Imidazole-containing phthalazine derivatives inhibit Fe-SOD performance in *Leishmania* species and are active *in vitro* against visceral and mucosal leishmaniasis

M. SÁNCHEZ-MORENO[1]*, F. GÓMEZ-CONTRERAS[2]*, P. NAVARRO[3], C. MARÍN[1], I. RAMÍREZ-MACÍAS[1], M. J. ROSALES[1], L. CAMPAYO[2], C. CANO[2], A. M. SANZ[2] *and* M. J. R. YUNTA[2]

[1] *Departamento de Parasitología, Facultad de Ciencias, Universidad de Granada, E-18071 Granada, Spain*
[2] *Departamento de Química Orgánica, Facultad de Química, Universidad Complutense, E-28040 Madrid, Spain*
[3] *Instituto de Química Médica, Centro de Química Orgánica M. Lora-Tamayo, CSIC, E-28006 Madrid, Spain*

(*Received 27 November 2014; revised 11 January 2015; accepted 1 February 2015; first published online 31 March 2015*)

SUMMARY

The *in vitro* leishmanicidal activity of a series of imidazole-containing phthalazine derivatives **1**–**4** was tested on *Leishmania infantum*, *Leishmania braziliensis* and *Leishmania donovani* parasites, and their cytotoxicity on J774·2 macrophage cells was also measured. All compounds tested showed selectivity indexes higher than that of the reference drug glucantime for the three *Leishmania* species, and the less bulky monoalkylamino substituted derivatives **2** and **4** were clearly more effective than their bisalkylamino substituted counterparts **1** and **3**. Both infection rate measures and ultrastructural alterations studies confirmed that **2** and **4** were highly leishmanicidal and induced extensive parasite cell damage. Modifications to the excretion products of parasites treated with **2** and **4** were also consistent with substantial cytoplasmic alterations. On the other hand, the most active compounds **2** and **4** were potent inhibitors of iron superoxide dismutase enzyme (Fe-SOD) in the three species considered, whereas their impact on human CuZn-SOD was low. Molecular modelling suggests that **2** and **4** could deactivate Fe-SOD due to a sterically favoured enhanced ability to interact with the H-bonding net that supports the antioxidant features of the enzyme.

Key words: *Leishmania infantum*, *Leishmania donovani*, *Leishmania braziliensis*, iron superoxide dismutase, imidazole, phthalazine derivatives.

INTRODUCTION

Leishmaniasis is a parasitic disease caused by protozoan parasites of the genus *Leishmania* and transmitted by sandflies commonly found in tropical and subtropical regions of the world. It is estimated that 350 million people are at risk of infection worldwide and the annual incidence of the disease has been calculated to range between 1·5 and 2 million with about 70 000 deaths each year (Seifert, 2011). Even those data are probably very conservative because of underreporting and misdiagnosis. There are different clinical manifestations of this disease. The most widespread, but also the least dangerous is cutaneous leishmaniasis (CL), which triggers localized self-healing skin lesions. In mucocutaneous leishmaniasis (ML), also known as espundia, the infection spreads in the mucous membranes of the mouth and nose, becoming fatal in some cases. However, the most serious among them is undoubtedly visceral leishmaniasis (VL), also known as Kala azar, which is often lethal if untreated, since the parasites infect the liver and spleen and cause anaemia by severely affecting the immune system (Ryan *et al*. 2010). Those clinical syndromes are caused by different *Leishmania* subspecies. The most representative are: *Leishmania major*, which originates CL and is found in North Africa, India, China or the Middle East; *Leishmania braziliensis*, one of the main species causing ML, most common in South America (Bolivia, Brazil and Peru); and the *Leishmania donovani* complex, responsible for the fearsome VL, which is mainly constituted by zoonotic *Leishmania infantum*, found in Europe, North Africa and Latin America and anthroponotic *L. donovani*, present in East Africa and the Indian subcontinent (Chappuis *et al*. 2007; Lukes *et al*. 2007).

Because leishmaniasis is a severe health problem, mainly (but not only) in developing countries, it is necessary to develop drugs that adequately treat and prevent the disease. The current list of available drugs is limited in number and clearly unsatisfactory due to toxicity, generation of resistance and/or the very high costs involved. Pentavalent antimonials, mainly meglumine antimoniate (glucantime) and sodium stibogluconate, have been the usual drugs of choice in the treatment of VL and CL for more

* Corresponding authors: Departamento de Parasitología, Facultad de Ciencias, Universidad de Granada, E-18071 Granada, Spain. Email: msanchem@ugr.es and Departamento de Química Orgánica, Facultad de Química, Universidad Complutense, E-28040 Madrid, Spain. Email: fercon@ucm.es

doi:10.1017/S0031182015000219

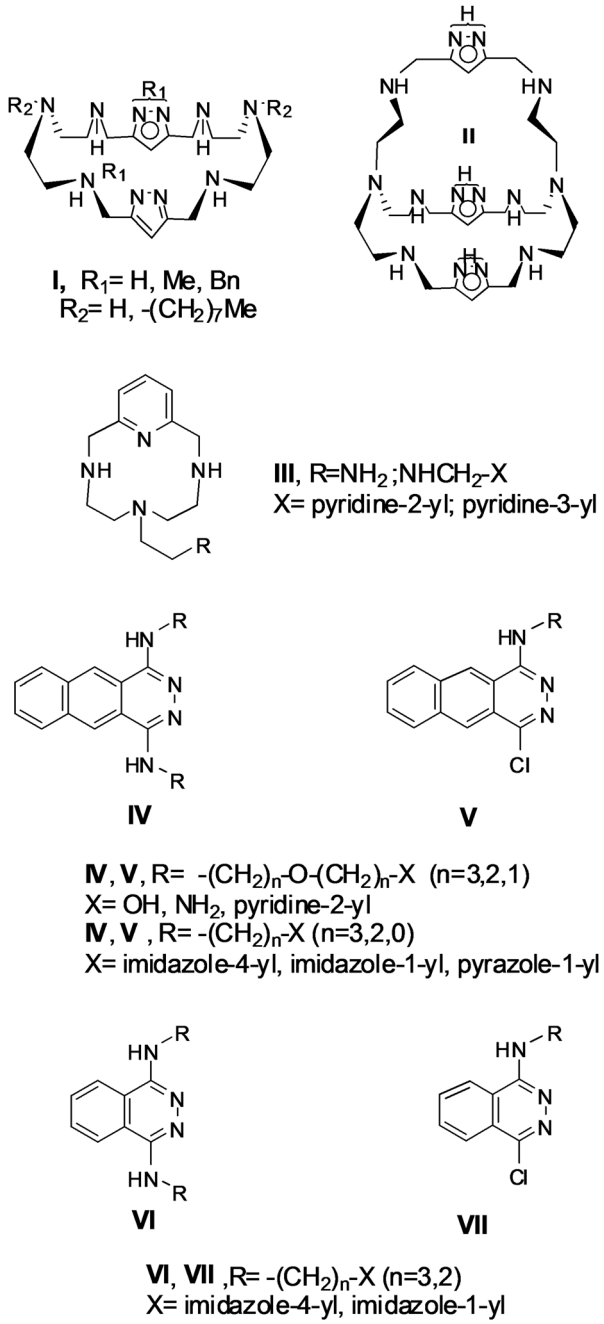

Fig. 1. Heterocyclic structures that inhibit Fe-SOD and show antiparasitic activity.

than 60 years. However, acquired resistance has been reported, especially in India, with failure rates of 65% and side-effects including serious cardiotoxicity in about 10% of patients (Croft *et al*. 2006; Seifert, 2011). Second-line drugs, such as pentamidine and amphotericin B have not experienced widespread use due to toxicity and cost. Pentamidine is associated with hypotension, hypoglycaemia and diabetes, and also cause nephrotoxicity. Miltefosine is used in the treatment of VL infections and is less harmful than pentamidine, but the ease with which resistant mutants are obtained is worrying (Ouellette *et al*. 2004). The most recent line of drugs are fluconazole, paromomycin and sitamaquine, but they also exhibit toxicity problems (i.e. hepatotoxicity with paromomycin or fluconazole, methaemoglobinaemia with sitamaquine) and it remains to be seen how these agents stand up in face of the pandemic of resistance (Loiseau *et al*. 2011; Freitas-Junior *et al*. 2012). Due to the growing resistance to chemotherapy, drawbacks shown by most of the compounds mentioned above, drug combinations are now being explored as a path to delay or prevent the emergence of resistance, increase efficacy or shorten the course of treatment (Freitas-Junior *et al*. 2012).

In search of new effective anti-leishmanial drugs able to broaden the narrow spectrum of compounds currently available, potential targets that perform functions essential for parasite survival are being investigated. Among them, antioxidant enzymes preventing oxidative stress that would otherwise lead to death of the parasite are in the spotlight. Within that group, trypanothione is currently receiving special consideration (Flohé, *2009*; Manta *et al*. 2013). However, another basic defence mechanism against oxidation is provided by the iron superoxide dismutase enzyme (Fe-SOD), which is located in the cytosol, the mitochondria and glycosomes of the parasite and plays an important role in the dismutation of harmful superoxide radicals into oxygen and hydrogen peroxide (Miller, 2004). One further advantage of Fe-SOD as a drug target is that it is not present in humans, who use Cu, Zn-SOD and Mn-SOD enzymes for the same purposes. Therefore, molecules able to inhibit selectively Fe-SOD could be promising candidates as leishmanicidal agents (Turrens, 2004). Since their prosthetic groups are essential in all processes regulated by enzymes, alterations in the active centre induced by dissociation of the iron atom or by modifications in the coordination geometry could effectively deactivate its antioxidant action and, therefore, influence both the growth and survival of the parasite cells.

In connection with this hypothesis, different series of macrocyclic or acyclic structures functionalized with nitrogenated heteroaromatic rings have been reported in previous work (Fig. 1, formulae **I–VII**). All these compounds exhibit remarkable affinity towards transition metals (Lamarque *et al*. 2001; Rodríguez-Ciria *et al*. 2007; Blasco *et al*. 2010); and are, simultaneously, powerful inhibitors of parasitic Fe-SOD, although poorer inhibitors of human CuZn-SOD. This is the case with pyrazole-containing macrocyclic and macrobicyclic polyamines **I** and **II** (Sánchez-Moreno *et al*. 2012*a*) and with pyridine-containing scorpiand-like azamacrocycles **III** (Olmo *et al*. 2013), in which their inhibitory ability against *T. cruzi* Fe-SOD runs in parallel with *in vitro* trypanocidal properties and *in vivo* activity against both acute and chronic phases of Chagas disease. Similar results have been obtained by testing different series of benzo[*g*]

https://doi.org/10.1017/S0031182015000219 Published online by Cambridge University Press

phthalazine and phthalazine derivatives functionalized with flexible side-chains ending with pyridine, pyrazole or imidazole moieties (**IV–VII**), some of which have been shown to significantly reduce parasite reactivation in immunosuppressed mice (Sánchez-Moreno *et al*. 2011, 2012*b*). In light of these findings, we proceeded to evaluate the activity of series **I–V** towards *L. braziliensis* and *L. infantum* parasites, as significant representatives of species causing CL and VL, respectively. It was found that the tested compounds exhibited a behaviour closely related to that found in *T. cruzi*: good *in vitro* activity, low toxicity against mammalian cells and inhibition of *Leishmania* Fe-SOD (Sánchez-Moreno *et al*. 2012*c*; Marín *et al*. 2013; Navarro *et al*. 2014). One point to highlight is that a noticeable structure-activity relationship was observed in the compounds studied in both the *Leishmania* and *T. cruzi* assays. This was particularly evident in assays performed with the 1,4-disubstituted pyridazine derivatives **IV–VII** on *T. cruzi* parasites (Sánchez-Moreno *et al*. 2011, 2012*b*): the monoalkylamino substituted compounds **V** and **VII** were always more active against the parasite and better inhibitors of Fe-SOD than their bisalkylamino substituted analogues **IV** and **VI.** This difference in behaviour has been tentatively explained by molecular modelling on the basis of an easier approach of **V** and **VII** to the active centre of *T. cruzi* Fe-SOD (Sánchez-Moreno *et al*. 2012*b*).

In this work, we intend to verify if derivatives of types **VI–VII** also present leishmanicidal activity against *L. infantum* and *L. braziliensis*, and if the structure-activity relationship patterns are also operative for these parasites. Furthermore, we have incorporated for the first time in our studies assays with *L. donovani*, since this parasite forming part of the *L. donovani* complex is the main causative agent of visceral leishmaniasis in underdeveloped countries and is regarded as a particularly harmful *Leishmania* subspecies.

MATERIALS AND METHODS

Chemistry

The synthesis of the 1,4-bis(alkylamino)- and 1-alkylamino-4-chlorophthalazine derivatives of types **VI** and **VII** with the structures shown in Fig. 2 was accomplished as described in reference (Sánchez-Moreno *et al*. 2012*b*). The bis(alkylamino) substituted derivatives **1** and **3** were obtained by simultaneous nucleophilic substitution at the C-1 and C-4 positions of the starting compound with 2-(imidazol-4-yl)ethylamine or 3-(imidazol-1-yl) propylamine in a 1:2 molar ratio under reflux with xylene, using triethylamine as the chlorine acceptor. The synthesis of the mono(alkylamino) substituted compounds **2** and **4** was achieved using a different

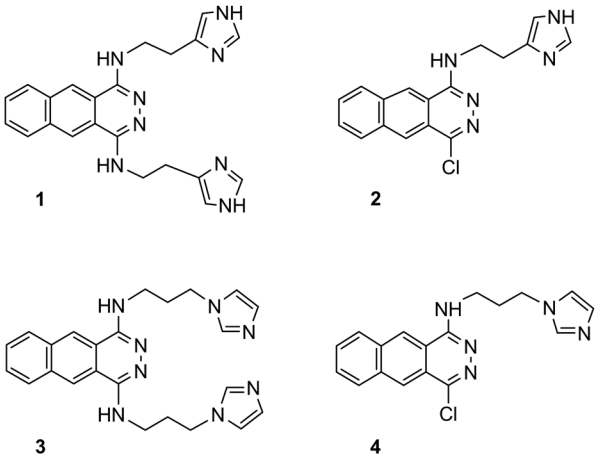

Fig. 2. Phthalazine derivatives tested against *Leishmania* species.

procedure in which the reaction was performed in 1:1 molar ratio under reflux with acetonitrile and triethylamine was replaced by sodium carbonate. That modification usually leads to better yields of the monosubstituted compounds in relation to the disubstituted analogues. Isolation of the compounds from the crude reaction mixtures was performed in all cases by column flash chromatography with a chloroform/methanol mixture of increasing polarity. Spectroscopic data were the same as those described in reference (Sánchez-Moreno *et al*. 2012*b*) and analytical data confirmed that the purity of the compounds was adequate for performing the planned biological tests.

Parasite strain and culture

Promastigote forms of *L. infantum* (MCAN/ES/2001/UCM-10), *L. braziliensis* (MHOM/BR/1975/M2904) and *L. donovani* (MHOM/PE/84/LC26) were cultured *in vitro* in medium trypanosomes liquid (MTL) together with 10% inactive fetal calf serum (FCS) kept in an air atmosphere at 28 °C, in Roux flasks (Corning, USA) with a surface area of 75 cm^2, according to the methodology described by González *et al*. (2005).

In vitro *activity assays*

The compounds to be tested were first dissolved in dimethylsulphoxide (DMSO, Panreac, Barcelona, Spain) at a concentration of 0·1% and then assayed for toxicity and inhibitory effects on parasite and mammalian cells growth as previously described (González *et al*. 2005).

Cell culture and cytotoxicity tests

The macrophage line J774·2 [European collection of cell cultures (ECACC) number 91051511] was

https://doi.org/10.1017/S0031182015000219 Published online by Cambridge University Press

derived in 1968 from a tumour in a female BALB/c mouse. The macrophages were grown in minimal essential medium (MEM) plus glutamine (2 mM) and 20% inactive FCS, with a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The cytotoxicity testing on macrophages was performed by flow cytometric analysis according to a method previously described (Marín *et al*. 2011). The percentage of viable cells was calculated with respect to the control culture. The IC_{50} was calculated using linear regression analysis from the Kc values of the concentrations employed.

Promastigote assay: extracellular forms

The compounds were dissolved in the culture medium to give final concentrations of 100, 50, 25, 10 and 1 μM. The effects of each compound against the promastigote forms at the different concentrations were tested at 72 h using a Neubauer haemocytometric chamber. The leishmanicidal effect was expressed as the IC_{50} value, i.e. the concentration required to result in 50% inhibition, calculated by linear regression analysis from the Kc values of the concentrations employed.

Amastigote assay: intracellular forms

J774·2 macrophage cells were grown and 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. The adherent macrophages were then infected with promastigotes of *L. infantum, L. braziliensis* and *L. donovani* in the stationary growth phase, at a ratio of 10:1 and maintained for 24 h at 37 °C in air containing 5% CO_2. Non-phagocytosed parasites were removed by washing, and the infected cultures were incubated with the testing compounds (concentrations ranging from 1 to 100 μM) and then cultured for 72 h in MEM plus glutamine (2 mM) and 20% inactive FCS. Compound activity was determined from the percentage reductions in amastigote number in treated versus untreated cultures in methanol-fixed and Giemsa-stained preparations. Values are the means of three separate determinations (Sánchez-Moreno *et al*. 2012*c*).

Infectivity assay

Adherent macrophage cells grown as described above, were then infected *in vitro* with promastigote forms of *L. infantum, L. braziliensis* or *L. donovani*, at a ratio of 10:1. The compounds to be tested (IC_{25} concentrations) were added immediately after infection, and incubated for 12 h at 37 °C in 5% CO_2. Non-phagocytosed parasites and compounds were removed by washing, and then the infected cultures were grown for 10 days in fresh medium. Cultures were washed every 48 h and fresh culture medium was added. Compound activity was determined on the basis of both the percentage of infected cells and the number of amastigotes per infected cell in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analysing more than 200 host cells distributed in randomly chosen microscopic fields. Values are the means of three separate determinations.

Ultrastructural alterations

The parasites were cultured at a density of 5×10^5 cells mL^{-1} in the corresponding medium, each of which contained a compound to be tested at the IC_{25} dose. After 72 h, the cultures were centrifuged at 400 ***g*** for 10 min, and the pellets produced were washed in phosphate-buffered saline (PBS) and then incubated with 2% (v/v) *p*-formaldehyde/glutaraldehyde in 0·05 M cacodylate buffer (pH 7·4) for 24 h at 4 °C. The pellets were then prepared for transmission electron microscopy (TEM) using a technique previously described (González *et al*. 2005).

Metabolite excretion

Cultures of *L. infantum, L. braziliensis* and *L. donovani* promastigotes (initial concentration 5×10^5 cells mL^{-1}) received the IC_{25} dose of each compound (except for control cultures). After incubation for 72 h at 28 °C the cells were centrifuged at 400 ***g*** for 10 min. The supernatants were collected to determine the excreted metabolites by ^{1}H NMR, and chemical shifts were expressed in ppm, using sodium 2,2-dimethyl-2-silapentane-5-sulphonate as the reference signal. The chemical displacements used to identify the respective metabolites were consistent with those described previously by some of the co-authors (Fernandez-Becerra *et al*. 1997).

Fe-SOD enzymatic inhibition

Parasites were collected in the logarithmic growth phase by centrifugation (400 ***g*** for 10 min at room temperature). The pellet obtained after centrifugation, was resuspended in 3 mL of STE buffer (0·25 M sucrose, 25 mM Tris–HCl, 1 mM EDTA, pH 7·8) and the cells were lysed by three cycles of sonication for 30 s each at 60 W. The sonicated homogenate was centrifuged at 1500 ***g*** for 5 min at 4 °C, and the pellet was washed three times in ice-cold STE buffer. This fraction was centrifuged (2500 ***g*** for 10 min at 4 °C) and the supernatant was collected. Afterwards, the supernatant was subjected to ice-cold ammonium sulphate precipitation between 35 and 85% salt concentration and the

https://doi.org/10.1017/S0031182015000219 Published online by Cambridge University Press

resulting precipitate was dissolved in 2·5 mL of distilled water and desalted by chromatography in Sephadex G-25 column (GE Healthcare Life Sciences®, PD 10 column) previously equilibrated with 2 mL of distilled water, taking it up to a final volume of 3·5 mL (Longoni *et al*. 2013). The protein content was quantified using the Sigma Bradford test, which uses bovine serum albumin (BSA) as a standard (Bradford, 1976). Iron and copper–zinc superoxide dismutases activities were determined using a previously described method (Beyer and Fridovich, 1987) that measures the reduction in nitroblue tetrazolium (NBT) by superoxide ions. According to the protocol, 845 μL of stock solution [3 mL of L-methionine (300 mg, 10 mL^{-1}), 2 mL of NBT (1·41 mg, 10 mL^{-1}) and 1·5 mL of Triton X-100 1% (v/v)] were added into each well, along with 30 μL of the parasite homogenate fraction, 10 μL of riboflavine (0·44 mg, 10 mL^{-1}), and an equivalent volume of the different concentrations of the compounds being tested. Seven different concentrations were used for each agent, from 0·1 to 100 μM. In the control experiment the volume was made up to 1000 μL with 50 mM potassium phosphate buffer (pH 7·8, 3 mL), and 30 μL of the parasite homogenate fraction were added to the mixtures containing the compounds. Then, the absorbance (A_0) was measured at 560 nm in a UV spectrophotometer. Afterward, each well was illuminated with UV light for 10 min under constant stirring and the absorbance (A_1) was measured again. The human CuZn-SOD and substrates used in these assays were obtained from Sigma Chemical Co. The resulting data were analysed using the Newman–Keuls test.

Molecular modelling

Molecular modelling studies were carried out using the AMBER (Case *et al*. 2005) method implemented in the HyperChem 8.0 package (Hypercube Inc., FL, USA), modified by the inclusion of appropriate parameters (Miranda *et al*. 2004). Starting structures for the phthalazine derivatives were built using HyperChem's capabilities. Their geometry was minimized to a maximum energy gradient of 0·1 cal/(Å mol) with the AMBER force field, using the Polak-Ribière (conjugate gradient) minimizer, and a 'simulated annealing' procedure was used to cover all conformational space. The most stable extended geometry was always used in all calculations of interaction with the enzyme. To mimic the conditions used in the activity measurements, i.e. water as solvent, all calculations were carried out *in vacuo* with a distance-dependent dielectric constant value. In the absence of explicit solvent molecules, a distance-dependent dielectric factor qualitatively simulates the presence of water, as it takes into account the fact that intermolecular electrostatic interactions should die off more rapidly with distance than in the gas phase (Revíriego *et al*. 2008). The same results can be obtained using a constant dielectric factor greater than 1. We chose to use a distance-dependent dielectric constant (ε = 4 Rij) as this was the method used to develop the AMBER force field (Cornell *et al*. 1995). Charge assignments for all atoms were done by means of ab initio calculations using the STO-3G basis set, as it is compatible with the AMBER force field, prior to energy minimization using AMBER. The Fe-SOD enzymes structure were obtained from the Brookhaven protein data bank (entry 2gpc for *Trypanosoma cruzi* and entry 4F2N for *Leishmania*, corresponding to *L. major*, as it is the only published structure for a *Leishmania* species) and their energies were minimized in the same way. Interaction studies were done starting from structures with the compound positioned in the border of each enzyme cavity. To study different possible interactions of the enzyme's iron atom with any of the imidazole nitrogen atoms, entry into the cavity was forced using a restraint on the selected N-Fe distance, starting from 0·15 nm and slowly decreasing this distance, allowing the complex to achieve the minimum energy conformation with no restraints on all the small driving steps, using the same conditions mentioned above.

RESULTS AND DISCUSSION

As explained above, previous studies have indicated that phthalazine derivatives containing imidazole rings attached to the side chains may be considered prospective chemotherapeutic drugs in the treatment of Chagas disease caused by *T. cruzi* parasites. We comment now on the results obtained concerning the antiparasitic activity of compounds **1**–**4** (Fig. 2) against three significant species of *Leishmania: L. infantum, L. braziliensis* and *L. donovani*). Pairs **1,3** and **2,4** differ in the number of imidazole-containing alkylamino groups attached to the pyridazine ring, whereas pairs **1,2** and **3,4** have different modes of connection of the flexible aliphatic chain to the imidazole ring and also different numbers of nitrogen atoms able to interact with the active centre of the enzyme.

In vitro antileishmanial evaluation

In a first step we assayed the *in vitro* biological activity of compounds **1**–**4** on both extra- and intracellular forms of the parasites. Extracellular forms are more commonly used due to the ease of working with them, but are less indicative of leishmanicidal activity. The use of intracellular forms is more cumbersome but gives more accurate results, as promastigotes are converted to amastigotes in vertebrate host cells (González *et al*. 2005). Intracellular

https://doi.org/10.1017/S0031182015000219 Published online by Cambridge University Press

Table 1. *In vitro* activity and toxicity found for the phthalazine derivatives **1**–**4** on extra- and intracellular forms of *Leishmania* spp

	Activity IC_{50} (μM)[a]						
	Leishmania infantum		*Leishmania braziliensis*		*Leishmania donovani*		
Comp.	Promast.	Amast.	Promast.	Amast.	Promast.	Amast.	Macrophage toxicity IC_{50} (μM)[b]
Glucantime	18·0 ± 3·1	24·2 ± 2·6	25·6 ± 1·6	30·4 ± 6·1	26·6 ± 5·4	33·3 ± 1·2	15·2 ± 1·0
1	21·3 ± 1·4	19·3 ± 1·1	15·2 ± 0·9	20·3 ± 2·1	30·0 ± 3·6	20·6 ± 1·3	300·4 ± 17·5
2	14·9 ± 0·7	5·1 ± 0·3	14·3 ± 0·3	12·7 ± 1·1	15·9 ± 0·8	8·8 ± 1·2	297·5 ± 22·5
3	24·5 ± 1·6	13·3 ± 0·6	25·7 ± 1·5	25·6 ± 2·3	28·1 ± 3·2	18·4 ± 1·6	131·1 ± 10·7
4	15·1 ± 1·0	14·6 ± 1·4	18·9 ± 0·6	25·4 ± 1·6	32·4 ± 2·7	7·1 ± 0·6	748·9 ± 36·9

Results are averages of three separate determinations.
[a] IC_{50} is the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at the concentrations employed (1, 10, 25, 50 and 100 μM).
[b] Against J774·2 macrophages after 72 h of culture.

Table 2. Selectivity index calculated for phthalazine derivatives **1**–**4** on extra- and intracellular forms of *Leishmania* spp

	SI[a]					
	Leishmania infantum		*Leishmania braziliensis*		*Leishmania donovani*	
Comp.	Promast.	Amast.	Promast.	Amast.	Promast.	Amast.
Glucantime	0·8	0·6	0·6	0·5	0·6	0·5
1	14·1 (18)	15·6 (26)	19·8 (33)	14·8 (30)	10·0 (17)	14·6 (29)
2	20·0 (25)	58·3 (97)	20·8 (35)	23·4 (47)	18·7 (31)	33·8 (68)
3	5·3 (7)	9·9 (16)	5·1 (9)	5·1 (10)	4·7 (8)	7·1 (14)
4	49·6 (62)	51·3 (85)	39·6 (66)	29·5 (59)	23·1 (39)	105·5 (211)

[a] Selectivity index = IC_{50} macrophages toxicity/IC_{50} activity on extracellular or intracellular forms of the parasite. In brackets: number of times the compound SI exceeded the reference drug SI.

assays were performed by infecting macrophage cells with promastigotes, which transformed into amastigotes within 1 day after infection. Table 1 shows the IC_{50} values obtained after 72 h of exposure when compounds **1**–**4** were tested on extra- and intracellular forms of *L. infantum, L. braziliensis* and *L. donovani*. Toxicity values against J774·2 macrophage after 72 h of culture were also calculated. Results obtained for the reference drug glucantime were included in all cases for comparison.

It was shown that the leishmanicidal activities against the more indicative intracellular forms of the parasites were higher than those found with glucantime with all four compounds tested, whereas the effect on extracellular forms was more random. On the other hand, a marked difference in the behaviour of the monoalkylamino substituted compounds **2** and **4** was found with respect to their bis(alkylamino) substituted analogues **1** and **3**, especially with the amastigote forms, since **2** and **4** were more active in most cases than their disubstituted counterparts, in particular against the two Donovani complex species. But more interesting were the toxicity data in mammalian cells, since all four compounds tested were found to be much less toxic for macrophages than the reference drug. Thus, compound **4** was 40-fold less cytotoxic than glucantime, and **1**–**2** was also about 20-fold less cytotoxic. Even the most cytotoxic compound, the bis(alkylamino) substituted derivative **3**, was 6·5-fold less cytotoxic than glucantime.

In order to obtain a more accurate picture of the features commented on above, we show in Table 2 the selectivity index values calculated from the data in Table 1, since they are very illustrative of the *in vitro* potential of the compounds tested with respect to the reference drug. The number of times that the SI of each compound exceeded the SI of glucantime is also shown in parenthesis. In this Table the differences between glucantime and the tested compounds are clearly revealed. All four compounds exhibited substantially better SI values than the reference drug in the three *Leishmania* species tested and, in the most remarkable case, the SI of the monosubstituted compound **4** on the intracellular form of *L. donovani* exceeded that of glucantime by 211-fold, a relevant data point which is by far the best SI value obtained with the different series of

https://doi.org/10.1017/S0031182015000219 Published online by Cambridge University Press

related heterocyclic systems tested by us against parasites in previous research (Sánchez-Moreno *et al*. 2011, 2012*a*,*b*,*c*; Navarro *et al*. 2014). The SI displayed in Table 2 are also very illustrative about the difference in behaviour of mono- and disubstituted compounds, which recurs in the three species and in both extra- and intracellular forms, since **2** and **4** always show substantially better results than **1** and **3**. In tests performed on the *L*. *infantum* species, the SI exceeded that of the reference drug by 25- and 97-fold for the extra- and intracellular forms in the case of **2**, respectively, and by 62- and 85-fold with **4**; on *L*. *braziliensis*, the respective values obtained were 35- and 47-fold with **2** and 66- and 59-fold with **4**; while on *L*. *donovani*, respective values of 31- and 68-fold with **2** and 39- and 211-fold with **4** were found. Interestingly, the best SI results for the more representative intracellular forms were obtained in *L*. *infantum* and *L*. *donovani*, two species forming part of the *L*. *donovani* complex, pointing towards a greater specificity towards parasites causing the particularly harmful visceral leishmaniasis in both its European and American versions.

In order to gain better insight into the activities of these compounds, their effect on the infectivity and intracellular replication of amastigotes was subsequently determined. Macrophage cells were grown and infected with promastigotes in the stationary phase. The parasites invaded the cells and underwent morphological conversion to amastigotes within 1 day after infection. On day 10, the rate of host cell infection reached its maximum (control experiment). We used the IC_{25} of each product as the test dosage. Different authors have claimed that compounds with SI values less than 20-fold those of the reference drug should be discarded as candidates for more advanced leishmanicidal tests due to their poor selectivity against mammalian cells (Nwaka *et al*. 2011). Since the bis(alkylamino) substituted compound **3** gave SI values of 9·9, 5·1 and 7·1 against intracellular forms, it was therefore not considered for infectivity assays.

As shown in Fig. 3A, when compounds **1, 2** and **4** were added to macrophages infected with *L*. *infantum* promastigotes, the infection rate decreased significantly with respect to the control and, furthermore, the three compounds were also remarkably more effective in decreasing infectivity than glucantime (55, 84 and 90% for **1, 2** and **4**, respectively, versus only 8% for the reference drug). A measure of the average number of amastigotes per infected macrophage (Fig. 3B) led to similar conclusions: all three compounds were more effective than glucantime (with only a 22% decrease), and amastigote numbers of 69, 88 and 85% were found for **1, 2** and **4** respectively. Infection rates (Fig. 3C) and amastigote numbers (Fig. 3D) obtained on *L*. *braziliensis* also showed that, in both cases, the three compounds were clearly more effective than glucantime, and also in both cases the order of effectiveness was **2**>**4**>**1**, since the infectivity rates calculated were: 72, 81, 76 and 22%, respectively. The decreases in amastigote numbers were: 60, 93, 83 and 32% for **1, 2, 4** and glucantime, respectively. Finally, similar results were found on *L*. *donovani*, with infectivity rates of 48, 83, 79 and 28% (Fig. 3E); and decreases in the number of amastigotes of: 53, 86, 95 and 33% (Fig. 3F) for **1, 2, 4** and glucantime, respectively. From all these data it can be concluded that: (a) all three compounds were substantially more active than glucantime against the three *Leishmania* species tested; (b) in full accordance with related data described for *T*. *cruzi* parasites (Sánchez-Moreno *et al*. 2011, 2012*a*,*b*), the monoalkylamino substituted derivatives were clearly more efficient than the bis(alkylamino) substituted compound **1**, reinforcing the previously proposed idea that a structure-activity relationship is operating in the mechanism of action of these series of compounds against all types of parasites tested.

Ultrastructural alterations

Since all these compounds showed remarkable leishmanicidal activity, major damage to parasite cells could be inferred. In order to confirm this, a TEM study on the promastigote forms of the three *Leishmania* species under consideration was performed. As expected, significant morphological alterations were observed compared with untreated control cells. Fig. 4 displays some of the most relevant structural features observed in control and treated cells of promastigotes of the three species, which are commented on below. As a general observation, the three tested compounds were responsible for the death of a high percentage of individuals and many other abnormalities such as vacuolization, disintegration of the cell cytoplasm and rupture of the parasites. Chart 1 corresponds to *L*. *infantum*, and it can be seen that compound **1** caused very swollen mitochondria and many empty or lipidic vacuoles. Something similar was observed with **2**, which triggered the formation of huge vacuoles filled with residues of parasite metabolism and swollen kinetoplasts. Compound **4** gave also devastating results, since most of the parasites were dead and appeared either totally empty of cytoplasmic content or included very few organelles, scant ribosomes and empty vacuoles. Mitochondria were swollen and showed irregular and disorganized crypts. Damage caused to *L*. *braziliensis* promastigotes is exemplified in Chart 2. After treatment with **1**, some parasites appeared dead and others showed serious alterations that included intensive vacuolization, few ribosomes, swollen mitochondria and plasmatic membrane undulations. Compound **2**

https://doi.org/10.1017/S0031182015000219 Published online by Cambridge University Press

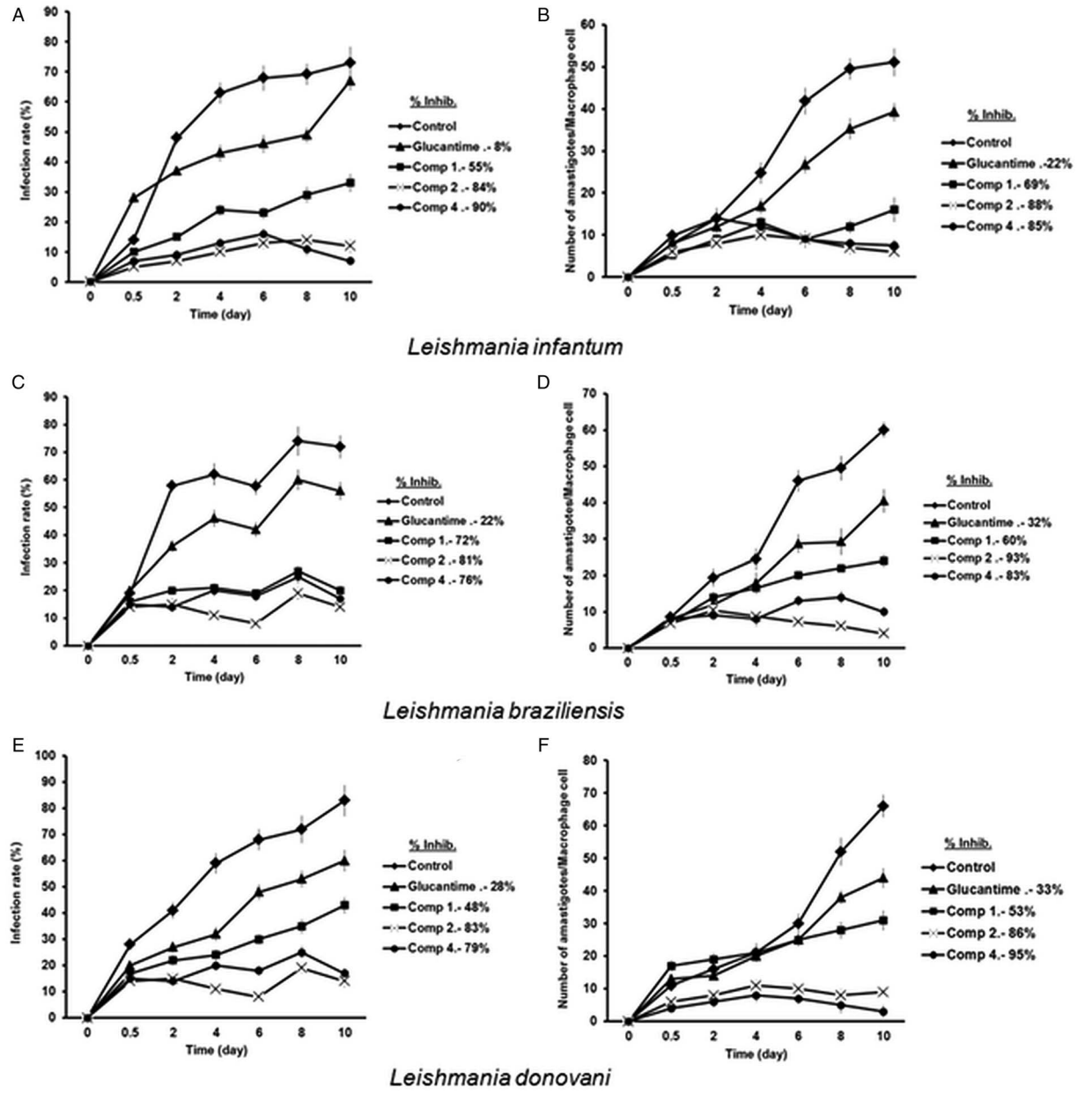

Fig. 3. Effect of imidazole-containing phthalazine derivatives **1, 2** and **4** on the infection and growth rates of *Leishmania* spp. species. (A), (C) and (E) Rates of infection of *L. infantum, L. braziliensis* and *L. donovani*, respectively; (B), (D) and (F) mean numbers of amastigotes of *L. infantum, L. braziliensis* and *L. donovani*, respectively, per infected J774 A.2 macrophage cell (at IC_{25} conc). Values are the means of three separate experiments.

altered the parasites in such a way that their appearance was absolutely unnatural, with strange and deformed shapes. Cytoplasmic organelles were mostly non-existent and the remainders were very electron-dense, with swollen mitochondria. In some cases, the cytoplasms were exclusively filled with huge vacuoles or associated myelin material. Most of these promastigotes, even those with some inner content, were probably dead. This extensive vacuolization was even more evident in parasites treated with compound **4**, in which huge vacuoles filled with metabolic residues were the norm. Finally, Chart 3 exemplifies the alterations produced in *L. donovani* promastigotes, which were especially devastating. After treatment with **1** many parasites were severely damaged or dead, and extensive remains of dead parasites and loose flagella appeared in the culture supernatant. The surviving parasites had very scant ribosomes, swollen and deformed mitochondria, and broken or discontinuous cytoplasmic membranes. A similar picture was obtained with compound **2**. Mitochondria without crypts, electron-dense vesicles corresponding to abnormal waste products, and membranes spaced apart from the nucleus were common. Beyond this, most of the kinetoplasts were unrecognizable. In the case of **4**, about 80% of the parasites were dead, and the remainders were deeply disturbed, as can be easily

https://doi.org/10.1017/S0031182015000219 Published online by Cambridge University Press

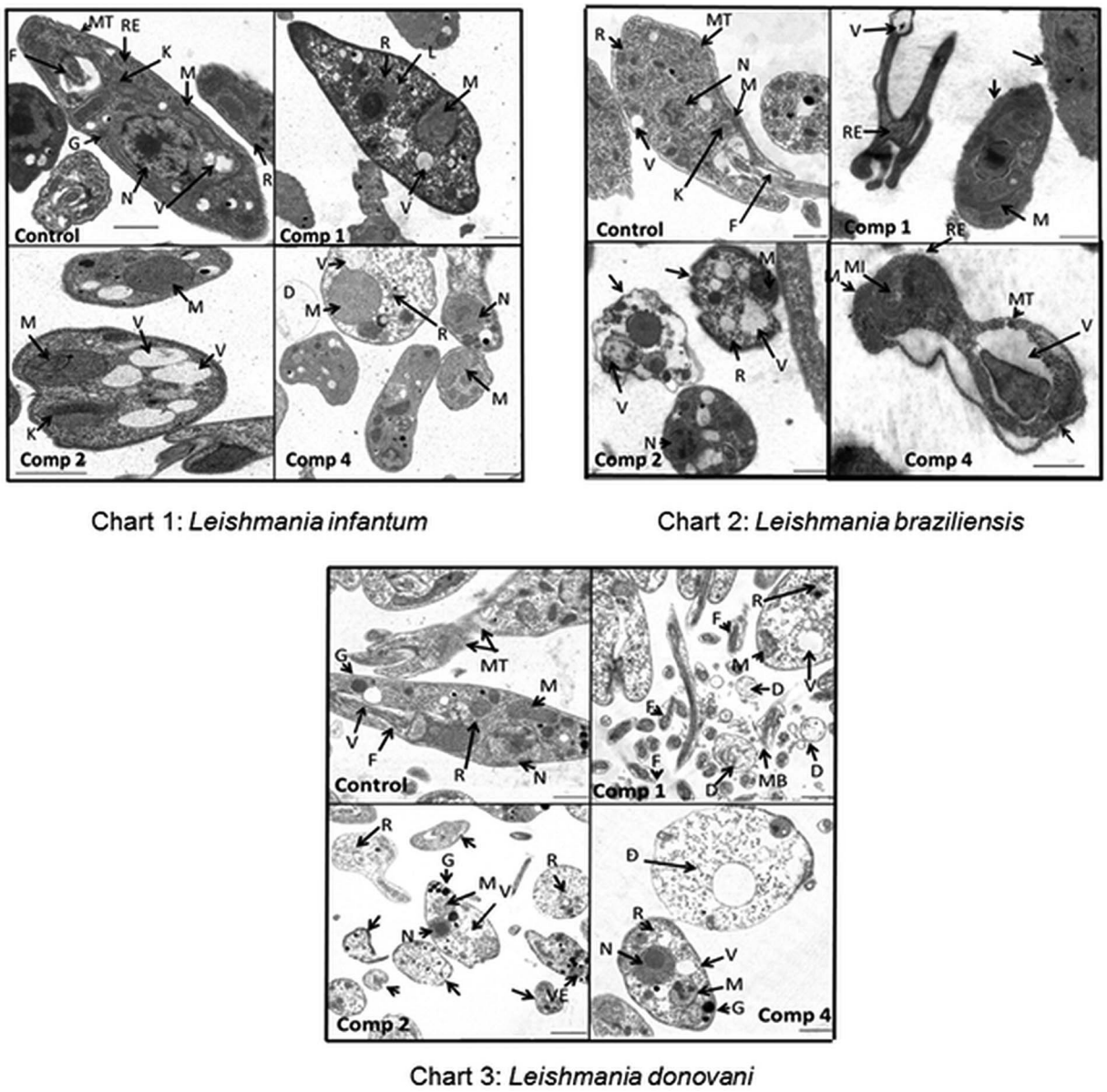

Fig. 4. Ultrastructural alterations observed by TEM in promastigotes of the three *Leishmania* species specified after treatment with compounds **1, 2** or **4**. Abbreviations: dead parasites (D), flagellum (F), glycosomes (G), kinetoplasts (K), lipidic vacuoles (L), mitochondria (M), cytoplasmic membranes broken (MB), myelin debris (MI), microtubules (MT), nucleus (N), ribosomes (R), endoplasmic reticula (RE), empty or swollen vacuoles (V), electron dense vesicles (VE), distorted membranes (single arrow). Scale bars are 1·0 mm in all cases.

appreciated in the fourth plate of Chart 3, which shows unrecognizable, swollen and content-lacking mitochondria, together with large electron-dense glycosomes. Although TEM images are partial pictures that do not allow accurate quantitative comparisons to be made, it remains clear from the images displayed in Fig. 4 that the three compounds tested caused great damage to promastigotes of the three *Leishmania* species investigated, in accordance with the leishmanicidal activity data detailed above.

Metabolite excretion effect

Since trypanosomatids are unable to completely degrade glucose to CO_2 under aerobic conditions, they excrete much of the hexose skeleton into the medium as partially oxidized fragments in the form of fermented metabolites. The nature and percentages of those excretion products depend on the pathway used for glucose metabolism by each species (Ginger, 2005; Bringaud *et al*. 2006). The final products of glucose catabolism in *Leishmania* are usually CO_2, succinate, acetate, D-lactate, L-alanine and, in some cases, ethanol (Cazzulo, 1992). Among them, succinate is the most relevant, because its main role is to maintain the glycosomal redox balance, allowing the reoxidation of NADH produced in the glycolytic pathway. Succinic fermentation requires only half of the phosphoenolpyruvate produced to maintain the NAD^+/NADH

https://doi.org/10.1017/S0031182015000219 Published online by Cambridge University Press

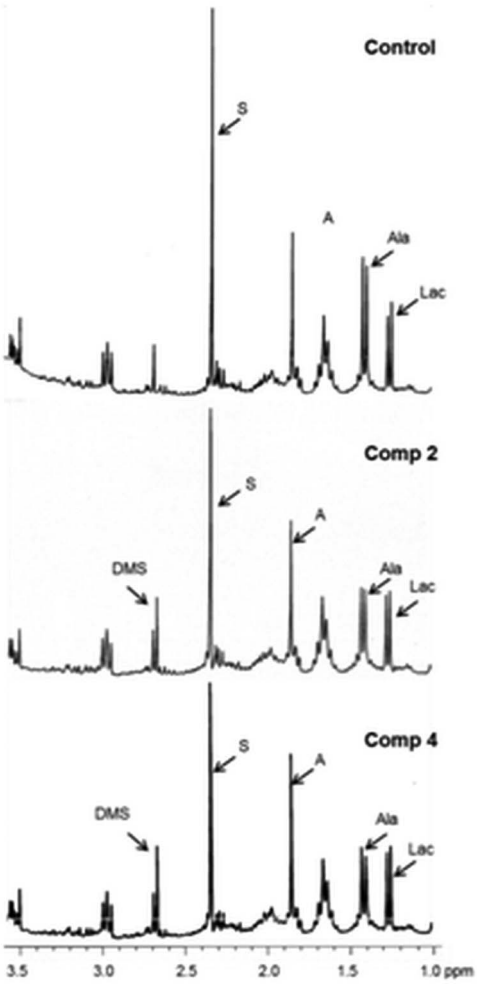

Fig. 5. ^{1}H NMR spectra showing metabolites excreted by promastigote forms of *L. infantum* either untreated or treated with compounds **2** and **4**, IC_{25} dosage. Lac: D-lactate; Ala: L-alanine; A: acetate; S: succinate.

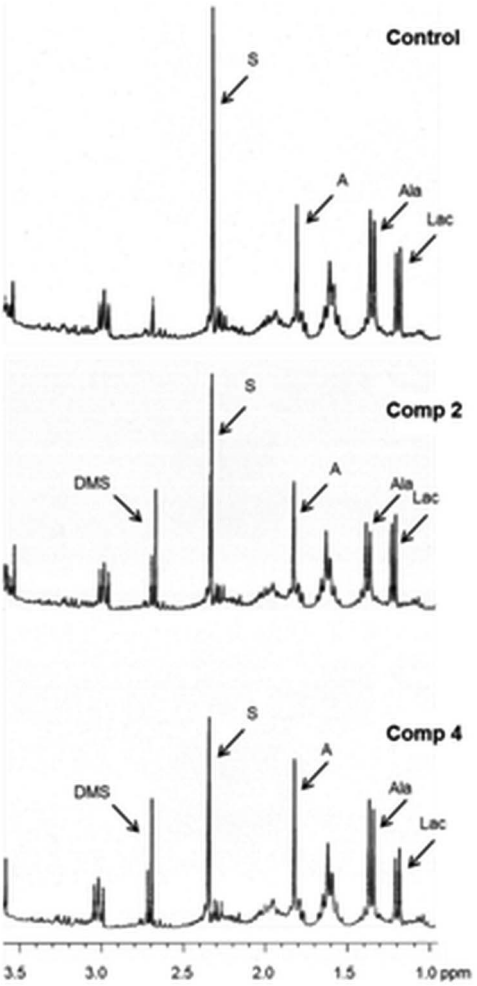

Fig. 6. ^{1}H NMR spectra showing metabolites excreted by promastigote forms of *L. braziliensis* either untreated or treated with compounds **2** and **4**, IC_{25} dosage. Lac: D-lactate; Ala: L-alanine; A: acetate; S: succinate.

balance, and the remaining pyruvate is converted inside the mitochondrion and the cytosol into acetate, D-lactate, L-alanine or ethanol, according to the degradation pathway followed by a specific species (Michels *et al*. 2006).

We reported above that the most active monoalkylamino substituted compounds **2** and **4** caused great damage to the mitochondria of the parasites in the three *Leishmania* species, so that their highly disruptive action presumably affected their glucose metabolism and, consequently, modified the percentages of the final excretion products formed. Therefore, we registered the ^{1}H NMR spectra of promastigotes from the *L. infantum*, *L. braziliensis* and *L. donovani* species after treatment with the two compounds with significant leishmanicidal activity, and the final excretion products were identified qualitatively and quantitatively. The spectra obtained were compared with those from promastigotes maintained in cell-free medium (control) for 4 days after inoculation with the parasites. Variations in the height of the signals corresponding to the most significant catabolites are displayed in Figs 5–7. The expected presence of acetate, D-lactate, L-alanine and succinate was confirmed in the control experiments performed on all three species, and the major metabolite was succinate in all cases, in agreement with data reported in the literature (Turrens, 1999). However, noteworthy differences were found in parasites treated with the phthalazine derivatives, whereas the presence of benznidazole did not lead to significant alterations in fuel metabolism (data not shown).

The most relevant finding was that the excretion of succinate, the key catabolite in glucose metabolism, was substantially inhibited by the two monoalkylamino substituted compounds in both *L. infantum* and *L. donovani*, components of the *L. donovani* complex (Table 3). In *L. infantum*, 31 and 35% reductions in the succinate peaks were observed for **2** and **4**, respectively, and 35%/32% reductions were found for the same compounds in *L. donovani* promastigotes. A similar behaviour was found in the *L. braziliensis* species, where

https://doi.org/10.1017/S0031182015000219 Published online by Cambridge University Press

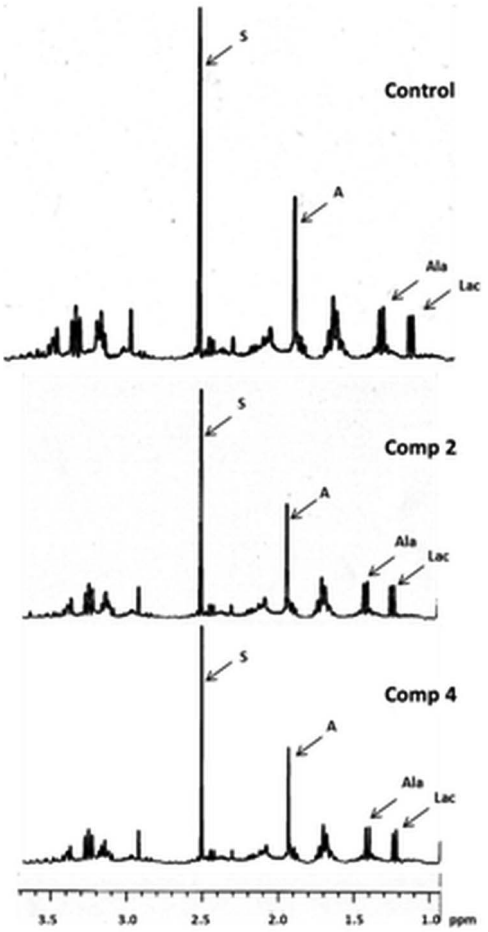

Fig. 7. ^{1}H NMR spectra showing metabolites excreted by promastigote forms of *L. donovani* either untreated or treated with compounds **2** and **4**, IC_{25} dosage. Lac: D-lactate; Ala: L-alanine; A: acetate; S: succinate.

compounds **2** and **4** caused decreases of 30 and 37%, respectively with respect to control. In fact, the relevant succinate inhibition values obtained agreed with the TEM evidence, indicating that these phthalazine derivatives severely damage the internal organelles of the parasites and, therefore, succinate excretion is substantially affected in all three species of parasites.

With regard to the less significant excretion products shown in Table 3, the formation of L-alanine, D-lactate and acetate were also remarkably inhibited in *L. donovani* by both **2** and **4**, which produced inhibitory values ranging from 22 to 29%. Although variations in the secondary excretion products were in general more erratic in *L infantum* and *L. braziliensis*, inhibition of the formation of L-alanine and D-lactate was always seen with compounds **2** and **4**, to a greater or lesser extent. The only exceptions found were increases of 9% and 20%, respectively, in the production of acetate when parasites of those two series were treated with compound **4**. In summary, the NMR data on catabolites are indicative of substantial alterations in glucose metabolism in the three species. It is feasible that the small size of these monoalkylamino substituted compounds allows them to easily penetrate the cristae (tubular invaginations of the inner mitochondrial membrane) (Von der Malsburg *et al*. 2011) leading to subsequent changes in the metabolic pathway. Concerning the variations in acetate production, it is well known that this end-product originates in the mitochondria of *Leishmania* species through a collateral pathway via pyruvate which is regulated by the pyruvate dehydrogenase complex (Bringaud *et al*. 2006), so that **2** and **4** could be interfering in a different way with that enzyme complex in both the *L. infantum* and *L. braziliensis* species.

SOD enzymatic inhibition in the Leishmania parasites and in human erythrocytes

Since we had previously found that the anti-*T. cruzi* activity of compounds **1**–**4** was always accompanied by inhibitory activity on the antioxidant enzyme Fe-SOD (Sánchez-Moreno *et al*. 2012*b*) we tested the effects of those compounds on Fe-SOD isolated from *L. infantum, L. braziliensis* and *L. donovani* over a range of concentrations from 0·1 to 100 μM. We used promastigote forms of both species, which excrete Fe-SOD as a mixture of isoenzymes when cultured in a medium lacking inactive FBS (Longoni *et al*. 2013). The inhibition data obtained are shown in Fig. 8, graphs A, B and C, and the corresponding IC_{50} values are included for easier evaluation of the graphs displayed. For comparison, graph D of Fig. 8 shows the effects of the same compounds on CuZn-SOD obtained from human erythrocytes. The most remarkable result was the significant inhibitory effect on Fe-SOD found for the highly leishmanicidal monoalkylamino substituted compounds **2** and **4** in the three species tested, whereas their inhibition of human CuZn-SOD was clearly lower. If we consider the IC_{50} calculated for *L. infantum*, inhibition of Fe-SOD by **2** and **4** was, respectively, 17·8- and 8·0-fold higher than inhibition of CuZn-SOD, with respective values of 8·9-, and 9·4-fold higher in the case of *L. braziliensis* and 7·0- and 10·4-fold higher values in the case of *L. donovani*. Even more, both compounds achieved 100% inhibition of Fe-SOD in the three *Leishmania* species at a 100 μM concentration, and values ranging from 60 to 100% at 25 μM. As expected on the basis of the infectivity graphs shown in Fig. 3, the bisalkylamino substituted compound **1** had a notably lower inhibitory activity for Fe-SOD, since between 60 and 70% inhibition was seen at a dose of 100 μM and its IC_{50} values were in all cases very high compared to those found for **2** and **4**. It also showed poor selectivity against the Fe-SOD of the *Leishmania* species with respect to the CuZn-SOD of human erythrocytes. All these data seem to confirm some kind of relation

https://doi.org/10.1017/S0031182015000219 Published online by Cambridge University Press

Table 3. Variations in the heights of peaks corresponding to catabolites excreted by *Leishmania* spp. promastigotes in the presence of compounds **2** and **4** with respect to the control[a]

	Leishmania infantum				*Leishmania braziliensis*				*Leishmania donovani*			
Comp.	Lac (%)	Ala (%)	A (%)	S (%)	Lac (%)	Ala (%)	A (%)	S (%)	Lac (%)	Ala (%)	A (%)	S (%)
2	−5	−38	−9	−31	−2	−30	−6	−30	−24	−25	−29	−35
4	−5	−36	+9	−35	−18	−2	+20	−37	−22	−29	−25	−32

[a] A: acetate; Ala: L-alanine; Lac: D-lactate; S, succinate; (−): peak inhibition; (+): peak increasing.

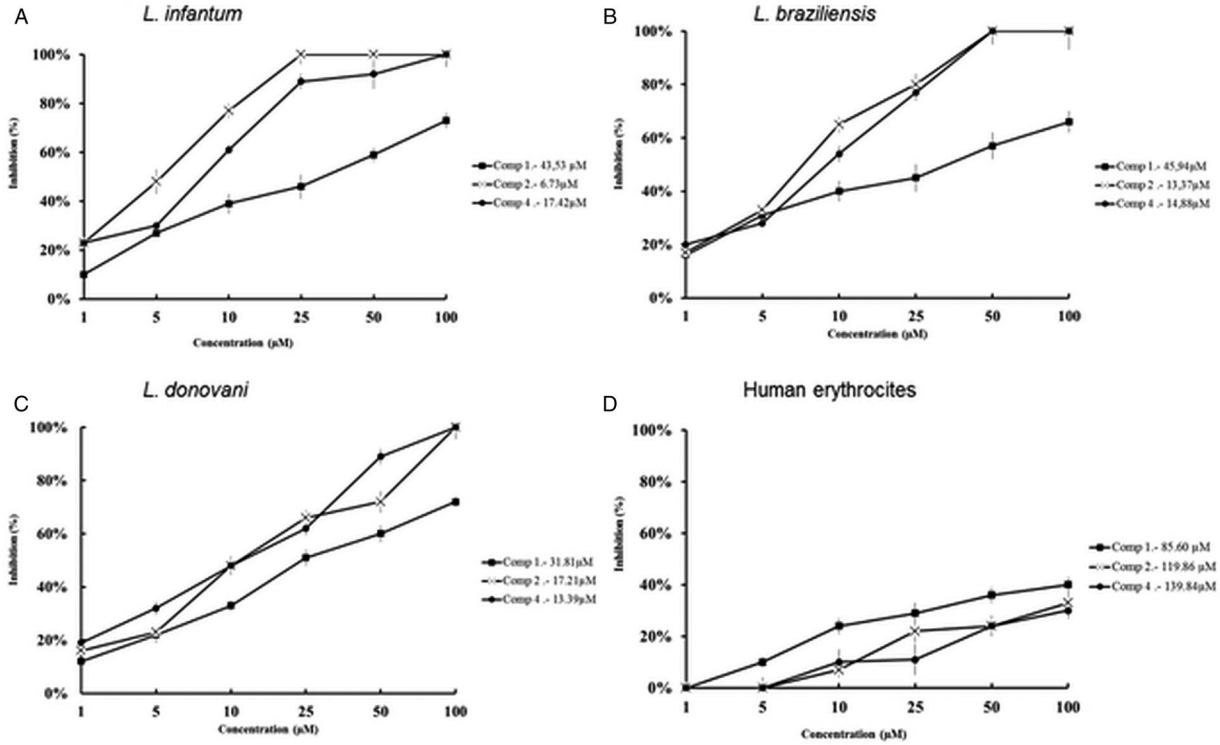

Fig. 8. (A–C) *In vitro* inhibition (%) of Fe-SOD of *L. infantum*, *L. braziliensis* and *L. donovani* promastigotes by compounds **1, 2** and **4**. (D) *In vitro* inhibition of CuZn-SOD in human erythrocytes by compounds **1, 2** and **4**. Values are the average of three separate determinations. Differences between the activities of the control homogenate and those incubated with the tested compounds were obtained according to the Newman–Keuls test. IC_{50} was calculated by linear regression analysis from the *K*c values at the concentrations employed (1, 10, 25, 50 and 100 μM).

between parasiticidal activity and inhibition of Fe-SOD, in accordance with results described in previous work (Sánchez-Moreno *et al*. 2011, 2012*b*,*c*; Navarro *et al*. 2014). Fe-SOD inhibition could also, at another level, be related to the catabolic changes discussed above, since a mitochondrial malfunction, originating from the redox stress produced by inhibition of the mitochondrion-resident Fe-SOD enzyme (Kirkinezos and Moraes, 2001), should result in severe alteration of pyruvate metabolism and a consequent decrease in the production of succinate.

On the other hand, a relationship between structural features and inhibition of Fe-SOD activity can also be deduced from the data shown in this work and in the above mentioned previous study, since the less bulky monoalkylamino substituted phthalazines are always more effective inhibitors than their bisalkylamino substituted counterparts. This could be in accordance with some kind of interaction of the compounds with the active sites of the enzyme in the *Leishmania* species considered. As has been pointed out, modification of Fe-SOD activity by a particular compound could be due to modifying the coordination geometry of the iron atoms, reorganizing the active sites of the H-bonding network (Yikilmaz *et al*. 2006) or reducing the cooperative interaction between the two monomers of the enzyme (Muñoz *et al*. 2005). In order to obtain more information about this matter, molecular modelling calculations on the mode of interaction of the tested compounds with the enzyme were performed. Fe-SOD enzymes are dimeric forms in which either of the two monomers contains

https://doi.org/10.1017/S0031182015000219 Published online by Cambridge University Press

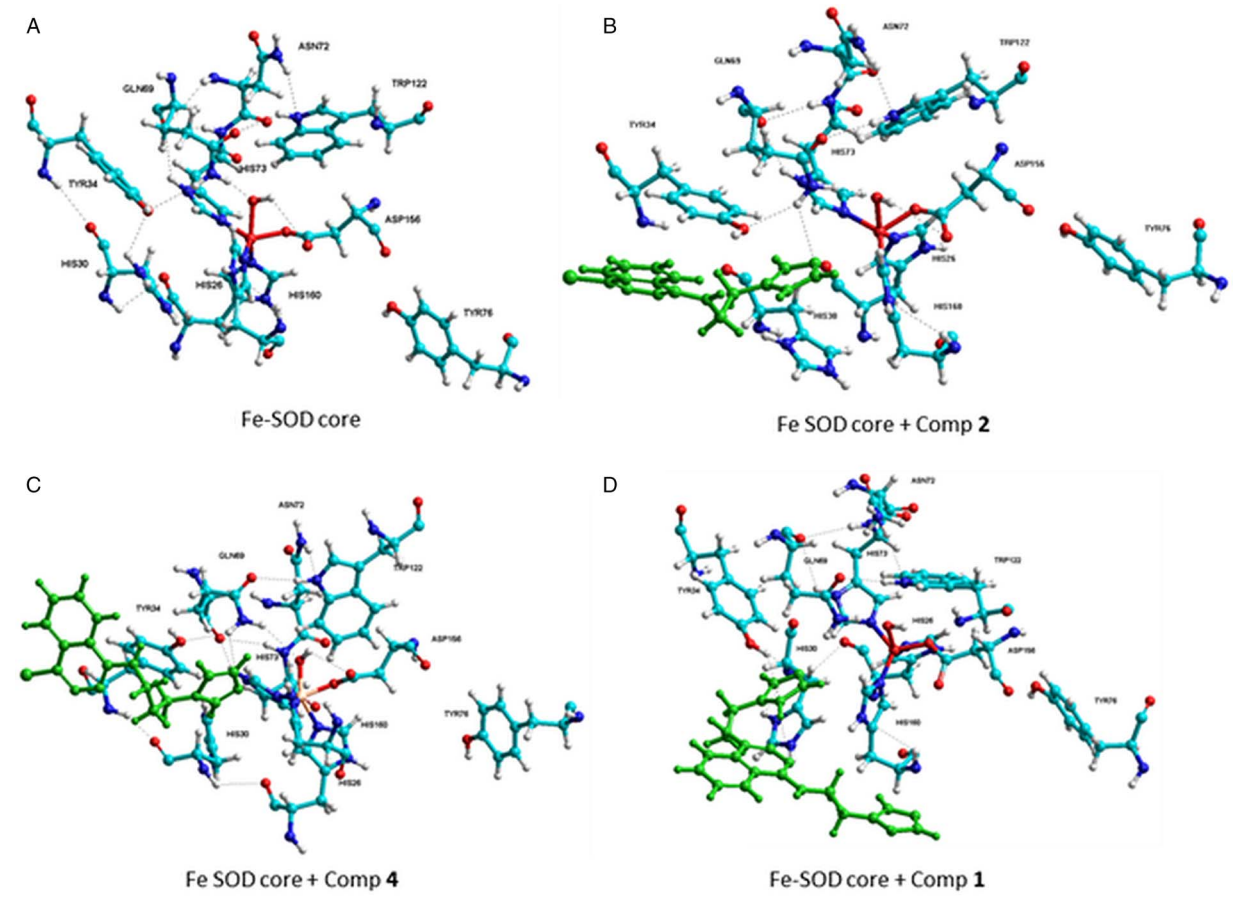

Fig. 9. (A) Molecular model of the free Fe-SOD enzyme active site with its supporting network of hydrogen bonds displayed as dashed lines. (B), (C) and (D) Molecular models of the Fe-SOD enzyme active site with compounds **2, 4** and **1**, respectively, embedded in the proximity of the iron atom showing the modified hydrogen-bonding networks.

an iron atom. While the CuZn-SOD enzyme of mammals has the metal active sites located deeper inside the monomers, the active sites of the parasite Fe-SOD are closer to the interface between the two subunits, so that the channel opening in the inner part of the dimer is the obvious place to locate a molecule when searching for an interaction with the iron environment (Abreu and Cabelli, 2010). Therefore, the phthalazine derivatives were positioned inside the cavity with the imidazole ring pointing towards the active centre core. On that basis, and using the AMBER force field implemented in HyperChem, we applied a methodology previously developed by us (Sánchez-Moreno *et al*. 2012*b*) to the structure of a *Leishmania* Fe-SOD enzyme model obtained from the Brookhaven Protein Data Bank 4F2N entry. Calculation of the most favoured distance between the iron atom of one subunit and the preferred imidazole nitrogen showed that the monoalkylamino substituted compounds could be located closer to the metal than their respective bisalkylamino substituted analogues (3·57 Å and 3·19 Å for **1** and **2**, 3·86 Å and 3·40 Å for **3** and **4**, respectively). These differences could be related to a more effective interaction of the imidazole rings of the less bulky compounds **2** and **4** with the active centre of the enzyme and match data noted above about Fe-SOD activity inhibition. As a consequence, we proceeded to study the modifications caused by the tested compounds on the hydrogen bonding network wrapping the metal atom. In Fe-SOD, the metal appears as a five-coordinated active site ligated by three histidine units, an aspartate group and an axial H_2O or HO^- ligand supported by an essential hydrogen bonding network comprising diverse amino acids (Miller *et al*. 2005). Specifically, the coordinated molecule engages in a H-bond with Asp156, and another H-bond engages with Gln69 (Yikilmaz *et al*. 2006). The H_2O/HO^- ligand and its hydrogen bonding partners are essential for tuning the antioxidant activity of Fe-SOD (Yikilmaz *et al*. 2002), since it acts as a proton donor/acceptor and facilitates the release of peroxide generated by the reduction of the substrate (Han *et al*. 2002). On this basis, we modelled the active site moiety of the Fe-SOD enzyme as shown in Fig. 9A, in which the hydrogen-bonding system is displayed as dashed lines, the H_2O ligand appears in the foreground and the key interactions with Gln69 and Asp156 are clearly seen. Then, the effect of the best Fe-SOD inhibitors, **2** and **4**, on the active site environment was estimated. Both

https://doi.org/10.1017/S0031182015000219 Published online by Cambridge University Press

compounds were steered to the active site with the imidazole ring pointing towards the metal ion in the most favoured disposition (Fig. 9B and C). As a consequence of the molecule's approach, the amino acids surrounding the iron atom were displaced apart from their initial positions and the hydrogen-bonding pattern appeared partially distorted. The histidine molecules lost some of their links to the surrounding amino acids, although the H_2O/HO^- ligand preserved its two interactions with Gln69 and Asp156. But the most relevant point was that a new hydrogen bond was created between one nitrogen atom at the imidazole moiety of **2** or **4** and the nitrogen of the amide group present in Gln69. Therefore, both monoalkylamine derivatives were linked to one of the amino acids essential in modulating the antioxidant activity of the enzyme. We think that these interactions of the tested compounds with the coordination sphere of the iron atom could explain in part the enzyme deactivation found experimentally.

As a point of comparison, the same treatment was given to the less inhibitory compound **1**, in which the only difference with respect to **2** was replacement of the chlorine atom at the pyridazine ring by a more bulky alkylamino substituent. The most favoured disposition is shown in Fig. 9D. It can be seen that, although the H-bonding net is again distorted and Gln69 is moved away from its usual location losing its link with the water ligand, the drug molecule is positioned further away from the metal environment and, as a consequence, there is no hydrogen bonding between **1** and GLN69 or any other amino acid in the coordination sphere, and the figure only shows an interaction with TYR34, located far away from the first coordination sphere. This fact seems to be in accordance with the inhibitory activity differences between **1** and the monoalkylamino substituted compounds. Therefore, steric hindrance originating from the substituents of the phthalazine system could be affecting its ability to inhibit the Fe-SOD enzyme.

From all the results detailed above, we conclude that the phthalazine derivatives **2** and **4** show interesting *in vitro* leishmanicidal activity that seems to be related to their inhibitory ability against the Fe-SOD of the parasite. On this basis, we think that they fulfil the requirements needed to justify a more detailed investigation as to the nature of the mechanisms involved in their patterns of activity, and that they could be considered candidates for studying antiparasitic activity at a higher level.

ACKNOWLEDGEMENTS

The authors are grateful to the transmission electron microscopy and nuclear magnetic resonance spectroscopy services of the CIC-University of Granada for their contribution to the studies on ultrastructural alterations and catabolism.

FINANCIAL SUPPORT

Financial support from the Spanish Ministerio de Ciencia e Innovacion (CTQ2009-14288-C04-01 and Consolider Ingenio CSD2010-00065), and from the Santander-Universidad Complutense Research Programme (GR35/10-A-921371) is also acknowledged.

REFERENCES

Abreu, I. A. and Cabelli, D. E. (2010). Figure 2 in superoxide dismutases: a review of the metal-associated mechanistic variations. *Biochimica et Biophysica Acta* **1804**, 263–274.

Beyer, W. F. and Fridovich, I. (1987). Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Analytical Biochemistry* **161**, 559–566.

Blasco, S., Verdejo, B., Clarés, M. P., Castillo, C. E., Algarra, A. G., Latorre, J., Máñez, M. A., Basallote, M. G., Soriano, C. and García-España, E. (2010). Hydrogen and copper ion induced molecular reorganizations in two new scorpiand-like ligands appended with pyridine rings. *Inorganic Chemistry*. **49**, 7016–7027.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

Bringaud, F., Riviere, L. and Coustou, V. (2006). Energy metabolism of trypanosomatids: adaptation to available carbon sources. *Molecular and Biochemical Parasitology* **149**, 1–9.

Case, D. A., Cheatham, T. E., Darden, T., Gohlke, H., Luo, R., Merz, K. M., Onufriev, A., Simmerling, C., Wang, B. and Woods, R. J. (2005). The Amber biomolecular simulation programs. *Journal of Computational Chemistry* **26**, 1668–1688.

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

Chappuis, F., Sundar, S., Hailu, A., Ghalib, H., Rijal, S., Peeling, R. W., Alvar, J. and Boelaert, M. (2007). Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nature Reviews Microbiology* **5**, 873–882.

Cornell, W.D., Cieplak, P., Bayly, C. I., Gould, I. R., Merz, K. M., Jr, Ferguson, D. M., Spellmeyer, D. C., Fox, T., Caldwell, J. W. and Kollman, P. A. (1995). A Second Generation Force Field for the Simulation of Proteins, Nucleic Acids, and Organic Molecules. *J. Am. Chem. Soc.* **117**, 5179–5197.

Croft, S. L., Sundar, S. and Fairlamb, A. H. (2006). Drug resistance in Leishmaniasis. *Clinical Microbiology Reviews* **19**, 111–126.

Fernandez-Becerra, C., Sánchez-Moreno, M., Osuna, A. and Opperdoes, F. R. (1997). Comparative aspects of energy metabolism in plant trypanosomatids. *Journal of Eukaryotic Microbiology* **44**, 523–529.

Flohé, L. (2009). In search of trypanocidal drugs. In *Antiparasitic and antibacterial drug discovery* (ed. Selzer, P. M.), pp. 211–227. Wiley CH Verlag GmbH & Co KGaA, Weinheim.

Freitas-Junior, L. H., Chatelain, E., Kim, H. A. and Siqueira-Neto, J. L. (2012). Visceral leishmaniasis treatment: what do we have, what do we need and how to deliver it? *International Journal for Parasitology Drugs and Drug Resistance* **2**, 11–19.

Ginger, M. (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. and Sánchez-Moreno, M. (2005). In vitro activity of C20-diterpenoid alkaloid derivatives in promastigotes and intracellular amastigotes of *Leishmania infantum*. *International Journal Antimicrobial Agents* **25**, 136–141.

Han, W. G., Lovell, T. and Noodleman, L. (2002). Coupled redox potentials in manganese and iron superoxide dismutases from reaction kinetics and density functional/electrostatics calculations. *Inorganic Chemistry* **41**, 20–218.

Kirkinezos, I. G. and Moraes, C. T. (2001). Reactive oxygen species and mitochondrial diseases. *Cell and Developmental Biology* **12**, 449–457.

Lamarque, L., Navarro, P., Miranda, C., Arán, V. J., Ochoa, C., Escartí, F., García-España, E., Latorre, J., Luis, S. V. and Miravet, J. F. (2001). Dopamine interaction in the absence and in the presence of Cu(II) ions with macrocyclic and macrobicyclic polyamines containing pyrazole units. Crystal structures of $[Cu_2(L_1)(H_2O)_2](ClO_4)_4$ and $[Cu_2(H_{-1}L_3)](ClO_4)$. *Journal of the American Chemical Society* **123**, 10560–10570.

Loiseau, P. M., Cojean, S. and Schrével, J. (2011). Sitamaquine as a putative antileishmanial drug candidate: from the mechanism of action to the risk of drug resistance. *Parasite* **18**, 115–119.

Longoni, S. S., Sánchez-Moreno, M., López, J. E. and Marín, C. (2013). *Leishmania infantum* secreted iron superoxide dismutase purification and its application to the diagnosis of canine Leishmaniasis. *Comparative Immunology, Microbiology and Infectious Disease* **36**, 499–506.

Lukes, J., Mauricio, I. L., Schönian, G., Dujardin, J. C., Soteriadou, K., Dedet, J. P., Kuhls, K., Tintaya, K. W. Q., Jirků, M., Chocholová, E., Haralambous, C., Pratlong, F., Oborník, M., Horák, A., Ayala, F. J. and Miles, M. A. (2007). Evolutionary and geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. *PNAS* **104**, 9375–9380.

Manta, B., Comini, M., Medeiros, A., Hugo, M., Trujillo, M. and Radi, R. (2013). Trypanothione: a unique bis-glutathionyl derivative in trypanosomatids. *Biochimica et Biophysica Acta* **1830**, 3199–3216.

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

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

Michels, P. A. M., Bringaud, F., Herman, M. and Hannaert, V. (2006). Metabolic functions of glycosomes in trypanosomatids. *Biochimica et Biophysica Acta* **1763**, 1463–1477.

Miller, A. F. (2004). Superoxide dismutases: active sites that save, but a protein that kills. *Current Opinion in Chemical Biology* **8**, 162–168.

Miller, A. F., Sorkin, D. L. and Padmakumar, K. (2005). Anion binding properties of reduced and oxidized iron-containing superoxide dismutase reveal no requirement for tyrosine 34. *Biochemistry* **44**, 5969–5981.

Miranda, C., Escartí, F., Lamarque, L., Yunta, M. J. R., Navarro, P., García-España, E. and Jimeno, M. L. (2004). New 1H-pyrazolecontaining polyamine receptors able to complex L-glutamate in water at physiological pH values. *Journal of the American Chemical Society* **126**, 823–833.

Muñoz, I. G., Morán, J. F., Becana, M. and Montoya, G. (2005). The crystal structure of an eukaryotic iron superoxide dismutase suggests intersubunit cooperation during catalysis. *Protein Science* **14**, 387–394.

Navarro, P., Sánchez-Moreno, M., Marín, C., García-España, E., Ramírez-Macías, I., Olmo, F., Rosales, M. J., Gómez-Contreras, F., Yunta, M. J. R. and Gutiérrez-Sánchez, R. (2014). In vitro leishmanicidal activity of pyrazole-containing polyamine macrocycles which inhibit the Fe-SOD enzyme of *L. infantum* and *L. braziliensis* species. *Parasitology* **141**, 1031–1043.

Nwaka, S., Besson, D., Ramirez, B., Maes, L., Matheeussen, A., Bickle, Q., Mansour, N. R., Yousif, F., Townson, S., Gokool, S., Cho-Ngwa, F., Samje, M., Misra-Bhattacharya, S., Murthy, P. K., Fakorede, F., Paris, J. M., Yeates, C., Ridley, R., Van Voorhis, W. C. and Geary, T. (2011). Integrated dataset of screening hits against multiple neglected disease pathogens. *PLoS Neglected Tropical Diseases* **5**, 658, e1412.

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

Ouellette, M., Drummelsmith, J. and Papadopoulou, B. (2004). Leishmaniasis: drugs in the clinic, resistance and new developments. *Drug Resistance Update* **7**, 257–266.

Reviriego, F., Navarro, P., García-España, E., Abelda, M. T., Frías, J. C., Domenech, A., Yunta, M. J. R., Costa, R. and Ortí, E. (2008). Diazatetraester 1H-pyrazole crowns as fluorescent chemosensors for AMPH, METH, MDMA (ecstasy), and dopamine. *Organic Letters* **10**, 5099–5102.

Rodríguez-Ciria, M., Sanz, A. M., Yunta, M. J. R., Gómez-Contreras, F., Navarro, P., Sanchez–Moreno, M., Boutaleb-Charki, S., Osuna, A., Castiñeiras, A., Pardo, M., Cano, C. and Campayo, L. (2007). 1,4-Bis(alkylamino)benzo[g]phthalazines able to form complexes of Cu(II) which as free ligands behave as SOD inhibitors and show efficient in vitro activity against *Trypanosoma cruzi*. *Bioorganic Medicinal Chemistry* **15**, 2081–2091.

Ryan, K. J., Ray, C. G., Ahmad, N., Lawrence Drew, W. and Plorde, J. J. (2010). *Sherris Medical Microbiology: an Introduction to Infectious Diseases*, 5th Edn. McGraw Hill, New York.

Sánchez-Moreno, M., Sanz, A. M., Gómez-Contreras, F., Navarro, P., Marín, C., Ramírez-Macías, I., Rosales, M. J., Olmo, F., García-Aranda, I., Campayo, L., Cano, C., Arrebola, F. and Yunta, M. J. R. (2011). *In vivo* trypanosomicidal activity of imidazole- or pyrazole-based benzo[*g*]phthalazine derivatives against acute and chronic phases of Chagas disease. *Journal Medicinal Chemistry* **54**, 970–979.

Sánchez-Moreno, M., Marín, C., Navarro, P., Lamarque, L., García-España, E., Miranda, C., Huertas, O., Olmo, F., Gómez-Contreras, F., Pitarch, J. and Arrebola, F. (2012*a*). In vitro and in vivo trypanosomicidal activity of pyrazole-containing macrocyclic and macrobicyclic polyamines: their action on acute and chronic phases of Chagas disease. *Journal Medicinal Chemistry* **55**, 4231–4243.

Sánchez-Moreno, M., Gómez-Contreras, F., Navarro, P., Marín, C., Olmo, F., Yunta, M. J. R., Sanz, A. M., Rosales, M. J., Cano, C. and Campayo, L. (2012*b*). Phthalazine derivatives containing imidazole rings behave as Fe-SOD inhibitors and show remarkable anti-*T. cruzi* activity in immunodeficient-mouse mode of infection. *Journal Medicinal Chemistry* **55**, 9900–9913.

Sánchez-Moreno, M., Gómez-Contreras, F., Navarro, P., Marín, C., Ramírez-Macías, I., Olmo, F., Sanz, A. M., Campayo, L., Cano, C., and Yunta, M. J. R. (2012*c*). In vitro leishmanicidal activity of imidazole- or pyrazole-based benzo[*g*]phthalazine derivatives against *L. infantum* and *L. braziliensis* species. *Journal Antimicrobial Chemotherapy* **67**, 387–397.

Seifert, K. (2011). Structures, targets and recent approaches in anti-leishmanial drug: discovery and development. *Open Medicinal Chemistry Journal* **5**, 31–39.

Turrens, J. F. (1999). More differences in energy metabolism between trypanosomatidae. *Parasitology Today* **15**, 346–348.

Turrens, J. F. (2004). Oxidative stress and antioxidant defences: a target for the treatment of diseases caused by parasitic protozoa. *Molecular Aspects of Medicine* **25**, 211–220.

Von der Malsburg, K., Müller, J. M., Bohnert, M., Oeljeklaus, S., Kwiatkowska, P., Becker, T., Loniewska-Lwowska, A., Wiese, S., Rao, S., Milenkovic, D., Hutu, D. P., Zerbes, R. M., Schulze-Specking, A., Meyer, H. E., Martinou, J. C., Rospert, S., Rehling, P., Meisinger, C., Veenhuis, M., Warscheid, B., van der Klei, I. J., Pfanner, N., Chacinska, A. and van der Laan, M. (2011). Dual role of mitofilin in mitochondrial membrane organization and protein biogenesis. *Developmental Cell* **21**, 694–707.

Yikilmaz, E., Xie, J., Brunold, T. C. and Miller, A. F. (2002). Hydrogen bond mediated tuning of the redox potential of the non-heme Fe site of superoxide dismutase. *Journal of the American Chemical Society* **124**, 3482–3483.

Yikilmaz, E., Rodgers, D. W. and Miller, A. F. (2006). The crucial importance of chemistry in the structure-function link: manipulating hydrogen bonding in iron-containing superoxide dismutase. *Biochemistry* **45**, 1151–1161.