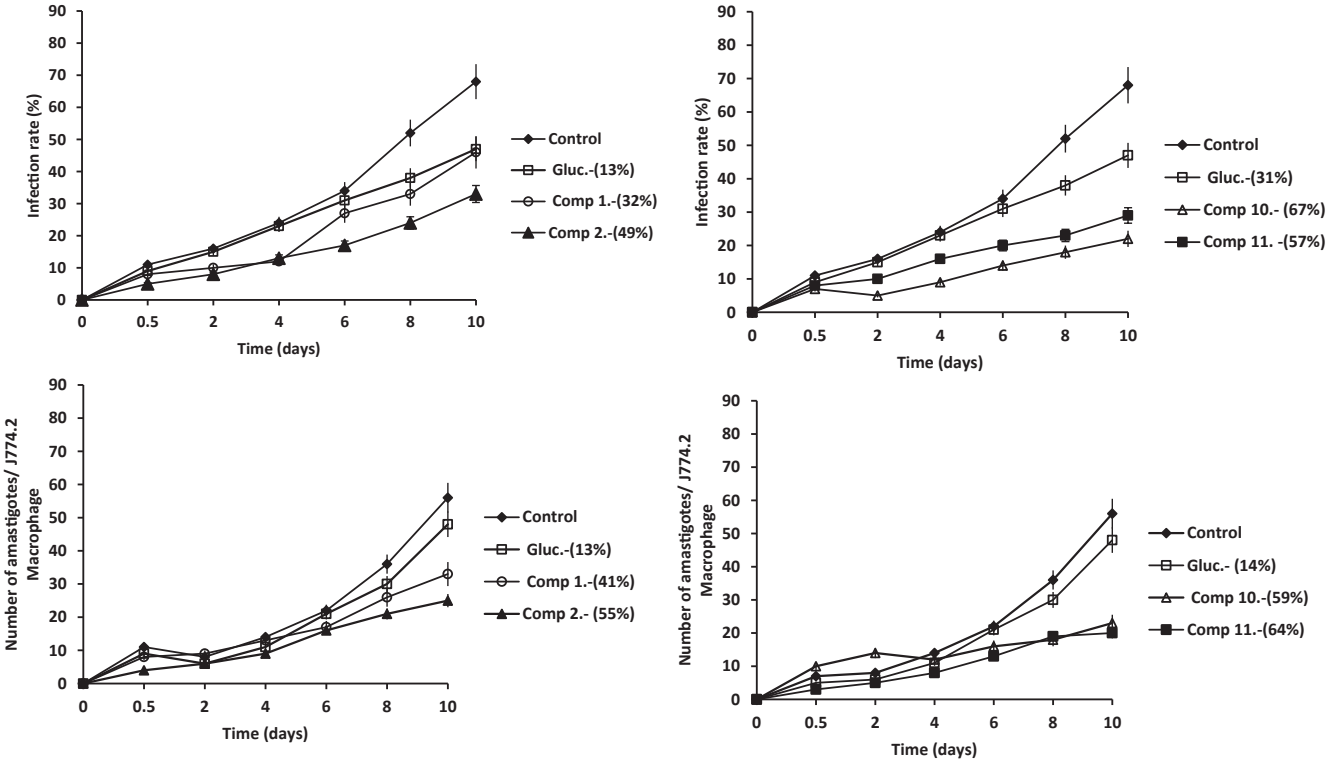

Fig. 4. Effect of 3-alkoxy-5-nitroindazole-derived ethylamines derivatives **1**, **2**, **10**, and **11** on the infection and growth rates and mean numbers of amastigotes per infected J774.2 macrophage cell (at IC_{25} concentration) of *L. donovani*. The inhibition percentage is indicated in parenthesis. Values are the means of three separate experiments. All compounds are statistically significant against glucantime at a P value < 0.05.

Table 4
Metabolite-excretion chart of *L. infantum* treated with an IC_{25} dosage of the drugs. Results are expressed as percentages of augmentation or reduction on production and excretion of each metabolite with respect to the control.

Compound	Succinate	Pyruvate	Acetate	L-Alanine	D-Lactate
1	243,1	–32,4	–22,5	−18,2	–23,8
2	−15,3	−5,4	−12,2	−12	−11,3
10	−24,7	−8,1	−3,4	−0,3	0,6
11	−31,6	−24	−24,4	−21,3	−17,6
12	−20	−12,7	−13,1	−12,7	−14

Table 5
Metabolite-excretion chart of *L. braziliensis* treated with an IC_{25} dosage of the drugs. Results are expressed as percentages of augmentation or reduction on production and excretion of each metabolite with respect to the control.

Compound	Succinate	Pyruvate	Acetate	L-Alanine	D-Lactate
1	19,5	31,4	20,7	28,2	2,9
2	24,1	38,7	27,1	34,5	25,7
9	13	34,7	18	31	1,5
10	51,3	79	60,8	67,2	57,5
11	47,7	74,3	60,2	67,3	56,5
12	14	46,6	36,9	33,6	5,5

Table 6
Metabolite-excretion chart of *L. donovani* treated with an IC_{25} dosage of the drugs. Results are expressed as percentages of augmentation or reduction on production and excretion of each metabolite with respect to the control.

Compound	Succinate	Pyruvate	Acetate	L-Alanine	D-Lactate
1	−6,2	−0,4	−1,3	−0,7	−3,1
2	13,4	37,7	24,9	46,9	10,1
9	4,8	2,9	0,6	4,1	−0,8
10	−3,8	9,1	4,5	5,9	0,9
11	599,8	40,1	51,2	47,1	12,6
12	51,6	31,5	30,9	29,9	−4,4

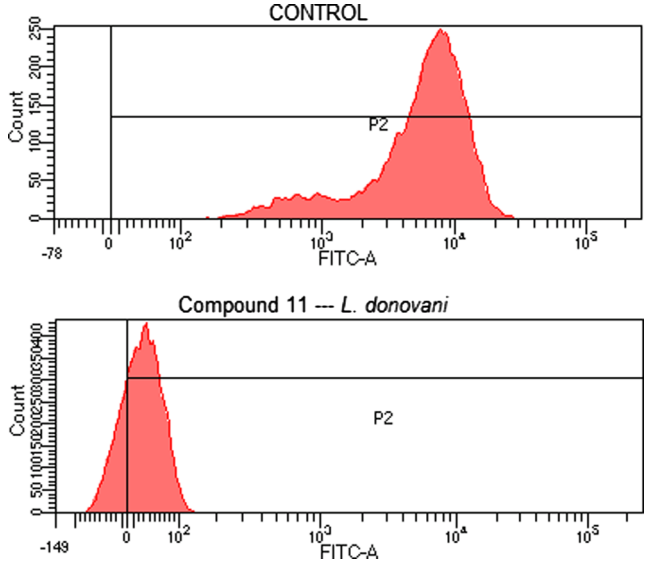

Fig. 5. Mitochondrial-membrane potential graphs for control and for compound **11** against *L. donovani. FITC-A stands for Rho 123.*

showed leishmanicidal activity were not effective at inhibiting Fe-SOD activity, indicating that their action mechanism was something other than inhibition of Fe-SOD activity. Alternative possible mechanisms, similar to those proposed for related antichagasic 5-nitroindazoles, based on the intracellular reduction of the nitro group, are discussed.

Due to their *in vitro* leishmanicidal activity, we continued performing experiments with the 6 drugs chosen in order to advance our understanding of their action mechanism, testing their metabolite alteration via ^{1}H NMR. This enabled us to detect different alterations in the parasite metabolic pathways, such as a general reduction of metabolite excretion in *L. infantum* (except succinate for compound **1**), an enhancement in metabolite excretion in *L. braziliensis*, and an slight increase for the drugs tested in *L. donovani*, except product **1**, which caused an exiguous reduction and the extraordinary surge in succinate production triggered by compound **11**. This finding led us to deduce that the mitochondria were completely inhibited, and therefore we performed the rhodamine assay, which corroborated our suspicions for that case, as it turned out to be the only drug that caused a mitochondrial-membrane depolarization, causing cell death from energetic collapse [30].

The results confirm the value of 5-nitroindazole derivatives in the field of antileishmanial agents. In the current case, however, the structures of the most active compounds are rather heterogeneous, including a primary amine (**2**), a secondary amine (**1**), two 1-(2-bromoethyl)indazoles (**9** and **10**) and two 1-vinyl derivatives (**11** and **12**). Consequently, we cannot establish of a clear structure-activity relationship.

For the immediate future we have planned the identification of the products arising from the bioreduction of our 5-nitroindazoles, as well as the synthesis of fluorescent derivatives allowing the labelling of the possible organelles that constitute their primary target. We also believe in the convenience of carrying out in vivo studies with animal models of leishmaniasis in order to deepen the knowledge of these products and their therapeutic potential.

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