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In vitro leishmanicidal activity of 1,3-disubstituted 5-nitroindazoles

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ABSTRACT

The antiprotozoal activity of some indazole-derived amines (**2**, **3**, **5–8**) as well as that of some simple structurally related 3-alkoxy-1-alkyl-5-nitroindazoles (**1**, **4**) against promastigote and amastigote forms of *Leishmania infantum* and *Leishmania braziliensis* is reported. In some cases, these compounds showed *in vitro* activities against the different morphological forms of *Leishmania* similar to or higher than those of the reference drug glucantime; this fact, along with low unspecific cytotoxicities against macrophages shown by some of them, led to good selectivity indexes (SI). The high efficiency of some 5-nitroindazoles against the mentioned protozoa was confirmed by further *in vitro* studies on infection rates. Complementary analyses by ^{1}H NMR of the changes on the metabolites excreted by parasites after treatment with the more active indazole derivatives in many cases showed the decreased excretion of succinate and increased levels of acetate, lactate and alanine, as well as, in some cases, the appearance of glycine and pyruvate as new metabolites. Damage caused by indazoles at the glycosomal or mitochondrial level are consistent with these metabolic changes as well as with the huge ultrastructural alterations observed by transmission electron microscopy (TEM), especially affecting the mitochondria and other cytoplasmic organelles.

1. Introduction

Leishmaniases are a group of parasitic diseases caused by different protozoan species of genus *Leishmania*, transmitted by phlebotomine sand flies. In humans, the disease presents as three main clinical forms, depending on the involved species of *Leishmania*: cutaneous, mucocutaneous and visceral leishmaniasis, with the latter being the most severe and life-threatening form. Leishmaniases are prevalent in tropical and subtropical areas; they currently affect 98 countries with 12 million cases, and a further 350 million people are presently at risk; the annual incidence is estimated at 1–1.5 million cases of cutaneous disease and 500,000 cases of visceral disease (Croft et al., 2006; Santos et al., 2008; Cavalli and Bolognesi, 2009; WHO, 2010).

Treatment of leishmaniases has been based for many years on pentavalent antimonials, which are still the first-line drugs. The second-line drugs include pentamidine, amphotericin B, miltefosine and paromomycin, and more recently, sitamaquine has shown very promising properties (Santos et al., 2008; Cavalli and Bolognesi, 2009; Espuelas et al., 2012; Singh et al., 2012). As in the case of other neglected tropical diseases, most of the current therapies are inadequate essentially due to several factors such as the low therapeutic indexes leading to high toxicities and unacceptable side-effects, the emergence of resistant parasites, high prices that are unaffordable for the affected countries, *etc.* These drawbacks of the current therapy, together with the fact that a vaccine is an unachieved goal, make the search for new drugs urgently needed (Cavalli and Bolognesi, 2009; Croft et al., 2006; Espuelas et al., 2012). In the last few years, many compounds showing leishmanicidal properties have been reported in the literature, and furthermore, several potential drug targets have been proposed and validated (Cavalli and Bolognesi, 2009; Espuelas et al., 2012; Singh et al., 2012). Nevertheless, owing to the low income of the affected population, investment in the development of new drugs against leishmaniases has not been financially attractive for pharmaceutical companies, and the interest of academic institutions is rather limited (Cavalli and Bolognesi, 2009; Espuelas et al., 2012).

In this context, in recent years, we have been studying the anti-chagasic (Arán et al., 2005; Boiani et al., 2009; Rodríguez et al., 2009a, 2009b) and leishmanicidal (Boiani

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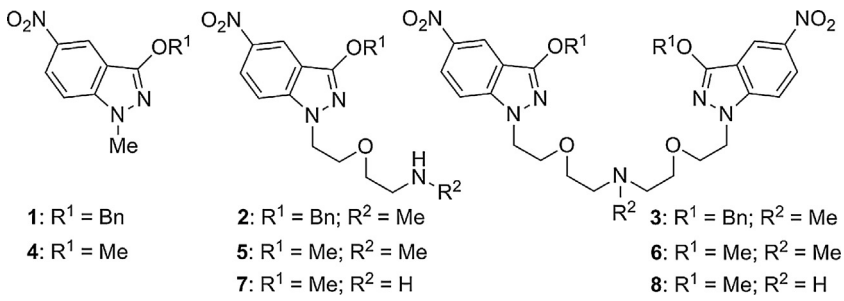

Fig. 1. Chemical structure of indazole derivatives **1–8** studied in the present work.

et al., 2009) properties of some 3-alkoxy-1-alkyl-5-nitroindazoles. Nitroheterocycles are not currently used for the treatment of leishmaniases (Raether and Hänel, 2003), but some compounds of this class, *e.g.* the anti-trypanosomal drug fexinidazole [2-acetoxy-*N*-(5-nitrothiazol-2-yl)benzamide] (Wyllie et al., 2012) or the broad-spectrum anti-parasitic compound nitazoxanide {1-methyl-2-[4-(methylthio)phenoxymethyl]-5-nitro-1*H*-imidazole} (Chan-Bacab et al., 2009), have recently been reported as potential anti-leishmanial drugs. In the present article, we report the leishmanicidal properties of a new family of 5-nitroindazole-derived primary, secondary and tertiary amines (**2**, **3**, **5–8**) recently prepared and studied as anti-chagasic agents (Muro et al., 2014); compounds **1** and **4** were also tested as simple models containing the 3-alkoxy-1-alkyl-5-nitroindazole scaffold (Fig. 1).

Leishmanicidal activity was first studied *in vitro* against *Leishmania infantum* and *Leishmania braziliensis* (promastigote and amastigote forms) as representative species causing visceral and cutaneous leishmaniasis, respectively; unspecific cytotoxicity against mammalian cells of all compounds, as well as infectivity assays for compounds **1**–**5**, were carried out using macrophages.

Furthermore, a ^{1}H NMR study has been conducted in order to observe changes in the nature and percentage of metabolites' excretion directed to obtain information about the effect of our compounds on the glycolytic pathway of parasites; finally, we have also studied the ultrastructural alterations of parasites treated with some of our compounds using transmission electron microscopy (TEM).

2. Materials and methods

2.1. Preparation of studied compounds

Simple 3-alkoxy-1-alkylindazoles **1** and **4** were prepared by the alkylation of 1-methyl-5-nitroindazol-3-ol, and 5-(3-alkoxyindazolyl)-3-oxapentylamines **2**, **3**, **5–8** were prepared from the required (3-alkoxyindazolyl)alkyl halides and ammonia or methylamine, as reported previously (Muro et al., 2014).

2.2. Parasite strains culture

Promastigote forms of *L. infantum* (MCAN/ES/2001/UCM-10) and *L. braziliensis* (MHOM/BR/1975/M2904) were routinely maintained in our laboratory by fortnightly passages in RPMI 1640 medium (Gibco, Spain) at 28 °C. For the studies described in Sections 2.4, 2.7 and 2.8, promastigotes were cultivated 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 cm^2, according to a previously described methodology (González et al., 2005).

2.3. Cell culture and cytotoxicity tests

J774.2 macrophages (ECACC number 91051511) originally obtained from a tumour in a female BALB/c mouse in 1968 (Koren et al., 1975) were grown in RPMI 1640 medium, supplemented with 20% inactivated foetal bovine serum in a humidified 95% air and 5% CO_2 atmosphere at 37 °C for two days. The cytotoxicity test for macrophages was performed according to a previously described methodology (Marín et al., 2011). After 72 h of treatment, cell viability was determined by flow cytometry. Thus, 100 μL/well of propidium iodide solution (100 mg/mL) was added and incubated for 10 min at 28 °C in darkness. Afterwards, 100 μL/well of fluorescein diacetate (100 ng/mL) was added and incubated under the same conditions. Finally, the cells were recovered by centrifugation at 400 × *g* for 10 min and the precipitate washed with phosphate buffered saline (PBS). Flow cytometric analysis was performed with a FACSVantage flow cytometer (Becton Dickinson). The viability percentage was calculated in comparison with the control culture. The IC_{50} was calculated using linear regression analysis from the K_c values corresponding to the concentrations employed (1, 5, 10, 25, 50 and 100 μM).

2.4. In vitro *activity: Promastigotes assays (extracellular forms)*

The obtained compounds and the reference drug (glucantime) were dissolved in dimethyl sulphoxide, which, at a final concentration of 0.01%, was shown to be nontoxic and without inhibitory effects on parasite growth (González et al., 2005). Compounds were added to the culture medium at dosages of 1, 5, 10, 25, 50 and 100 μM. The effects of each compound against promastigotes of *L. infantum* and *L. braziliensis* were tested at 72 h using a Neubauer haemocytometric chamber.

2.5. In vitro *activity: Amastigotes assays (intracellular forms)*

J774.2 macrophages were grown in RPMI 1640 medium, supplemented with 20% inactivated foetal bovine serum, and kept in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were seeded at a density of 1×10^4 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 promastigote forms of *L. infantum* and *L. braziliensis* in the stationary phase, at a ratio of 10:1 for 24 h. The non-phagocytosed parasites were removed by washing, and then the drugs (1, 5, 10, 25, 50 and 100 μM) were added. Macrophages were incubated with the drugs for 72 h at 37 °C in 5% CO_2. Drug activity was determined on the basis of the 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.

2.6. In vitro *activity: Infection assays*

J774.2 macrophages were grown under the same conditions expressed in the amastigotes assay for two days. Afterwards, the cells were infected *in vitro* with promastigote forms of *L. infantum* and *L. braziliensis* in the stationary phase, at a ratio of 10:1. Immediately after infection, the studied drugs were added at their IC_{25} concentrations in order to see their effects but minimising the number of dead parasite cells, and the cultures were incubated for 12 h at 37 °C in 5% CO_2. IC_{25} values of compounds **1–5** were, respectively, 3.05, 0.89, 2.75, 6.51 and 2.30 μM for *L. infantum* promastigotes, and 1.15, 1.85 and 2.25 μM for **1–3** in the case of *L. braziliensis* promastigotes. The non-phagocytosed parasites and drugs were removed by washing, and then the infected cultures were grown for 10 days in fresh medium. Fresh culture medium was added every 48 h. The drug activity was determined from the percentage of infected cells and the mean number of amastigotes per infected cell. These parameters were determined by analysing 200 host cells distributed in randomly chosen microscopic fields.

2.7. *Metabolites excretion study*

Cultures of *L. infantum* and *L. braziliensis* promastigotes in MTL medium (initial concentration 5×10^5 cells/mL) received IC_{25} concentrations (see Section 2.6) of the studied compounds (except for control cultures). After incubation for 96 h at 28 °C, the cells were centrifuged at 400 × *g* for 10 min. The supernatants were collected and, after the addition of D_2O (10% v/v), their ^1H NMR spectra were recorded at 25 °C on a Varian Inova Unity 300 MHz instrument using pre-saturation for water suppression and the following parameters: spectral width 10 ppm, acquisition time 1.998 s, relaxation delay 20 s, number of transients 80; software: VnmrJ 1.1B. Chemical shifts were expressed in parts per million (ppm, δ scale), using sodium 2,2-dimethyl-2-silapentane-5-sulfonate (45 μM) as the reference. The chemical shifts used to identify the respective metabolites were consistent with those that were previously reported (Singha et al., 1996; Fernández-Becerra et al., 1997; Marín et al., 2013a).

2.8. *Ultrastructural alterations*

Promastigotes of *L. infantum* and *L. braziliensis* were cultured at a density of 5×10^5 cells/mL in MTL medium containing the compounds tested at their IC_{25} concentrations (see Section 2.6). After 96 h, these cultures were centrifuged at 400 × *g* for 10 min, and the pellets produced were washed in PBS and then mixed with 2% (v/v) paraformaldehyde/glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 4 h at 4 °C. Following this, the pellets were prepared for transmission electron microscopy (TEM) studies using a previously described technique (Ramírez-Macías et al., 2012a).

3. Results and discussion

3.1. In vitro *activity: Promastigotes and amastigotes assays*

Initial *in vitro* activity studies were carried out on promastigote forms (extracellular sand fly vector stage), which, in contrast to the amastigote forms, can be easily maintained in axenic culture; further assays were performed by infecting macrophages with promastigotes, which were rapidly converted into amastigotes after infection. Intracellular amastigotes mainly reside and multiply in macrophages and are responsible for the pathologies associated with the disease, thus being the real target of anti-leishmanial chemotherapy (Hussain et al., 2014; Marín et al., 2013b).

The *in vitro* activity of compounds **1–8** as well as that of the standard drug glucantime (meglumine antimoniate) on promastigote and amastigote forms (Bates, 2007; Wheeler et al., 2011) of *L. infantum* and *L. braziliensis*, the unspecific cytotoxicity against macrophages and the corresponding selectivity indexes (SI) are presented in Table 1. All derivatives were assayed at concentrations of 1–100 μM, and the obtained data allowed the IC_{50} values shown in Table 1 to be calculated. The leishmanicidal effect is expressed as the IC_{50}, *i.e.* the concentration required to give 50% of growth inhibition, calculated by linear regression analysis from the K_c values corresponding to the concentrations employed. Results gathered in Table 1 are the averages of four separate experiments.

Activities against the two morphological forms of both species of *Leishmania* range from three times to one third of those of the reference drug. In most cases, 3-benzyloxy derivatives are more active than the corresponding 3-methoxy analogues (compare **1** *vs.* **4**, **2** *vs.* **5** and **3** *vs.* **6**) and the activities of primary amine **7** are usually lower than those of the corresponding *N*-methyl derivative **5**. Nevertheless, there is no clear correlation between the activities and the structures of indazole- and bisindazole-derived compounds (**2** *vs.* **3**, **5** *vs.* **6** and **7** *vs.* **8**). On the other hand, there are great differences in toxicity against macrophages; however, it is not easy to establish a relationship between the structures of compounds and their unspecific toxicities, but primary amine **7** and, especially, secondary amine **8** are by far the more toxic compounds.

These different toxicities are of course reflected in the corresponding selectivity indexes, which were higher in most cases for amastigotes than for promastigotes (Table 1). Thus, 3-benzyloxy derivatives **1–3**, with SI values exceeding 12–43 times those of glucantime depending on the *Leishmania* species and on the parasite stage are the best compounds, followed by 3-methoxy derivatives **4–6**. Only the SI values of compounds **7** and **8** are very poor, similar to those of the standard drug glucantime.

Additionally, the effect of the most active indazole derivatives on the propagation of *L. infantum* (compounds **1–5**) and *L. braziliensis* (compounds **1–3**) in macrophages was studied by measuring the rates of infection and the average number of amastigotes per macrophage present during a 10-day experiment. Values represented in Fig. 2 are the means of three separate experiments; in all cases, experimental values fall within two standard deviations from the mean. Cultured macrophages were infected with promastigotes of both species of *Leishmania*, which were converted in the invaded cells into amastigotes within 1 day. In the case of *L. infantum*, the percentages of infection after 12 h were 14% for the untreated control, and 27, 30, 28, 12 and 10% for compounds **1**, **2**, **3**, **4** and **5**, respectively; similarly, for *L. braziliensis*, the percentages of infection were 19% in the case of untreated control, and 28, 30 and 16% for compounds **1**, **2** and **3**, respectively. This initially increased rate of infection probably induced by some of the tested compounds is not usual (Ramírez-Macías et al., 2011, 2012b, 2012c), but has previously been observed for some benzo[*g*]phthalazine derivatives after 24 h of treatment (Sánchez-Moreno et al., 2012).

During the mentioned 10-day period, the rate of infection of the host cells in the absence of drugs (control) gradually increased, reaching 73% invasion for *L. infantum* (Fig. 2A) and 72% for *L. braziliensis* (Fig. 2C) at the end.

For *L. infantum*, similar experiments in the presence of glucantime and compounds **1–5** at their IC_{25} concentrations were carried out (Fig. 2A). All compounds were more effective than the reference drug (40% reduction in relation to the untreated control), displaying infection rate decreases between 56 and 81%; special mention deserve compounds **5**, **2** and **4** with values of 81, 75 and 73%, respectively (Fig. 2A). In relation to the number of amastigotes per macrophage (Fig. 2B), related results were obtained; all indazole derivatives were more efficient than glucantime (47% reduction with respect to the control), but, in this case, compounds **1**, **2** and **5**, with decreases of 85, 78 and 74%, respectively, exhibited the best results.

Table 1
In vitro activity, unspecific cytotoxicity and selectivity index (SI) found for 5-nitroindazole derivatives **1–8** and glucantime (reference drug) on extra- and intracellular forms of *L. infantum* and *L. braziliensis*.

Compounds	IC_{50} (μM)[a]				Macrophages toxicity IC_{50} (μM)[a]	SI[b]			
	L. infantum		*L. braziliensis*			*L. infantum*		*L. braziliensis*	
	Promastigote forms	Intracellular amastigote forms	Promastigote forms	Intracellular amastigote forms		Promastigote forms	Intracellular amastigote forms	Promastigote forms	Intracellular amastigote forms
Glucantime	18.0 ± 1.3	24.2 ± 2.6	25.6 ± 1.7	30.4 ± 6.1	15.2 ± 1.0	0.8	0.6	0.6	0.5
1	16.9 ± 0.6	12.1 ± 0.7	13.9 ± 0.8	12.9 ± 1.1	178.8 ± 11.7	10.5 (13)	14.8 (25)	12.9 (21)	13.9 (28)
2	11.7 ± 1.0	10.9 ± 0.4	15.0 ± 0.3	9.1 ± 0.3	196.7 ± 17.3	16.8 (21)	17.9 (30)	13.1 (22)	21.6 (43)
3	18.7 ± 0.7	13.3 ± 0.6	17.3 ± 1.3	16.4 ± 0.9	179.3 ± 10.8	9.6 (12)	13.5 (22)	10.4 (17)	10.9 (22)
4	33.2 ± 1.1	24.8 ± 1.8	37.8 ± 2.6	36.2 ± 4.1	300.0 ± 21.0	9.0 (11)	12.1 (20)	7.9 (13)	8.3 (17)
5	21.7 ± 0.9	12.4 ± 1.2	17.6 ± 0.8	38.2 ± 2.5	155.3 ± 10.6	7.2 (9)	12.5 (21)	4.1 (7)	8.8 (18)
6	26.7 ± 1.5	27.1 ± 2.2	30.4 ± 1.5	37.1 ± 3.2	205.5 ± 16.2	7.7 (10)	7.6 (13)	6.8 (11)	5.5 (11)
7	41.9 ± 3.2	24.8 ± 0.3	45.6 ± 2.8	39.5 ± 2.8	66.7 ± 4.3	1.6 (2)	2.7 (5)	1.5 (2)	1.7 (3)
8	52.1 ± 2.5	41.7 ± 2.6	29.2 ± 1.4	55.6 ± 2.7	14.4 ± 1.2	0.3 (1)	0.3 (1)	0.5 (1)	0.3 (1)

[a] IC_{50} = 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) = IC_{50} macrophages/IC_{50} 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.

In the case of *L. braziliensis*, compounds **1**–**3**, subjected to similar studies (Fig. 2C and D), showed to be more effective than the reference drug in both tests. In fact, the rate of infection decreased in relation to the control, showing compounds **2**, **1** and **3**, with reductions in the infection rate of 79, 72 and 64% respectively, a much higher efficiency than glucantime (44% reduction) (Fig. 2C). In connection with the average number of amastigotes per macrophage (Fig. 2D), glucantime showed a reduction of only 26% relative to the control, while compounds **1, 2** and **3** significantly increased this value to 79, 71 and 65%, respectively.

According to our studies, compounds **1** and **2** are always among the most active in the four described anti-leishmanial infection trials, and show the highest SI values found in Table 1. We can also highlight here the remarkable leishmanicidal activity of the easily available 3-benzyloxy-1-methylindazole **1**; this compound clearly shows that the presence of amino groups at the end of the chain at position 1 of the indazole system is not strictly necessary for activity and that this class of compounds, *i.e.*, 3-alkoxy-1-alkylindazoles containing simple alkyl substituents at N-1 and 3-O, are interesting candidates for further studies.

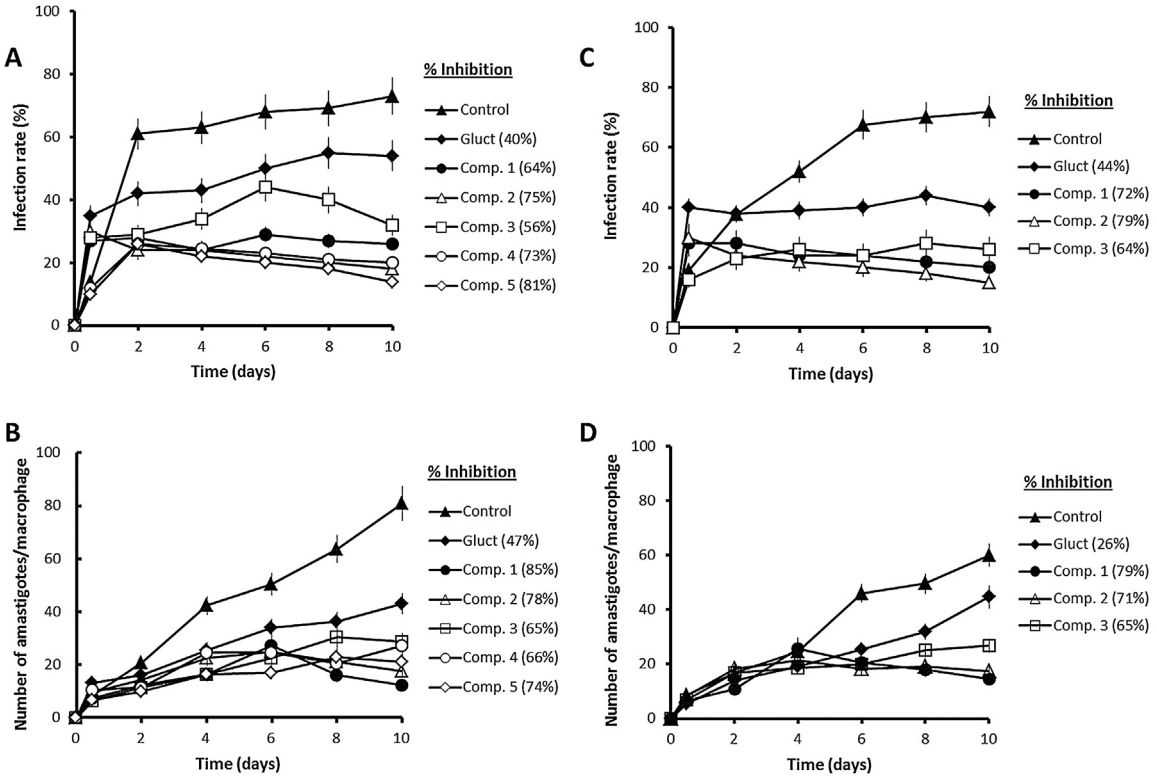

Fig. 2. Effect of glucantime (Gluct) and 5-nitroindazole derivatives (IC_{25} concentrations) on the infection rate and growth of *L. infantum* (A and B) and *L. braziliensis* (C and D): (A and C) rate of infection; (B and D) mean number of amastigotes per infected macrophage. % Inhibition refers to the percentage decrease of the mentioned infection parameters in relation to the untreated control at the end of the experiment.

Activities against *L. amazonensis*, *L. braziliensis* and *L. infantum* for related 3-alkoxy-1-[ω-(dialkylamino)alkyl]indazoles have been published (Boiani et al., 2009); as in our current case, 3-benzyloxy derivatives were more active than the corresponding 3-methoxy analogues, but the data provided by the mentioned authors are heterogeneous (changes in the *Leishmania* strains, differences in the way of expressing leishmanicidal activity, IC_{50} and SI values not determined, *etc.*) and a precise comparison with those obtained in this article is not possible. Unfortunately, this situation is rather frequent in the field of anti-parasitic drugs, as has been stressed, *e.g.* for anti-chagasic or anti-malarial agents, by some authors (Sykes and Avery, 2013).

In relation to the mode of action of our compounds, it has been suggested (Rodríguez et al., 2009a, 2009b) that anti-chagasic nitroindazoles act, as other nitroheterocycles, upon intracellular nitro-reduction followed by redox cycling leading to reactive oxygen species (ROS) or through the production of electrophilic metabolites that are able to damage essential biomolecules of trypanosomatid parasites (Maya et al., 2003, 2007). It seems that nitroreductases are, effectively, as in the case of fexinidazole (Wyllie et al., 2012), essential for the transformation of anti-parasitic nitro compounds into the reactive species responsible for activity. However, a recent report has shown that the mode of action usually claimed for nifurtimox, *i.e.*, the induction of oxidative stress in *Trypanosoma cruzi*, cannot be sustained (Boiani et al., 2010) and, in fact, the concrete mechanism of action after the reduction of NO_2 group of anti-chagasic drugs nifurtimox and benznidazole has recently been reviewed (Hall et al., 2011; Hall and Wilkinson, 2012).

3.2. Metabolism studies by ^{1}H NMR

Trypanosomatid protozoa are unable to completely degrade glucose to CO_2 under aerobic conditions and, accordingly, a considerable part of the skeleton of hexose is excreted into the medium as partially oxidised fragments (Bringaud et al., 2006; Cazzulo, 1992). Most glycolytic enzymes responsible for their characteristic catabolism are located in the organelle called the glycosome (Ginger, 2005; Michels et al., 2006). Depending on the pathways used for glucose metabolism by the different species of trypanosomatids and their life cycle stage, variations in the nature and percentage of catabolism products can be observed, but CO_2, succinate, acetate, lactate, pyruvate, L-alanine and ethanol are usually detected (Bringaud et al., 2006; Cazzulo, 1992).

To gain information concerning the effect of our indazole derivatives on glucose metabolism in the parasites, the final excretion products were identified. These data were obtained by recording the ^{1}H NMR spectra of cultures of promastigotes of *L. infantum* and *L. braziliensis* after treatment with the studied compounds at their IC_{25} concentrations and separation of the parasite cells by centrifugation. The results were compared with a control of promastigotes maintained in a cell-free medium for four days after inoculation with the parasite, showing the characteristic signals of the CH_3 groups of acetate (singlet, $\delta = 1.95$ ppm), alanine (doublet, $\delta = 1.47$ ppm), lactate (doublet, $\delta = 1.32$ ppm) and pyruvate (singlet, $\delta = 2.30$ ppm), and CH_2 signals of glycine (singlet, $\delta = 3.50$ ppm) and succinate (singlet, $\delta = 2.48$ ppm) (Supplementary material, Figs. S1 and S2).

For *L. infantum*, when parasites were treated with compounds **1–3**, succinate excretion was inhibited (*ca.* 24%), levels of alanine remained unchanged and those of lactate and acetate increased *ca.* 12% and 30–58%, respectively (Table 2; Supplementary material, Fig. S1). After treatment with compounds **4** and **5**, succinate excretion was slightly inhibited or remained unchanged, respectively; on the other hand, increases of acetate [especially for compound **5** (74%)], alanine (*ca.* 27%) and lactate levels (*ca.* 54%) were observed together with the emergence of glycine as a new metabolite.

Inhibition of succinate excretion caused by compounds **1–4** may indicate the disruption of glycosomal or mitochondrial enzymes involved in its biosynthesis (Bringaud et al., 2006). This inhibition of succinate production can explain the usually observed increased levels of lactate, alanine and acetate. In fact, if the processing of phosphoenolpyruvate (PEP) to succinate at the glycosomal level is inhibited, this may force the catabolism of PEP towards pyruvate and pyruvate-derived metabolites such as L-alanine and L-lactate at the cytosolic level, and acetate at the mitochondrial level. In relation to lactate, we have not established the chirality of the product observed by NMR. In trypanosomatids, L-lactate proceeds from the glycolytic pathway and, in *Leishmania* in particular, D-lactate comes from methylglyoxal detoxification (Bringaud et al., 2006). We assume that most of the lactate observed by NMR comes from the glycolytic route; in fact, in our study, lactate levels increase as those of other pyruvate-derived metabolites such as acetate or, in some cases, alanine usually do. Regarding the appearance of glycine as a new metabolite after treatment with compounds **4** and **5**, this amino acid has been detected in cultures of *T. brucei* procyclic trypomastigotes (Bringaud et al., 2006), as well as in some *L. donovani* strains (Singha et al., 1996). It has been assumed that glycine appears in trypanosomatids as a result of L-threonine catabolism at the mitochondrial level, but it is difficult to interpret the presence of glycine in our case taking into account that the role of this metabolic pathway is unclear (Bringaud et al., 2006).

Somewhat related effects were observed when promastigotes of *L. braziliensis* were treated with compounds **1–3**; succinate levels decreased in all cases and those of lactate and alanine increased (for **1** and **3**) or remained unchanged (for **2**). On the other hand, levels of acetate exhibited an erratic behaviour, showing increases for **3** and, especially, for **1** (212%), while they decreased after treatment with compound **2**. The glycine signal mentioned appeared after treatment with compounds **2** and **3**, and a new peak identified as pyruvate was detected in the presence of **1** and **2** (Table 2; Supplementary material, Fig. S2).

As previously commented for *L. infantum*, the strong inhibition of succinate production caused by compounds **1** and **2** may be responsible for the high levels of pyruvate observed which, despite its partial intracellular conversion to acetate, alanine and lactate, is secreted into the medium. For compound **2**, the decrease in the production of acetate may be correlated with a low mitochondrial permeability to pyruvate or with the inhibition of some of the mitochondrial enzymes involved in the biosynthesis of acetate from cytosolic pyruvate (Bringaud et al., 2006).

The observed metabolic changes are consistent with the huge ultrastructural alterations observed by TEM, especially with damage to the mitochondria and cytoplasmic organelles (Section 3.3).

3.3. Ultrastructural alterations

Ultrastructural alterations induced on *L. infantum* and *L. braziliensis* promastigotes by indazole derivatives **1–5** at their IC_{25} concentrations have been studied by transmission electron microscopy (TEM).

5-Nitroindazole derivatives were very effective against *L. infantum* (**1–5**) and *L. braziliensis* (**1–3**), as shown in Figs. 3 and 4, respectively, where ultrastructural alterations and the destruction of promastigotes are obvious.

In the case of *L. infantum* (Fig. 3), compound **1** induced the formation of a large number of vacuoles (V), some of them filled with granular and membranous structures. Other apparent effects were the presence in some promastigotes of bloated and unstructured kinetoplast (K) and swollen mitochondria (M), as well as barely electron-dense cytoplasm devoid of other organelles. Compound **2**

Table 2
Variation in the height of the peaks corresponding to catabolites excreted by *L. infantum* and *L. braziliensis* promastigotes in the presence of 5-nitroindazole derivatives **1–5** with respect to the control test[a].

Compounds	*L. Infantum*						*L. braziliensis*					
	Lac (%)	Ala (%)	A (%)	Pyr (%)	S (%)	Gly (%)	Lac (%)	Ala (%)	A (%)	Pyr (%)	S (%)	Gly (%)
1	+11	=	+30	und	−24	und	+30	+40	+212	+100	−45	und
2	+14	=	+58	und	−23	und	=	=	−40	+70	−40	+100
3	+13	=	+32	und	−25	und	+15	+26	+72	und	−20	+100
4	+55	+25	+22	und	−15	+100	–	–	–	–	–	–
5	+53	+29	+74	und	=	+100	–	–	–	–	–	–

[a] Lac, lactate; Ala, L-alanine; A, acetate; Pyr, pyruvate; S, succinate; Gly, glycine. (−) peak decrease; (+) peak increase; (=) no difference detected; (und) undetected.

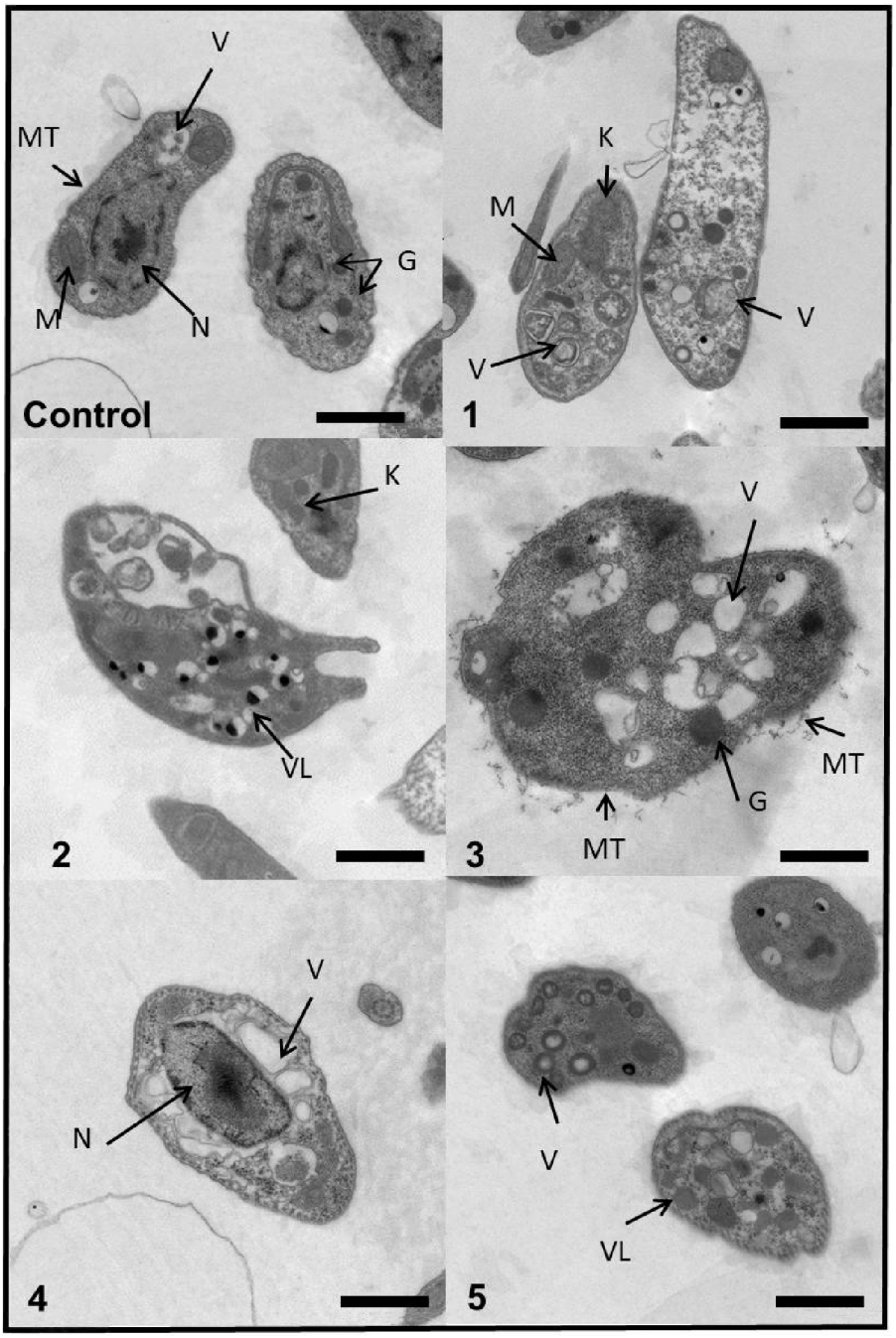

Fig. 3. Ultrastructural alterations by TEM in promastigotes of *L. infantum* either untreated (control) or treated with 5-nitroindazole derivatives **1–5** (IC_{25} concentrations), showing organelles with their characteristics: parasite nucleus (N); kinetoplast (K); lipidic vacuoles (VL); vacuoles (V); mitochondrion (M); glycosomes (G); cytoskeleton microtubules (MT). Control: untreated parasites; (1) parasites treated with compound **1**; (2) parasites treated with compound **2**; (3) parasites treated with compound **3**; (4) parasites treated with compound **4**; (5) parasites treated with compound **5**. In all cases, the bar corresponds to 1 μm.

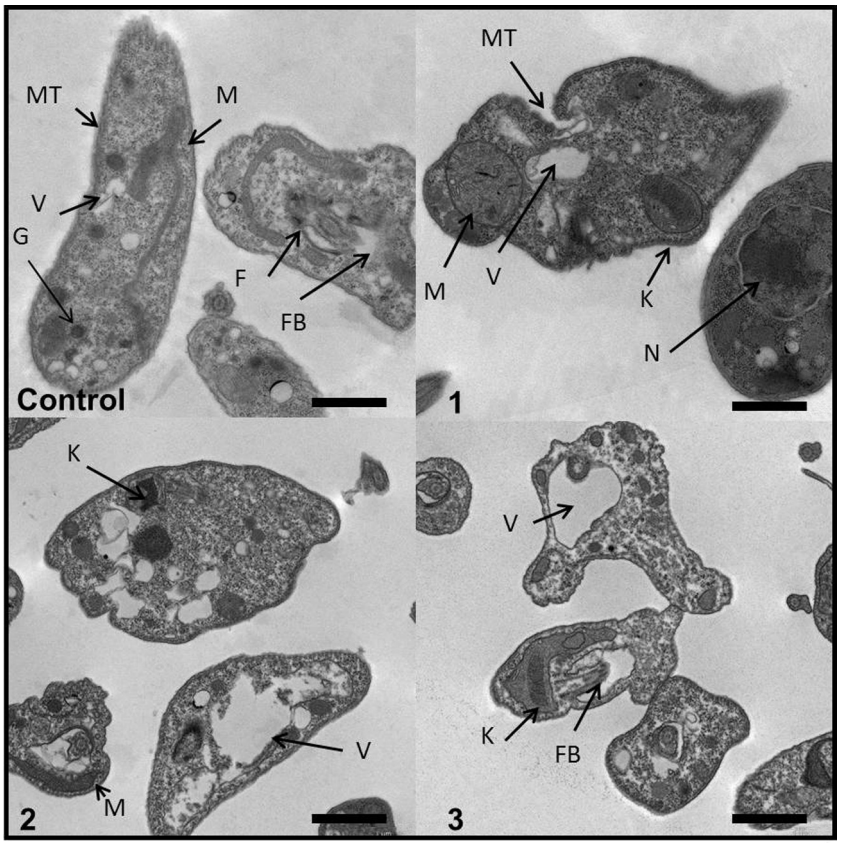

Fig. 4. Ultrastructural alterations by TEM in promastigotes of *L. braziliensis*, either untreated (control) or treated with 5-nitroindazole derivatives **1–3** (IC_{25} concentrations), showing organelles with their characteristics: parasite nucleus (N); kinetoplast (K); vacuoles (V); mitochondrion (M); glycosomes (G); cytoskeleton microtubules (MT); flagellum (F); large flagellar pockets (FB). Control: untreated parasites; (1) parasites treated with compound **1**; (2) parasites treated with compound **2**; (3) parasites treated with compound **3**. In all cases, the bar corresponds to 1 μm.

induced the death of most promastigotes of *L. infantum*, so that the culture medium was filled with odd shapes that are merely remnants of destroyed parasites. In some of them apparently retaining the usual morphology, the ultrastructure was completely abnormal, with vesicles of electron-dense material. Compound **3** led to the death of most parasites, whose debris appeared spread throughout the culture medium (figure not shown). The breakup of the cytoplasmic membrane of some of them, with loss of microtubules (MT), was also observed. The cytoplasm of most parasites treated with compound **3** contained exclusively empty vacuoles (V) or membranous structures of unknown origin and glycosomes. Compound **4** produced alterations in both the cytoplasm and the nucleus of parasites (N). The latter appeared oversized, occupying most of the cytoplasmic space of the parasite. Vacuolisation (V) was intense and mitochondria of the parasites were almost unrecognisable.

Compound **5** was less effective, but also induced major changes in promastigotes, whose content were basically very abundant vacuoles (V), some of them of lipidic type (VL), empty vacuoles or vacuoles with unknown content. Some promastigotes presented a more electron-dense contour, but were empty inside.

In the case of *L. braziliensis* (Fig. 4), the most effective compounds **1–3** induced, in relation to the control cultures, strong changes in the morphology of promastigotes which could induce the death of many of them. Parasites treated with compound **1** showed strongly electron-dense and compact aspects, with altered cytoplasmic organelles and nucleus. Subpellicular microtubules (MT), kinetoplast (K), mitochondria (M) and nucleus (N) appeared strongly swollen and some parasites were full of vacuoles, of lipidic type or empty. Many promastigotes also had cracks in the cytoplasmic membrane.

When parasites were treated with indazole **2**, various alterations occurred in the morphology of promastigotes, seriously affecting their viability. Thus, we observed parasites with disorganised cytoplasm, grainy and full of empty vacuoles (V) or with membranous structures, which, in many cases, occupied most of the cytoplasm. Lipidic vacuoles, altered and with granular appearance, were also observed.

Mitochondria (M) were swollen and kinetoplasts (K) also swollen, with an electron-dense appearance and disruption of its typical helical structure. Other parasite cells had an altered, very electron-dense cytoplasm, with vacuoles containing granular or membranous structures, and a cytoskeleton devoid of microtubules. Finally, compound **3** also induced similar effects in promastigotes, with changes in the aspect of cytoplasm, to granular and vacuolated, and the appearance of membranous associations, possibly arising from the destruction of other cytoplasmic structures. Slightly altered kinetoplasts (K), with discontinuities in their helical structure, were also observed in some promastigotes.

These data suggest that the severe damage caused by our compounds in organelles such as mitochondria or glycosomes are probably responsible for the remarkable metabolic changes observed in both species *of Leishmania*.

Very similar abnormalities, although less relevant (parasites size reduction, cytoplasmic disorganisation, intense vacuolisation, undulation of the plasma membrane, *etc.*), were observed after the treatment of both *L. infantum* and *L. braziliensis* with glucantime (images not shown).

4. Conclusions

There are several drugs for the treatment of leishmaniases, but most of them have a number of serious drawbacks; therefore, it is necessary to develop safer, more efficient and cheaper new drugs, preferably with novel modes of action.

In this regard, although nitroheterocycles are currently used for the treatment of certain protozooses, *e.g.*, nifurtimox and benznidazole for Chagas disease, metronidazole for trichomonosis, *etc.* (Raether and Hänel, 2003), their use in leishmaniases is poorly explored (Boiani et al., 2009; Chan-Bacab et al., 2009; Wyllie et al., 2012). In the present work, biological evaluation of the studied indazoles has shown that some compounds exhibit adequate *in vitro* leishmanicidal activity against the two studied morphological stages of *L. infantum* and *L. braziliensis*. The establishment of a clear structure–activity relationship is difficult, but the more lipophilic 3-benzyloxy derivatives show, in general, higher activities than the corresponding 3-methoxy analogues, and primary amines are less active than the corresponding *N*-methyl derivatives. On the other hand, the large changes observed in the cytotoxicity of indazoles against macrophages led to dramatic differences in the values of selectivity indexes in relation to those of the anti-leishmanial standard drug glucantime; thus, compounds **1** and **2** stand out for their high effectiveness, further confirmed through additional infectivity assays.

A ^{1}H NMR study showed great changes in the metabolites excreted by *L. infantum* and *L. braziliensis* after treatment with some of the studied indazoles.

In general, the excretion of succinate decreased and the levels of acetate, lactate and alanine were usually increased. In some cases, glycine and pyruvate, which were not found in the control cultures, appeared as new metabolites. Most of these metabolic changes are consistent with damage caused by indazole derivatives at the glycosomal or mitochondrial level. A TEM study of ultrastructure of parasites showed huge alterations induced by indazoles, especially affecting the mitochondria and other cytoplasmic organelles.

Much work remains to be done in order to increase our knowledge in the field of structure–activity relationships but, despite the limitations of the current study, it confirms that 3-alkoxy-1-alkyl-5-nitroindazole derivatives, whose activity against *T. cruzi* has already been proven (Arán et al., 2005; Boiani et al., 2009; Rodríguez et al., 2009a, 2009b; Muro et al., 2014), are also promising for the treatment of other diseases caused by trypanosomatid protozoa such as leishmaniases.

In the future, in order to better understand the structure–activity relationships, we have planned the synthesis of other substituted 5-nitroindazoles, including fluorescent derivatives that are potentially able to label the organelles constituting their primary target, as well as the identification of products arising from the bioreduction of 5-nitroindazoles mediated by nitroreductases. Performing some *in vivo* studies with animal models of leishmaniasis would also be convenient in order to obtain further insights into the therapeutic potential of these compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2015.04.028

References

Arán, V.J., Ochoa, C., Boiani, L., Buccino, P., Cerecetto, H., Gerpe, A., González, M., Montero, D., Nogal, J.J., Gómez-Barrio, A., Azqueta, A., López de Ceráin, A., Piro, O.E., Castellano, E.E., 2005. Synthesis and biological properties of new 5-nitroindazole derivatives. Bioorg. Med. Chem. 13, 3197–3207.

Bates, P.A., 2007. Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. Int. J. Parasitol. 37, 1097–1106.

Boiani, L., Gerpe, A., Arán, V.J., Torres de Ortiz, S., Serna, E., Vera de Bilbao, N., Sanabria, L., Yaluff, G., Nakayama, H., Rojas de Arias, A., Maya, J.D., Morello, J.A., Cerecetto, H., González, M., 2009. *In vitro* and *in vivo* antitrypanosomatid activity of 5-nitroindazoles. Eur. J. Med. Chem. 44, 1034–1040.

Boiani, M., Piacenza, L., Hernández, P., Boiani, L., Cerecetto, H., González, M., Denicola, A., 2010. Mode of action of nifurtimox and *N*-oxide-containing heterocycles against *Trypanosoma cruzi*: is oxidative stress involved? Biochem. Pharmacol. 79, 1736–1745.

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

Cavalli, A., Bolognesi, M.L., 2009. Neglected Tropical Diseases: multi-target-directed ligands in the search for novel lead candidates against *Trypanosoma* and *Leishmania*. J. Med. Chem. 52, 7339–7359.

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

Chan-Bacab, M.J., Hernández-Núñez, E., Navarrete-Vázquez, G., 2009. Nitazoxanide, tizoxanide and a new analogue [4-nitro-*N*-(5-nitro-1,3-thiazol-2-yl)benzamide; NTB] inhibit the growth of kinetoplastid parasites (*Trypanosoma cruzi* and *Leishmania mexicana*) *in vitro*. J. Antimicrob. Chemother. 63, 1292–1293.

Croft, S.L., Sundar, S., Fairlamb, A.H., 2006. Drug resistance in leishmaniasis. Clin. Microbiol. Rev. 19, 111–126.

Espuelas, S., Plano, D., Nguewa, P., Font, M., Palop, J.A., Irache, J.M., Sanmartín, C., 2012. Innovative lead compounds and formulation strategies as newer kinetoplastid therapies. Curr. Med. Chem. 19, 4259–4288.

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

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

González, P., Marín, C., Rodríguez-González, I., Hitos, A.B., Rosales, M.J., Reina, M., Díaz, J.G., González-Coloma, A., Sánchez-Moreno, M., 2005. *In vitro* activity of C_{20}-diterpenoid alkaloid derivatives in promastigotes and intracellular amastigotes of *Leishmania infantum*. Int. J. Antimicrob. Agents 25, 136–141.

Hall, B.S., Bot, C., Wilkinson, S.R., 2011. Nifurtimox activation by trypanosomal type I nitroreductases generates cytotoxic nitrile metabolites. J. Biol. Chem. 286, 13088–13095.

Hall, B.S., Wilkinson, S.R., 2012. Activation of benznidazole by trypanosomal type I nitroreductases results in glyoxal formation. Antimicrob. Agents Chemother. 56, 115–123.

Hussain, H., Al-Harrasi, A., Al-Rawahi, A., Green, I.R., Gibbons, S., 2014. Fruitful decade for antileishmanial compounds from 2002 to late 2011. Chem. Rev. 114, 10369–10428.

Koren, H.S., Handwerger, B.S., Wunderlich, J.R., 1975. Identification of macrophage-like characteristics in a cultured murine tumor line. J. Immunol. 114, 894–897.

Marín, C., Ramírez-Macías, I., López-Céspedes, A., Olmo, F., Villegas, N., Díaz, J.G., Rosales, M.J., Gutiérrez-Sánchez, R., Sánchez-Moreno, M., 2011. *In vitro* and *in vivo* trypanocidal activity of flavonoids from *Delphinium staphisagria* against Chagas disease. J. Nat. Prod. 74, 744–750.

Marín, C., Clares, M.P., Ramírez-Macías, I., Blasco, S., Olmo, F., Soriano, C., Verdejo, B., Rosales, M.J., Gomez-Herrera, D., García-España, E., Sánchez-Moreno, M., 2013a. *In vitro* activity of scorpiand-like azamacrocycle derivatives in promastigotes and intracellular amastigotes of *Leishmania infantum* and *Leishmania braziliensis*. Eur. J. Med. Chem. 62, 466–477.

Marín, C., Longoni, S.S., Sánchez-Moreno, M., 2013b. Leishmania. In: Liu, D. (Ed.), Molecular Detection of Human Parasitic Pathogens. CRC Press, Taylor & Francis Group, Boca Raton, FL, pp. 91–108.

Maya, J.D., Bollo, S., Nuñez-Vergara, L.J., Squella, J.A., Repetto, Y., Morello, A., Périé, J., Chauvière, G., 2003. *Trypanosoma cruzi*: effect and mode of action of nitroimidazole and nitrofuran derivatives. Biochem. Pharmacol. 65, 999–1006.

Maya, J.D., Cassels, B.K., Iturriaga-Vásquez, P., Ferreira, J., Faúndez, M., Galanti, N., Ferreira, A., Morello, A., 2007. Mode of action of natural and synthetic drugs against *Trypanosoma cruzi* and their interaction with the mammalian host. Comp. Biochem. Physiol. A—Mol. Integr. Physiol. 146, 601–620.

Michels, P.A.M., Bringaud, F., Herman, M., Hannaert, V., 2006. Metabolic functions of glycosomes in trypanosomatids. Biochim. Biophys. Acta—Mol. Cell Res. 1763, 1463–1477.

Muro, B., Reviriego, F., Navarro, P., Marín, C., Ramírez-Macías, I., Rosales, M.J., Sánchez-Moreno, M., Arán, V.J., 2014. New perspectives on the synthesis and

antichagasic activity of 3-alkoxy-1-alkyl-5-nitroindazoles. Eur. J. Med. Chem. 74, 124–134.
Raether, W., Hänel, H., 2003. Nitroheterocyclic drugs with broad spectrum activity. Parasitol. Res. 90 (Suppl. 1), S19–S39.
Ramírez-Macías, I., Marín, C., Salas, J.M., Caballero, A., Rosales, M.J., Villegas, N., Rodríguez-Dieguez, A., Barea, E., Sánchez-Moreno, M., 2011. Biological activity of three novel complexes with the ligand 5-methyl-1,2,4-triazolo[1,5-*a*]pyrimidin-7(4*H*)-one against *Leishmania* spp. J. Antimicrob. Chemother. 66, 813–819.
Ramírez-Macías, I., Marín, C., Chahboun, R., Messouri, I., Olmo, F., Rosales, M.J., Gutiérrez-Sánchez, R., Alvarez-Manzaneda, E., Sánchez-Moreno, M., 2012a. *In vitro* and *in vivo* studies of the trypanocidal activity of four terpenoid derivatives against *Trypanosoma cruzi*. Am. J. Trop. Med. Hyg. 87, 481–488.
Ramírez-Macías, I., Maldonado, C.R., Marín, C., Olmo, F., Gutiérrez-Sánchez, R., Rosales, M.J., Quirós, M., Salas, J.M., Sánchez-Moreno, M., 2012b. *In vitro* antileishmania evaluation of nickel complexes with a triazolopyrimidine derivative against *Leishmania infantum* and *Leishmania braziliensis*. J. Inorg. Biochem. 112, 1–9.
Ramírez-Macías, I., Marín, C., Chahboun, R., Olmo, F., Messouri, I., Huertas, O., Rosales, M.J., Gutiérrez-Sánchez, R., Alvarez-Manzaneda, E., Sánchez-Moreno, M., 2012c. *In vitro* evaluation of new terpenoid derivatives against *Leishmania infantum* and *Leishmania braziliensis*. Mem. Inst. Oswaldo Cruz 107, 370–376.
Rodríguez, J., Gerpe, A., Aguirre, G., Kemmerling, U., Piro, O.E., Arán, V.J., Maya, J.D., Olea-Azar, C., González, M., Cerecetto, H., 2009a. Study of 5-nitroindazoles' anti-*Trypanosoma cruzi* mode of action: electrochemical behaviour and ESR spectroscopic studies. Eur. J. Med. Chem. 44, 1545–1553.
Rodríguez, J., Arán, V.J., Boiani, L., Olea-Azar, C., Lavaggi, M.L., González, M., Cerecetto, H., Maya, J.D., Carrasco-Pozo, C., Speisky Cosoy, H., 2009b. New potent 5-nitroindazole derivatives as inhibitors of *Trypanosoma cruzi* growth: synthesis, biological evaluation, and mechanism of action studies. Bioorg. Med. Chem. 17, 8186–8196.
Sánchez-Moreno, M., Gómez-Contreras, F., Navarro, P., Marín, C., Ramírez-Macías, I., Olmo, F., Sanz, A.M., Campayo, L., Cano, C., Yunta, M.J.R., 2012. *In vitro* leishmanicidal activity of imidazole- or pyrazole-based benzo[*g*]phthalazine derivatives against *Leishmania infantum* and *Leishmania braziliensis* species. J. Antimicrob. Chemother. 67, 387–397.
Santos, D.O., Coutinho, C.E.R., Madeira, M.F., Bottino, C.G., Vieira, R.T., Nascimento, S.B., Bernardino, A., Bourguignon, S.C., Corte-Real, S., Pinho, R.T., Rodrigues, C.R., Castro, H.C., 2008. Leishmaniasis treament—a challenge that remains: a review. Parasitol. Res. 103, 1–10.
Singh, N., Kumar, M., Singh, R.K., 2012. Leishmaniasis: current status of available drugs and new potential drug targets. Asian Pac. J. Trop. Med. 5, 485–497.
Singha, U.K., Bhakuni, V., Ali, V., Roy, R., 1996. *Leishmania donovani*: metabolite mapping of promastigotes using proton nuclear magnetic resonance spectroscopy. Mol. Cell. Biochem. 162, 17–22.
Sykes, M.L., Avery, V.M., 2013. Approaches to protozoan drug discovery: phenotypic screening (Miniperspectives series on phenotypic screening for antiinfective targets). J. Med. Chem. 56, 7727–7740.
Wheeler, R.J., Gluenz, E., Gull, K., 2011. The cell cycle of *Leishmania*: morphogenetic events and their implications for parasite biology. Mol. Microbiol. 79, 647–662.
WHO (World Health Organization), 2010. Control of the leishmaniases. In: WHO Technical Report Series n. 949 (Report of a Meeting of the WHO Expert Committee on the Control of Leishmaniases). WHO (World Health Organization), Geneva, Switzerland.
Wyllie, S., Patterson, S., Stojanovski, L., Simeons, F.R.C., Norval, S., Kime, R., Read, K.D., Fairlamb, A.H., 2012. The anti-trypanosome drug fexinidazole shows potential for treating visceral leishmaniasis. Sci. Transl. Med. 4, 119re1.