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Original article

# *In vitro* and *in vivo* antitrypanosomatid activity of 5-nitroindazoles

Lucia Boiani [a], Alejandra Gerpe [a], Vicente J. Arán [b], Susana Torres de Ortiz [c], Elva Serna [c], Ninfa Vera de Bilbao [c], Luis Sanabria [c], Gloria Yaluff [c], Héctor Nakayama [c], Antonieta Rojas de Arias [c], Juan Diego Maya [d], J. Antonio Morello [d], Hugo Cerecetto [a,*], Mercedes González [a,**]

[a] *Departamento de Química Orgánica, Facultad de Ciencias-Facultad de Química, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay*
[b] *Instituto de Química Médica, CSIC, Juan de la Cierva, 3, Madrid, España, Spain*
[c] *Instituto de Investigaciones en Ciencias de la Salud, Departamento de Medicina Tropical, Universidad Nacional de Asunción, Río de la Plata y Lagerenza, Asunción, Paraguay*
[d] *Facultad de Medicina, ICBM, Universidad de Chile, P.O. Box 70086, 7, Santiago de Chile, Chile*

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**Abstract**

Previously, we have identified a series of 5-nitroindazoles with good antiprotozoal activities, against *Trypanosoma cruzi* epimastigotes and *Trichomonas vaginalis*. Most of them have shown very low unspecific toxicity on macrophage cell lines. In the present work, we assayed these compounds on *T. cruzi* bloodstream trypomastigotes and *Leishmania* promastigotes (*Leishmania amazonensis*, *Leishmania braziliensis* and *Leishmania infantum*). Derivatives **1**, **2**, **7** and **8** displayed remarkable trypanocidal activity (>80% lysis) equivalent to gentian violet. Derivatives **2** and **10**, as Pentamidine, caused the complete lysis of promastigotes of *Leishmania*. An oxidative stress-mediated mechanism of action was confirmed for derivatives **1**, **10** and **12** on *T. cruzi* epimastigotes. Supported by the *in vitro* activities, derivatives **1** and **2** were submitted to *in vivo* assays using an acute model of Chagas' disease and a short-term treatment. None of the animals treated with derivatives **1** and **2** died, unlike the untreated control and Benznidazole groups.


*Keywords:* Nitroindazoles; Chagas' disease; Leishmaniasis; Therapy

## 1. Introduction

The protozoan order kinetoplastida includes the genus *Trypanosoma* and *Leishmania,* both digenean parasites that alternate between two different hosts: a mammal and an insect host. These kinetoplastid parasites are the causative agents of Chagas' disease (American trypanosomiasis) and Leishmaniasis, respectively. These diseases affect around 30 million people worldwide [1,2]. Drugs currently used in the treatment of Chagas' disease are two nitroaromatic heterocycles, Nifurtimox (Nfx, recently discontinued by Bayer) and Benznidazole (Bnz, Rochagan, Roche), introduced empirically over three decades ago [3,4]. Both drugs are active in the acute phase of the disease but efficacy is very low in the established chronic phase. What is more, differences in drug susceptibility among different *Trypanosoma cruzi* strains lead to varied parasitological cure rates according to the geographical area [5]. The drugs of choice for the treatment of leishmaniasis are sodium stibogluconate (Pentostam), meglumine antimoniate (Glucantime), pentamidine (Ptd) and liposomal amphotericin B, but these sometimes meet with failure [6,7]. Currently, WHO/TDR is developing a research program with Miltefosine (hexadecylphosphorylcholine), a very promising leishmanocidal

* Corresponding author. Tel.: +598 2 525 86 18x216; fax: +598 2 525 07 49.
** Corresponding author. Tel.: +598 2 525 86 18; fax: +598 2 525 07 49.
*E-mail addresses:* hcerecet@fq.edu.uy (H. Cerecetto), megonzal@fq.edu.uy (M. González).

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drug, but new therapeutic alternatives should be found in order to increase the pharmaceutical arsenal [8].

The biological activity of nitroheterocycle antiparasitic drugs is dependent upon the nitro reduction process. The suggested mode of action is due to the intracellular nitro reduction followed by redox cycling yielding reactive oxygen species (ROS) and the formation of active intermediate species that can cause cellular damage directly by reaction with various biological macromolecules, or indirectly by generation of highly reactive hydroxyl radical [9,10]. Previously, we have synthesized a series of new 5-nitroindazole derivatives (Table 1) and its antiprotozoal properties and unspecific cytotoxicities against J774 macrophages have been studied. Some of these compounds displayed very interesting activities against *T. cruzi* epimastigote form, in the same order of the reference drugs (Nfx and Bnz). Since at the *T. cruzi* active concentrations the nitroindazole derivatives showed slight unspecific cytotoxicities against macrophages, it could be stated that the displayed antiparasitic activities are not consequence of the unspecific cytotoxicity [11,12]. In Table 1 it is marked whether these 5-nitroindazole derivatives possess unspecific cytotoxicity against macrophages or not at a concentration of 100 μg/mL at 24 and 48 h of contact. Moreover, the macrophage survival percentage for non-toxic compounds is 100 at the assay concentration.

In the present work, 13 5-nitroindazole derivatives (Table 1) were examined for trypanocidal effect on *T. cruzi* blood trypomastigotes and the antiproliferative *in vitro* activity against *Leishmania* spp. (promastigote form). In order to confirm an oxidative stress-mediated mechanism of action, we studied the compound effects on the epimastigote form of *T. cruzi* oxygen consumption. Finally, the most active compounds against *T. cruzi*, which showed very low unspecific cytotoxicity in macrophages cell lines, were submitted to *in vivo* biological evaluation using a murine model of acute Chagas' disease.

## 2. Chemicals

The synthesis of the compounds, 3-alkoxy- or 3-hydroxy-1-[ω-(dialkylamino)alkyl]-5-nitroindazole derivatives, **1**–**13**, was described elsewhere [11] and their structures are shown in Table 1. Benznidazole (Rochagan; Roche, Río de Janeiro, Brazil), gentian violet (GV) and Pentamidine (Pentamidine Isethionate; May & Baker Lab., England) were used as reference drugs in the different assays.

## 3. Pharmacology

### 3.1. In vitro trypanocidal activity

The lytic effect on mouse blood trypomastigotes (CL-Brener clone) was analyzed (Table 2) for derivatives **1**–**13** at 250 μg/mL [13]. GV was used as the reference drug. Derivatives showed differential lytic effect and presented similar behaviour as that observed for the antiproliferative epimastigote activities [11].

### 3.2. In vitro leishmanocidal activity

Leishmanocidal activity was assayed *in vitro* in three different *Leishmania* species (*L. braziliensis*, *L. amazonensis* and *L. infantum)* using a qualitative methodology (Table 3). Ptd was used as the reference drug. It was found that only two of the studied 5-nitroindazol derivatives (**2** and **10**) possess interesting leishmanocidal activities.

### 3.3. Effect on the parasitic respiration

In order to confirm an oxidative stress-mediated mechanism of action, we studied the effect on the parasitic oxygen consumption of some 5-nitroindazole derivatives (**1**, **2**, **8**, **10** and **12**). Respiration measurements were carried out polarographically treating *T. cruzi* (epimastigote form, Tulahuen 2 strain) with different concentrations of the studied compounds as shown in Table 4 [14].

### 3.4. In vivo anti-T. cruzi evaluation

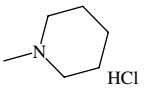
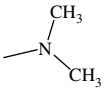
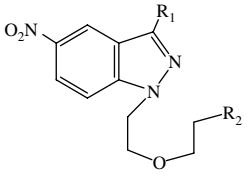
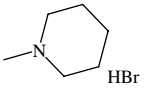
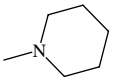
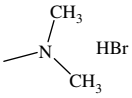
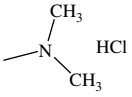
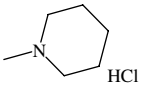
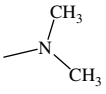
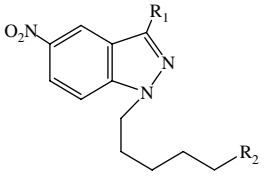
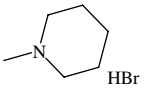
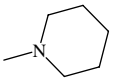
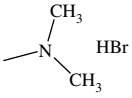
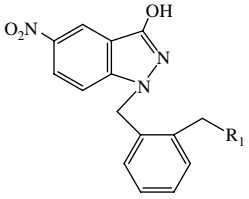
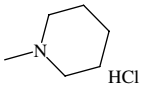
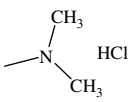
The best *in vitro* anti-*T. cruzi* and the least toxic derivatives, compounds **1** and **2**, were evaluated *in vivo* in a murine model of acute Chagas' disease. Bnz was used as the *in vivo* active reference drug. In this preliminary study, male mice were infected with blood trypomastigotes and treatment began 12 days post-infection with oral administration of each compound during 10 days [15]. Three different parameters were gathered to evaluate the *in vivo* activity, weekly parasitemia, weekly animals' survival percentage, and anti-*T. cruzi* antibody level at 30 and 60 days post-infection (Figs. 1 and 2).

## 4. Results and discussion

### 4.1. In vitro trypanocidal activity

Since transmission of *T. cruzi* by blood transfusion is becoming a source of concern in countries free of vectorial transmission, the need for new agents for chemoprophylaxis has been pointed out [13]. Despite the high efficacy of GV, a phenylmethane dye currently used in the treatment of blood, there are some restrictions to its use. After evaluation of the lytic effect on CL-Brener clone trypomastigote it was found that at the doses assayed, derivatives **1**, **2**, **7** and **8** (Table 2) showed a remarkable activity, as compared to the reference drug. Whereas derivatives **3**, **6** and **12** showed moderate activity, over 50% of lysis, the other compounds, with the exception of derivative **13**, showed some degree of activity against blood trypomastigote. Compound **2** and **8** were also very active against epimastigote form of *T. cruzi* ($IC_{50}$ values of 16.4 and 12.5 μM, respectively) [11]. However, derivatives **1** and **7** were poorly active against the insect stage of the parasite, probably due to difference between parasite stages or as a consequence of the different culture conditions (e.g.: 24 h at 4 °C for trypomastigote, 120 h at 28 °C for epimastigote) [11].

Table 1
Chemical structures of the evaluated compounds and unspecific toxicity

| | Compound | $-R_1$ | $-R_2$ | Unspecific toxicity[a] |
|---|---|---|---|---|
| | **1** | $-OCH_3$ |  | Non[b] |
| | **2** | $-OBn$ |  | Non |
|  | **3** | $-OH$ |  | Non |
| | **4** | $-OBn$ |  | Non |
| | **5** | $-OH$ |  | Non |
| | **6** | $-OCH_3$ |  | Non |
| | **7** | $-OCH_3$ |  | Yes |
| | **8** | $-OBn$ |  | Yes |
|  | **9** | $-OH$ |  | Non |
| | **10** | $-OBn$ |  | Yes |
| | **11** | $-OH$ |  | Non |
|  | **12** |  | | Non |
| | **13** |  | | Non |

[a] Unspecific toxicity was evaluated on J774 macrophages at 24 and 48 h [11].
[b] Non-toxic compound: it did not display toxicity up to 100 μg/mL at 24 and 48 h.

Table 2
*In vitro* trypanocidal activity (trypomastigote form)

| Compound | % Lysis[a,b] |
|---|---|
| **1** | 85 |
| **2** | 87 |
| **3** | 53 |
| **4** | 44 |
| **5** | 18 |
| **6** | 77 |
| **7** | 100 |
| **8** | 100 |
| **9** | 36 |
| **10** | 34 |
| **11** | 29 |
| **12** | 75 |
| **13** | 0 |
| **GV** | 100 |

[a] % Lysis = percentage of parasite lysis at 250 μg/mL of the compound.
[b] Results are the mean of three independent experiments with a SD less than 10% in all cases.

### 4.2. In vitro leishmanocidal activity

Three different *Leishmania* species were used to study the leishmanocidal effect of derivatives **1**, **2**, **7**, **9**, **10**, and **12** (Table 3). While *L. braziliensis* and *L. amazonensis* are the main causative agents of mucocutaneous and cutaneous Leishmaniasis in Latin America, *L. infantum* is responsible of cases of visceral Leishmaniasis in the Mediterranean region [2]. Among the analyzed compounds, only the 5-nitroindazole derivatives **2** and **10** were as active against promastigote form of *Leishmania* spp. as the reference drug, Ptd. Compound **2** also exhibited interesting activity against epimastigote form of *T. cruzi* and against other protozoa of human health concern, *Trichomonas vaginalis* [11]. Metronidazole, the drug currently used in trichomoniasis treatment, also bear a nitro group in its heterocyclic structure, and has proved to be active on *in vivo* models of visceral [16] and cutaneous leishmaniasis [17].

### 4.3. Effect on the parasitic respiration

Derivatives **1**, **2**, **8**, **10** and **12** were selected to be tested for their capacity to inhibit parasitic respiration. These derivatives were chosen taking into account their anti-*T. cruzi* activities, **1** and **12** being inactive compounds against epimastigote form

Table 3
*In vitro* leishmanocidal activity (promastigote form)

| Compound | *L. amazonensis*[a,b] | *L. infantum*[a,b] | *L. braziliensis*[a,b] |
|---|---|---|---|
| **1** | <60 | <60 | <60 |
| **2** | 100 | 100 | 100 |
| **7** | <60 | <60 | <60 |
| **9** | <60 | <60 | <60 |
| **10** | 100 | 100 | 100 |
| **12** | <60 | <60 | <60 |
| Ptd | 100 | 100 | 100 |

[a] Percentage of lysis after 72 h of treatment with 100 μg/mL of the compound.
[b] Results are the mean of three independent experiments with a SD less than 10% in all cases.

Table 4
Effects of 5-nitroindazole derivatives on oxygen uptake in *T. cruzi* epimastigotes

| Compound | $IC_{50}$ (μM)[a] | Concentration in the assay (mM) (times of $IC_{50}$ concentration) | Oxygen uptake (%)[b,c,d] |
|---|---|---|---|
| **1** | >50 | 0.258 (>5) | 118 ± 5 |
| | | 0.515 (>10) | 130 ± 5 |
| | | 1.030 (>21) | 155 ± 5 |
| **2** | 16.4 | 0.181 (11) | 66 ± 5 |
| | | 0.362 (22) | 10 ± 5 |
| | | 0.426 (26) | 10 ± 5 |
| | | 0.543 (33) | 11 ± 5 |
| | | 0.724 (44) | 9 ± 5 |
| **8** | 12.5 | 0.156 (12) | 99 ± 5 |
| | | 0.325 (26) | 12 ± 5 |
| | | 0.467 (37) | 11 ± 5 |
| | | 0.622 (50) | 7 ± 5 |
| **10** | 6.6 | 0.091 (14) | 109 ± 5 |
| | | 0.172 (26) | 111 ± 5 |
| | | 0.272 (41) | 100 ± 5 |
| | | 0.362 (55) | 20 ± 5 |
| **12** | >50 | 1.032 (>21)[e] | 109 ± 5 |

[a] $IC_{50}$ = concentration (in μM) that inhibits 50% of *T. cruzi* growth (CL-Brener clone) [11].
[b] Percent rate of oxygen consumption compared with that of control (C) cells in Tulahuen 2 strain.
[c] Values correspond to means of three independent experiments with a SD less than 5% in all cases.
[d] Control respiration was 31.5 nanoatoms of oxygen per min and per mg of protein.
[e] Superior doses were not assayed due to solubility problems.

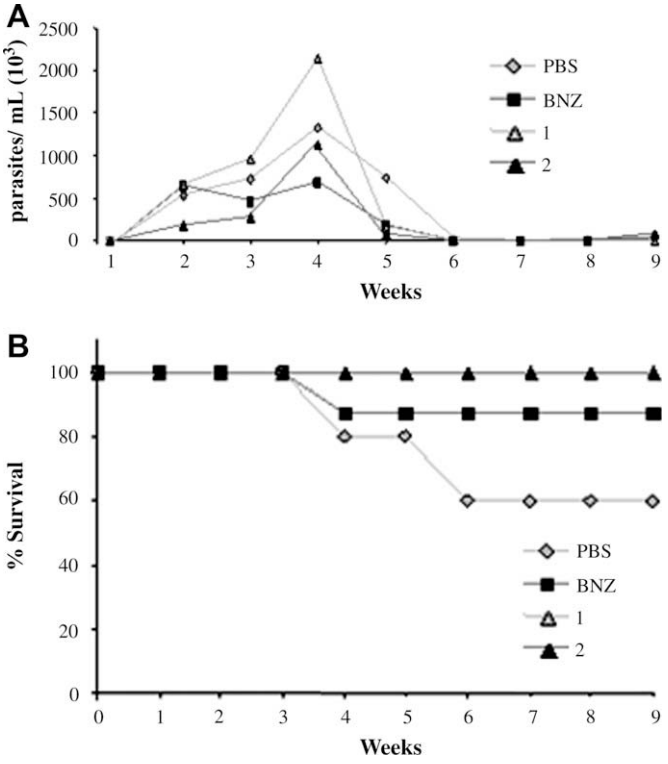



Fig. 1. Parasitemia (A) and survival expressed as percentage of living animals (B). Group treated with 50 mg/kg/d of Bnz (■), group treated with 30 mg/kg/d of **1** (▲), group treated with 30 mg/kg/d of **2** (▲), and control group (◆).

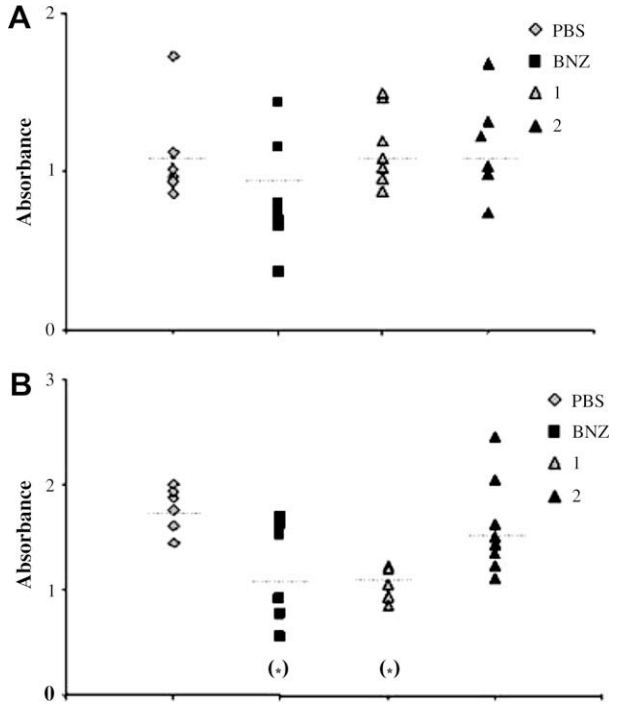



Fig. 2. Dispersion diagrams of antibody levels in control (treated with PBS solution) animals and those receiving Bnz, **1** and **2** treatments at 30 (A) and 60 days (B). Group treated with 50 mg/kg/d of Bnz (■), group treated with 30 mg/kg/d of **1** (▲), group treated with 30 mg/kg/d of **2** (▲), and control group (◆). (*) $p < 0.05$ as compared with the control.

and **10** being inactive against trypomastigote form. Derivatives **2** and **8** inhibited oxygen uptake in a concentration-dependent manner (for derivative **12** it was not possible to perform concentration-response studies because **12** precipitates in the experimental conditions). Some of the assayed derivatives (**1**, **10**, and **12**) increased oxygen consumption, between 10 and 60% with respect to untreated control cells (Table 4). While, the derivatives **2** and **8** inhibited respiration close to 90% at $IC_{50}$ equivalent concentration (see Section 6). No redox-cycling effect was observed for any of the studied nitroindazoles. According to the profile observed in the effect on the parasitic respiration by the tested 5-nitroindazole derivatives it do not depend of the nitro moiety. Interestingly it seems to be related to the amino side chain, the three derivatives that increase oxygen consumption possess a piperidine moiety and the other two that inhibit oxygen consumption bear a dimethylamino group. These mechanistic differences could be explained in terms of the different electronic and lipophilic characteristics of these two amino substituents. As it was observed for the previously studied trypanocidal activities where the HOMO energy was important and where these orbital frontiers were located mainly in this lateral amino substituent, the same could apply to the effect on the cellular respiration [11].

### 4.4. In vivo anti-T. cruzi evaluation

Parasite levels were similar among experimental groups having derivative **2** better parasitemia's profile than **1** and Bnz (Fig. 1A). None of the treated animals with the 5-nitroindazole derivatives died while in the control and Bnz groups survival fraction was 60.0 and 87.5%, respectively (Fig. 1B). Because the experiment lasted 60 days, it was not possible to obtain a negative serology (Fig. 2). In the first serology, 30 days post-infection, the four groups had the same level of anti-*T. cruzi* antibodies (Fig. 2A); however, 60 days post-infection both groups treated with derivative **1** and Bnz, showed a significantly ($p < 0.05$) lowering of anti-*T. cruzi* antibodies compared with control group (Fig. 2B). Overall, the 5-nitroindazole **1** showed a higher performance than the reference drug (Bnz) in this assay.

## 5. Conclusions

Thirteen 5-nitroindazole derivatives, previously developed as potential antiprotozoal drugs, were *in vitro* tested against trypomastigote form of *T. cruzi* (in mice blood), and six of them were also tested on promastigote form of three *Leishmania* species (*L. braziliensis*, *L. amazonenesis* and *L. infantum*). Most of the compounds showed promising trypanocidal activity on the bloodstream form of the parasite, excluding derivative **13**. Two of them, derivatives **7** and **8**, were as effective as the reference drug GV. Moreover, derivatives **2** and **10** caused a complete lysis of all the *Leishmania* promastigote form of the studied species, as observed with Ptd.

In addition, we have studied the effect on the oxygen consumption of *T. cruzi* in the presence of some derivatives, two of the tested compounds inhibited oxygen uptake in a concentration-dependent manner (derivatives **2** and **8**) and three derivatives increased oxygen consumption (derivatives **1**, **10** and **12**) without redox-cycling. These facts indicate different anti-*T. cruzi* mechanisms of action for the studied compounds showing that the only presence of a nitroaromatic group does not allow the compound to act as ROS producer.

Finally, supported by the *in vitro* trypanocidal activity showed by the 5-nitroindazoles at the present work and preceding data, as well on previous unspecific cytotoxicity studies, and possible different mode of action, two compounds – derivatives **1** and **2** – were submitted to *in vivo* evaluation using a murine model of acute Chagas' disease. Taking into account that in this initial *in vivo* screening the amount of tested compounds was very different, 78 mmol/kg/day for derivatives **1** and **2** and 192 mmol/kg/day for Bnz, some conclusions could be depicted. On the one hand, regarding to animal survival rate and the anti-*T. cruzi* antibodies levels after treatment, derivative **1** showed a higher performance than the reference drug, Bnz. On the other hand, according to parasitemia, animal survival rate and antibodies levels, derivative **2** *in vivo* behaviour was as good as Bnz one.

All of these results showed that derivatives **1** and **2** are excellent lead compounds for further structural modifications and deeper *in vitro* and *in vivo* biological studies as antiparasitic agents.

## 6. Experimental protocols

### 6.1. Chemistry

All starting materials were commercially available research-grade chemicals and were used without further purification. The compounds were prepared as previously reported [11]. All solvents were dried and distilled prior to use. All the organic reactions were carried out in a nitrogen atmosphere. Elemental analyses were performed on a Fisons EA 1108 CHNS-O analyzer on vacuum-dried samples (over phosphorous pentoxide at 3–4 mm Hg, 24 h at room temperature). Infrared spectra were recorded on a Perkin Elmer 1310 or a Bomen MB 102 apparatus, using potassium bromide tablets. $^{1}$H NMR, $^{13}$C NMR spectra and HMSQC and HMBC experiments were recorded on a Bruker DPX-400 (at 400 and 100 MHz) instrument.

### 6.2. Pharmacology

#### 6.2.1. In vitro anti-T. cruzi activity (trypomastigote form)

*T. cruzi* trypomastigotes (CL-Brener clone) were obtained from infected mice blood. Compounds were prepared in DMSO, and PBS was used to obtain a final concentration of 250 μg/mL. Blood containing $10^6$ parasites and the compounds at a 90/10 ratio were incubated in 96-well flat bottom microplates for 24 h at 4 °C. Parasites were counted by the method of Brener using an OLYMPUS BH-2 microscope. GV was included as a positive control, and PBS was used as negative control [13].

#### 6.2.2. In vitro anti-leishmania activity (promastigote form)

*L. amazonensis* (MHOM/BR/PH8), *L. infantum* (MHOM/FR/91/LEM 2259), and *L. braziliensis* (MHOM/BR/75/M-2903) promastigotes were grown at 28 °C in Schneider's medium supplemented with 20% (v/v) foetal calf serum. Parasites were seeded ($10^7$ parasites/mL) in 96-well flat bottom microplates, and compounds were prepared in a minimum amount of DMSO or Tween 80 using Schneider's medium to reach a final concentration of 100 μg/mL. After 72 h of incubation at 28 °C, parasites were observed using an inverted microscope OLYMPUS IMT-2 and compared to control (no compound added). Ptd was included as a positive control. The control where no lysis is observed represents the 0% whereas the positive control (Ptd) represents the 100% of lysis.

#### 6.2.3. Oxygen uptake

Tulahuen strain *T. cruzi* epimastigotes were harvested by 500*g* centrifugation, followed by washing and re-suspension in 0.05 M sodium phosphate buffer, pH 7.4, and containing 0.107 M sodium chloride. Respiration measurements were carried out polarographically with a Clark no. 5331 electrode (Yellow Springs Instruments) in a 53 YSI model (Simpson Electric Co). The chamber volume was 2 mL and the temperature was 28 °C. The amount of parasite used was equivalent to 2 mg of protein. The $IC_{50}$ equivalent concentration corresponds to the final concentration used in the oxygen uptake experiments. This concentration was calculated considering that the $IC_{50}$ (culture growth experiments) was determined using $3 \times 10^6$ parasites/mL, equivalent to 0.0375 mg protein/mL as the initial parasite mass; $80 \times 10^6$ parasites/mL, equivalent to 1 mg protein/mL, was used in the oxygen uptake experiments. In order to maintain the parasite mass–drug ratio constant in these two types of experiments, the original $IC_{50}$ was corrected by this 26-fold parasite mass increase in the oxygen uptake experiment. Values are expressed as mean ± SD for three independent experiments. No effect of DMSO alone was observed [18,19].

#### 6.2.4. In vivo anti-T. cruzi activity (acute model)

BALB/c male mice (30 days old, 25–30 g) bred under specific pathogen-free (SPF) conditions, were infected by intraperitoneal injection of $10^3$ blood trypomastigotes (CL-Brener clone). One group of 10 animals was used as control (PBS), and three groups of eight animals were treated with the two compounds and Bnz, respectively. First parasitemia was done 5 days post-infection (week 1) and the treatment begun 7 days after. Compounds were administered orally, as aqueous solution in PBS, at 30 mg/kg/day for 5-nitroindazole derivatives and 50 mg/kg/day for Bnz, during 10 days. Parasitemia in the control and treated mice was determined once weekly after the first administration for 60 days in tail-vein blood and the mortality rate was recorded. All the sera obtained after centrifugation of the blood extracted from infected mice were tested twice by ELISA (enzyme linked immuno assay) at 30 and 60 days post-infection. A locally produced ELISA kit (Chagas test, IICS, Asunción, Paraguay) was used following the procedure recommended by the manufacturer (IICS Production Department, Asunción-Paraguay). The antibodies analyzed are IgG1, IgG2a and IgG2b. The optical density values were obtained in an ELISA plate reader (Titerek Unistan I). Wilconxon test was used in order to compare the levels of anti-*T. cruzi* antibodies among experimental groups [14]. The experimental protocols with animals were evaluated and supervised by the local Ethics Committee and the research adhered to the Principles of Laboratory Animal Care [20].

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