

In vitro Leishmanicidal and Trypanosomicidal Properties of Imidazole-Containing Azine and Benzoazine Derivatives

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Leishmaniasis and Chagas diseases are two of the most important parasitic diseases in the world. Both belong to the category of Neglected Tropical Diseases, and they cannot be prevented by vaccination. Their treatments are founded in outdated drugs that possess many pernicious side-effects and they're not easy to administer. With the aim of discovering new compounds that could serve as anti-trypanosomal drugs, an antiparasitic study of a synthetic compound family has been conducted. A series of new 1,4-bis(alkylamino)- and 1-alkylamino-4-chloroazine and benzoazine derivatives 1–4 containing imidazole rings have been synthesized and identified.

Their structures showed a possible interest based on previous work. Their *in vitro* anti-*Leishmania infantum*, anti-*L. braziliensis*, anti-*L. donovani* and anti-*T. cruzi* activity were tested, as well as the inhibition of Fe-SOD enzymes. It was found that some of them exhibited quite relevant values indicative of being worthy of future more detailed studies, as most of them showed activity to more than only one parasite species, especially compound 3c was active for the three studied *Leishmania* species and also for *T. cruzi*, which is a very interesting trait as it covers a wide spectrum.

Leishmaniasis and Chagas disease count among the most extended tropical infections caused by protozoan parasites. Both diseases are globalized.^[1] *Trypanosoma cruzi* is responsible for Chagas disease, that consists of two clinical phases: acute and chronic. In its chronic phase is responsible of severe cardiac and gastrointestinal injuries,^[2] whereas more than twenty *Leishmania* species and subspecies are the cause of leishmaniasis, which encompasses a wide spectrum of diseases producing injuries at the cutaneous, mucocutaneous and visceral levels.

Visceral leishmaniasis is the most severe form among them, being responsible of thousands of deaths every year. It is caused by several subspecies included in the *L. donovani* complex. Cutaneous and mucocutaneous leishmaniasis are, however, more extended, and are prevalent in Latin America, where they are caused mainly by the *L. mexicana*, *L. braziliensis*, and *L. panamensis* species.^[3] Chagas disease can also reactivate the parasitemia when the host underwent immunosuppression. Some patients who were thought to be cured suffered a reactivation when treated with anticancer chemotherapy, had a liver or kidney transplant, or were diagnosed with AIDS. This reactivation also occurred with meningoencephalitis and/or acute myocarditis. The infection persists in patients with chronic chagasic cardiopathy that have a heart transplant, only a temporary remission of parasitemia takes place when treated with benznidazole.^[9–11] Due to the large number of people affected by these tropical infections every year, great efforts have been made to find and design therapeutic agents with leishmanicidal and/or antichagasic properties. Historically, two heterocyclic compounds (benznidazole and nifurtimox) have been broadly used against Chagas and both are useful in the treatment of the acute phase, but not so when the disease reaches the chronic stage, because of low effectiveness and undesirable side effects.^[12–14] Many other drugs have been tested in recent times with a wide variety of results,^[15] but none of them are as extended in the clinical practice as the aforementioned compounds, and extensive research remains in search of new effective, safe and easy to synthesize molecules.

A similar picture is obtained when considering the current anti-leishmania treatments. Pentavalent antimonials have been the matter of choice for many years, but resistance of the

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parasite against those drugs is now widespread in many countries, mainly in the Indian subcontinent. More recently, molecules like amphotericin B or miltefosine have shown great efficacy against leishmaniasis, but their syntheses are expensive, and their use is not exempt of therapeutic complications.^[16,17] Therefore, the design of new molecules effective and without negative side-effects against those parasitic diseases remains as a goal for many research groups.

The research of novel agents against diseases caused by trypanosomatid is centered against certain targets, mainly in metabolic pathways or key survival parasitic enzymes. In recent years, cysteine proteinase, hypoxanthine-guanine phosphoribosyl transferase, trypanothione reductase, sterol metabolism, kinetoplast DNA sites and such have been the main studied targets.^[18]

The present work is focused on iron superoxide dismutase (Fe-SOD), due to its specificity, as the equivalent molecule found in mammals appears linked to copper or zinc atoms (Cu/Zn-SOD). Malfunction of this enzyme compromises parasite survival greatly, as it is the parasite main defence against oxidative burst (and consequent generation of reactive oxygen species).^[19] For this reason, this molecule is an interesting target for study, as the interaction of prosthetic groups with the active sites containing the metallic ion deactivate the enzyme and its antioxidant effect.

In connection with this matter, our group has been working in the last years in the synthesis and antiparasitic properties of different series of phthalazine derivatives functionalized with imidazole or pyrazole rings at the end of sidechains attached to

the pyridazine moiety.^[20–24] According to the antiparasitic activity reported previously for many azole compounds, some of these series containing the imidazole moiety have shown interesting activity data against *T. cruzi* and/or different *Leishmania* species, and those properties seem to be related to the simultaneous presence of both the pyridazine and the imidazole heterocyclic systems, clearly showing structure dependence for both trypanocidal and leishmanicidal features.

In previous work we found that the imidazole and pyrazole derivatives inhibited the Fe-SOD activity on *T. cruzi*,^[21] and *Leishmania sp.* species,^[23] while their impact on human CuZn-SOD was not significant.

In this work we ponder the effect of modifications in the structure of the azine moiety on the leishmanicidal and trypanosomicidal properties in several series of related structures. Four groups of compounds were tested in vitro against three *Leishmania* species: *L. braziliensis*, as a representative parasite among those causing mucocutaneous leishmaniasis, *L. donovani* as a representative agent for visceral Leishmaniasis and *L. infantum* as a representative of cutaneous Leishmaniasis, and also against *T. cruzi*. All compounds tested (Figure 1) include the imidazole ring at the end of a sidechain linked to the exocyclic amino group but differ in the disposition of the nitrogen atoms located in the azine or benzoazine moiety, in the number of methylene groups conforming the sidechain, in the attachment point of the sidechain to imidazole or benzimidazole moieties or in the presence/absence of chlorine atoms attached to the azine ring.

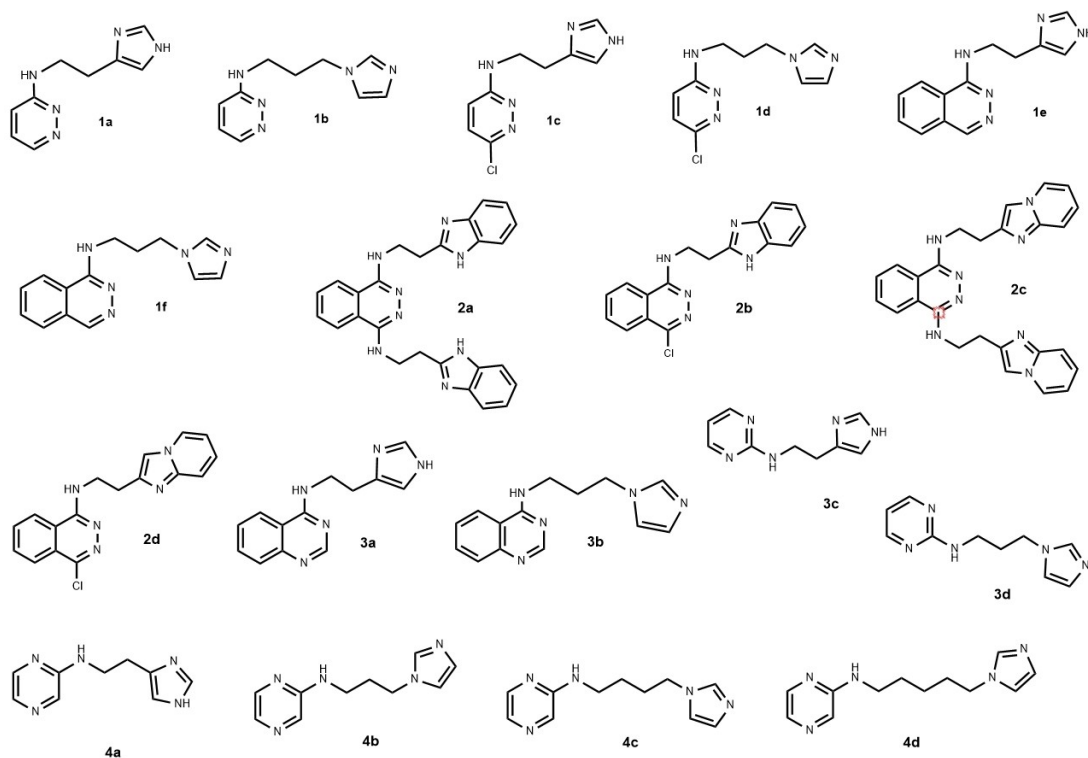


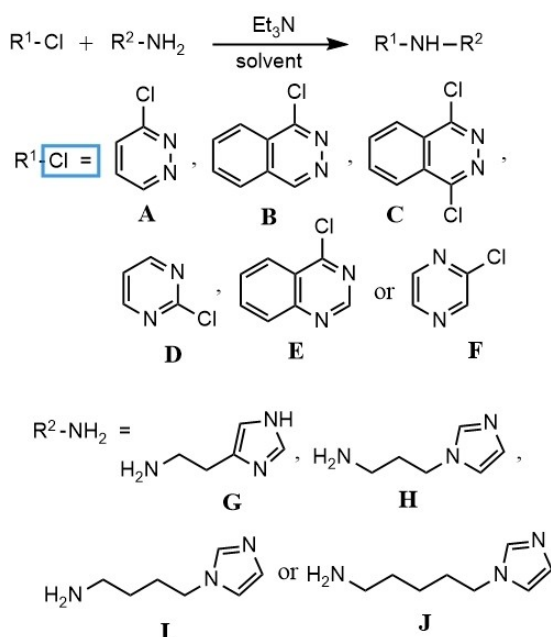
Figure 1. Imidazole-containing azine derivatives tested against *T. cruzi* and *Leishmania* species in this study.

Results and Discussion

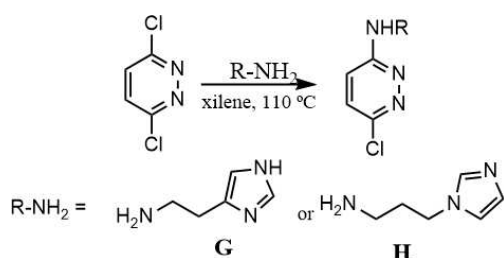
Chemistry

The preparation of compounds **1 a–b**, **1 e–f**, **3 a–d** and **4 a–d** was performed in every case through nucleophilic substitution of the chlorine atom at the corresponding monochloroazine or monochlorobenzoazine by the required imidazole-containing aliphatic amine^[20–22] (Scheme 1) while compounds **1 c–d** were prepared in a similar way using 2,4-dichloroazine with only one chlorine atom substitution (Scheme 2). Xylene or dry ethanol were alternatively used as the solvent depending on the solubility features of reactants and products. In most cases, trimethylamine was added.

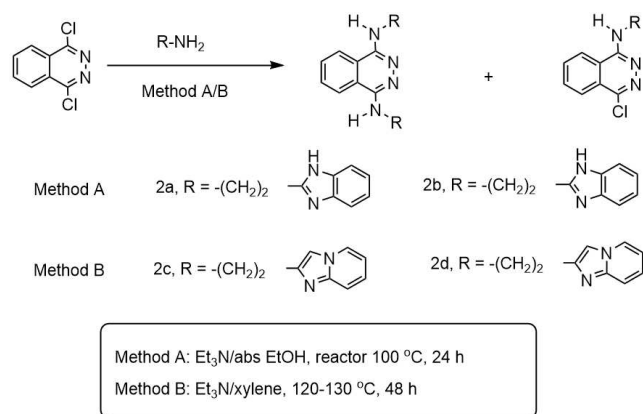
Compounds **2 a–d** were prepared from 1,4-dichlorophthalazine according to the methodology shown in Scheme 3, following conditions related to those previously established by our group for the synthesis of similar compounds.^[20–22] The mono- and bis(alkylamino)substituted derivatives **2 a** and **2 b** were obtained by nucleophilic substitution at the C-1 or simultaneously at C-1 and C-4 positions of the starting



Scheme 1. Synthesis of compounds **1 a–b**, **1 e–f**, **3 a–d** and **4 a–d**.



Scheme 2. Synthesis of compounds **1 c–d**.



Scheme 3. Preparation of the phthalazine derivatives **2 a–d**.

compound with 2-(1*H*-benzo[*d*]imidazol-2-yl)ethylamine at 100 °C in a reactor, with dry ethanol as the solvent, for 24 hours, using triethylamine as the chlorine acceptor (method A).^[21] The synthesis of the **2 c** and **2 d** was achieved in a similar way using 2-(imidazo[1,2-*a*]pyridin-2-yl)ethylamine by performing the reaction under reflux of toluene during 48 hours (method B).^[24] Isolation of the compounds from the crude reaction mixtures was performed by column flash chromatography with a chloroform/methanol 8:2 mixture.

All of the newly synthesized compounds were unequivocally identified by their analytical, ESI mass spectra, IR, ¹H NMR, and ¹³C NMR spectroscopic data, as shown in the Experimental Section. Heteronuclear multiple quantum coherence (gHMQC) experiments were performed for an accurate assignment of the ¹H and ¹³C NMR spectra signals, obtaining especially useful results.

The mono- and bis(alkylamino)substitution products **2 a–d** were easily differentiated in both ¹H and ¹³C NMR spectra on the basis of signals corresponding to the ring atoms of the phthalazine system.^[21] Protons H₅ and H₈, lost their equivalence in the monosubstituted compounds **2 b** and **2 d**, as the H₈ proton, close to the alkylamine chain, was deshielded with respect to the proton neighbouring the chlorine atom (H₅). Accordingly, the carbon atom attached to the alkylamine substituent (C₁) was always deshielded with respect to the carbon neighbouring the chlorine atom in the ¹³C NMR spectra.

In vitro leishmanicidal and trypanosomicidal evaluation

The *in vitro* activity, toxicity and selectivity index of compounds **1 a–f**, **2 a–d**, **3 a–d** and **4 a–d** were evaluated against extra and intracellular forms of *L. infantum* (MCAN/ES/2001/UCM-10), *L. braziliensis* (NHOM/BR/1975/M2909), *L. donovani* (MHOM/PE/84/LC26), and *T. cruzi* (IRHOD/CO/2008/SN3) strains as described in the experimental section. Although extracellular forms are more commonly used due to ease of working with them, they are less indicative of antiparasitic activity, since *Leishmania* extracellular forms are only transitory in vertebrate hosts and in any trypanosomatid infection, amastigotes last longer in the

host and are responsible of cell destruction and thus, symptoms.^[25] Therefore, intracellular assays were performed by infecting macrophage cells of the leishmanial species with promastigotes, which were transformed into amastigotes within 1 day of infection and then treated with the compounds.^[24] Compounds 1–4 were also tested against extracellular epimastigote and axenic amastigote forms of *T. cruzi* SN3 strains. Finally, the cytotoxicity of compounds 1–4 was evaluated using J774.2 macrophages for *Leishmania sp* assays and mammalian Vero cells in *T. cruzi* cases as the cellular model. Selectivity indexes were calculated in all cases and results obtained for the reference drug used in each case were included for comparison. In order to get a picture of compounds effectiveness, the followed criteria were a IC_{50} close to 10 μ M and a Selectivity Index (SI) higher than 20 for *Leishmania* species and higher than 50 for *T. cruzi*.^[26]

Tested compounds were less cytotoxic than BZN in *T. cruzi* assays and most of the tested compounds were also less cytotoxic than Glucantime in *Leishmania* assays, being compounds 2c and 2d the only exception, although no clear correlation between differences in their structures and measured toxicity could be found.

Regarding the *in vitro* data, the more informative selectivity index (SI) values were calculated, being shown in Tables 1–4. When analysing the calculated number of times that the SI of each compound exceeded the SI of Glucantime or BZN (in brackets), very good results were found, mainly for compounds 3c and 3d, which achieved high values in all the amastigote forms. These values are shown in bold type in the Tables.

The specificity index (SPI) was calculated for results in *Leishmania*. Obtained results were between the range of $0.4 < SPI < 2$, (data not shown) which indicate that the studied compounds were effective against both forms.^[27]

The following step in the activity study, alterations caused by the most active compounds among the eighteen synthesized on the infectivity was determined. J774.2 macrophage cells were then infected *in vitro* with promastigote forms of *L. infantum*, *L. braziliensis* or *L. donovani* at a ratio of 10:1, and the drugs were tested in IC_{25} concentrations. Drug activity shown in 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.

On day 10, the rate of host cell infection reached its maximum in the control experiment. When the selected compounds were added to the infected cells, the infection rate significantly decreased with respect to the control for all of the compounds tested, leading to inhibition values varying from 44% to 72%. In case of *L. infantum*, the five tested compounds (1a, 1f, 3c, 3d and 4b) were more effective than Glucantime, which showed a 29% inhibition, with 47%, 47%, 51%, 56% and 44% inhibition, respectively (Figure 2).

Similarly, in case of *L. braziliensis* the same tested compounds (1a, 1f, 3c, 3d and 4b) showed 65%, 72%, 53%, 49% and 58% respectively, while glucantime showed a 31% inhibition (Figure 3).

For *L. donovani* case, the tested compounds were four (3b, 3c, 3d and 4d) showing 65%, 72%, 44% and 50% inhibition respectively, again more effective than glucantime which showed a 47% inhibition (Figure 4).

Intracellular replication of *T. cruzi* amastigotes was determined in a similar way (at a ratio of 10:1, and the drugs in IC_{25} concentrations). Only two of the tested compounds (3b and 3c) were more effective than Benznidazole, since the two compounds, showing inhibition values of 40% and 57%, were

Table 1. *In vitro* activity, toxicity and selectivity index for the 1a–f, 2a–d, 3a–d and 4a–d derivatives on extra- and intracellular forms of *Leishmania infantum*.

Products	Activity IC_{50} [μ M] ^[a]		Toxicity in macrophages IC_{50} [μ M] ^[b]	Selectivity ^[c]	
	Promastigote forms	Amastigote forms		Promastigote forms	Amastigote forms
Glucantime	18.0 ± 3.1	24.2 ± 2.6	15.2 ± 1.0	0.8	0.6
1a	21.6 ± 1.8	18.6 ± 0.8	305.1 ± 15.8	16 (23)	16 (33)
1b	33.6 ± 2.4	nd	18.6 ± 1.0	1 (1)	nd
1c	19.9 ± 1.1	nd	17.1 ± 1.7	1 (1)	nd
1d	15.3 ± 0.5	nd	19.3 ± 1.1	1 (1)	nd
1e	10.2 ± 0.0	7.4 ± 0.3	63.9 ± 3.6	6 (9)	9 (17)
1f	11.1 ± 0.4	10.7 ± 0.7	210.7 ± 10.3	19 (38)	20 (39)
2a	19.9 ± 1.4	nd	18.7 ± 1.0	1(1)	nd
2b	24.5 ± 2.5	nd	25.2 ± 1.7	1(1)	nd
2c	18.7 ± 1.4	nd	2.4 ± 0.1	0	nd
2d	21.8 ± 2.3	nd	2.4 ± 0.3	0	nd
3a	27.7 ± 3.0	nd	65.2 ± 3.3	2 (3)	nd
3b	14.7 ± 0.6	11.9 ± 0.7	45.9 ± 2.8	3 (4)	4 (6)
3c	12.5 ± 0.4	9.3 ± 0.6	95.9 ± 4.6	8(11)	10 (21)
3d	16.3 ± 0.2	14.7 ± 0.7	160.1 ± 9.5	10 (14)	11 (22)
4a	20.8 ± 2.4	nd	21.9 ± 1.5	1 (1)	nd
4b	1.6 ± 0.2	2.7 ± 0.1	23.8 ± 1.9	15 (21)	9 (18)
4c	9.6 ± 0.8	5.8 ± 0.2	22.3 ± 2.3	2 (3)	4 (8)
4d	5.8 ± 0.3	4.0 ± 0.4	22.0 ± 1.7	4 (5)	5 (11)

Results are averages of four 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 and 200 μ M). [b] Against J774.2 macrophages after 72 h of culture. [c] 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.

Table 2. In vitro activity, toxicity and selectivity index for the 1a–f, 2a–d, 3a–d and 4a–d derivatives on extra- and intracellular forms of *Leishmania braziliensis*.

Products	Activity IC ₅₀ [μM] ^[a]		Toxicity in macrophages IC ₅₀ [μM] ^[b]	Selectivity ^[c]	
	Promastigote forms	Amastigote forms		Promastigote forms	Amastigote forms
Glucantime	25.6 ± 1.7	30.4 ± 6.1	15.2 ± 1.0	0.6	0.5
1a	20.6 ± 2.1	16.9 ± 1.6	305.1 ± 15.8	15 (25)	18 (36)
1b	30.5 ± 2.7	nd	18.6 ± 1.0	1 (1)	nd
1c	28.1 ± 0.4	nd	17.1 ± 1.7	1 (1)	nd
1d	13.5 ± 0.3	nd	19.3 ± 1.1	1 (2)	nd
1e	14.5 ± 1.3	8.1 ± 0.4	63.9 ± 3.6	4 (7)	8 (16)
1f	17.8 ± 1.4	15.0 ± 0.9	210.7 ± 10.3	12 (20)	14 (28)
2a	5.8 ± 0.2	4.4 ± 0.3	18.7 ± 1.0	3 (5)	4 (8)
2b	6.1 ± 0.5	5.3 ± 0.4	25.2 ± 1.7	4 (7)	5 (9)
2c	14.7 ± 0.6	nd	2.4 ± 0.1	0 (0)	nd
2d	26.6 ± 5.4	nd	2.4 ± 1.3	0 (0)	nd
3a	29.1 ± 2.3	18.9 ± 1.6	65.2 ± 3.3	2 (4)	3 (7)
3b	7.6 ± 0.5	6.6 ± 0.6	45.9 ± 2.8	6 (10)	7 (14)
3c	1.1 ± 0.1	1.9 ± 0.5	95.9 ± 4.6	87 (145)	50 (101)
3d	9.9 ± 1.2	8.3 ± 0.7	160.1 ± 9.5	16 (27)	19 (38)
4a	23.5 ± 1.7	nd	21.9 ± 1.5	1 (1)	nd
4b	1.5 ± 0.2	2.3 ± 0.2	23.8 ± 1.9	16 (26)	10 (21)
4c	10.7 ± 1.1	7.7 ± 0.5	22.3 ± 2.3	2 (3)	3 (6)
4d	3.7 ± 0.1	4.1 ± 0.3	22.0 ± 1.7	6 (10)	5 (11)

Results are averages of four separate determinations. [a] IC₅₀: is the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at the concentrations employed (1, 10, 25 and 200 μM). [b] Against J774.2 macrophages after 72 h of culture. [c] Selectivity index = IC₅₀ macrophages toxicity/IC₅₀ activity on extracellular or intracellular forms of the parasite. In brackets: number of times the compound SI exceeded the reference drug SI.

Table 3. In vitro activity, toxicity and selectivity index for the 1a–f, 2a–d, 3a–d and 4a–d derivatives on extra- and intracellular forms of *Leishmania donovani*.

Products	Activity IC ₅₀ [μM] ^[a]		Toxicity in macrophages IC ₅₀ [μM] ^[b]	Selectivity ^[c]	
	Promastigote forms	Amastigote forms		Promastigote forms	Amastigote forms
Glucantime	26.6 ± 5.4	33.3 ± 1.2	15.2 ± 1.3	0.7	0.5
1a	87.6 ± 6.3	nd	305.1 ± 15.8	4 (6)	nd
1b	64.3 ± 4.7	nd	18.6 ± 1.0	0	nd
1c	57.4 ± 4.1	nd	17.1 ± 1.7	0	nd
1d	20.6 ± 2.3	nd	19.3 ± 1.1	1 (1)	nd
1e	9.6 ± 1.3	10.8 ± 1.1	63.9 ± 3.6	7 (9)	6 (12)
1f	52.0 ± 3.6	44.7 ± 5.6	210.7 ± 10.3	4 (6)	5 (10)
2a	4.4 ± 0.2	nd	18.7 ± 1.0	4 (6)	nd
2b	10.7 ± 0.4	nd	25.2 ± 1.7	2 (3)	nd
2c	22.4 ± 1.3	nd	2.4 ± 0.1	0	nd
2d	11.9 ± 0.7	nd	2.4 ± 1.3	0	nd
3a	20.4 ± 2.0	nd	65.2 ± 3.3	3 (5)	nd
3b	2.0 ± 0.4	2.7 ± 0.3	45.9 ± 2.8	23 (33)	17 (34)
3c	2.0 ± 0.6	3.4 ± 0.9	95.9 ± 4.6	48 (69)	28 (56)
3d	9.4 ± 2.7	7.3 ± 1.7	160.1 ± 9.5	17 (24)	22 (44)
4a	76.1 ± 5.3	nd	21.9 ± 1.5	0	nd
4b	3.6 ± 0.4	2.5 ± 0.0	23.8 ± 1.9	7 (9)	9 (19)
4c	7.4 ± 0.4	5.5 ± 0.3	22.3 ± 2.3	3 (4)	4 (8)
4d	3.8 ± 0.3	1.9 ± 0.0	22.0 ± 1.7	6 (8)	11 (23)

Results are averages of four separate determinations. [a] IC₅₀: is the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at the concentrations employed (1, 10, 25 and 200 μM). [b] Against J774.2 macrophages after 72 h of culture. [c] Selectivity index = IC₅₀ macrophages toxicity/IC₅₀ activity on extracellular or intracellular forms of the parasite. In brackets: number of times the compound SI exceeded the reference drug SI.

found to be more effective than BZN, which only achieved 27% inhibition on day 10. (Figure 5)

Inhibitory effect on *L. braziliensis*, *L. donovani*, *L. infantum* and *T. cruzi* Fe-SOD enzymes

Previously we had found that benzo[g]phthalazine and phthalazine derivatives containing *sp*² or *sp*³ nitrogen atoms in the side chains and other pyrazole-containing related structures were able to inhibit the activity of the Fe-SOD enzyme of three of these parasites, *T. cruzi*, *L. infantum* and *L. braziliensis*. In all

Table 4. In vitro activity, toxicity and selectivity index for the 1a-f, 2a-d, 3a-d and 4a-d derivatives on extra- and intracellular forms of *Trypanosoma cruzi* SN3 strains.

Comp.	Activity IC ₅₀ [μM] ^[a]			Vero cells ^[b] toxicity IC ₅₀ [μM]	Selectivity index ^[c]		
	Epimastigote forms	Amastigote forms	Trypomastigote forms		Epimastigote forms	Amastigote forms	Trypomastigote forms
BNZ	15.8 ± 1.1	23.3 ± 4.6	6.2 ± 0.7	13.6 ± 0.9	0.9	0.6	2.2
1a	30.6 ± 2.4	nd	nd	225.7 ± 6.1	7 (8)	nd	nd
1b	29.7 ± 1.6	nd	nd	43.6 ± 2.7	1 (2)	nd	nd
1c	30.5 ± 2.7	nd	nd	39.6 ± 0.9	1 (1)	nd	nd
1d	11.8 ± 0.6	nd	nd	33.2 ± 2.8	3 (3)	nd	nd
1e	16.5 ± 0.6	14.7 ± 1.1	15.9 ± 1.2	349.5 ± 7.7	21 (23)	24 (39)	22 (10)
1f	101.7 ± 4.1	44.3 ± 2.7	40.8 ± 3.1	156.0 ± 4.6	1 (2)	3 (6)	4 (2)
2a	33.5 ± 1.6	nd	nd	38.7 ± 1.7	1 (1)	nd	nd
2b	30.0 ± 0.8	nd	nd	35. ± 2.3	1 (1)	nd	nd
2c	20.1 ± 1.3	nd	nd	19.6 ± 0.7	1 (1)	nd	nd
2d	19.6 ± 0.8	nd	nd	23.8 ± 2.1	1 (1)	nd	nd
3a	25.9 ± 1.2	6.7 ± 0.8	4.3 ± 0.3	111.6 ± 4.8	4 (5)	17 (28)	26 (12)
3b	9.4 ± 0.7	2.4 ± 0.0	1.3 ± 0.6	121.3 ± 6.3	13 (14)	50 (84)	93 (42)
3c	9.4 ± 1.0	4.7 ± 1.0	4.0 ± 0.2	292.5 ± 7.2	31 (35)	62 (124)	73 (33)
3d	9.6 ± 0.9	8.0 ± 0.7	6.5 ± 0.8	158.8 ± 6.2	16 (18)	20 (33)	24 (11)
4a	24.6 ± 1.7	nd	nd	31.0 ± 2.6	1 (1)	nd	nd
4b	15.7 ± 0.5	nd	nd	26.8 ± 0.9	2 (2)	nd	nd
4c	17.9 ± 1.1	nd	nd	29.8 ± 1.6	2 (2)	nd	nd
4d	23.3 ± 0.7	nd	nd	37.2 ± 1.2	2 (2)	nd	nd

Results are average of four determinations. [a] IC₅₀: is the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at the concentrations employed (1, 10, 25 and 200 μM). [b] Against Vero cells after 72 h of culture. [c] Selectivity index = IC₅₀ Vero cell toxicity/IC₅₀ activity on extracellular or intracellular forms of the parasite. In brackets: number of times the compound SI exceeded the reference drug SI.

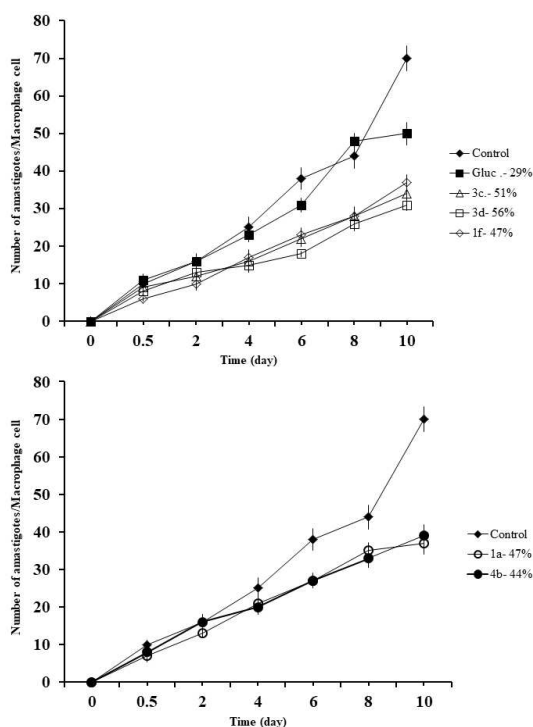


Figure 2. Effect of compounds 1a, 1f, 3c, 3d and 4b compared to reference drug Glucantime (G.) on the amastigote count per infected cell by *L. infantum*. Values are the means of three separate experiments.

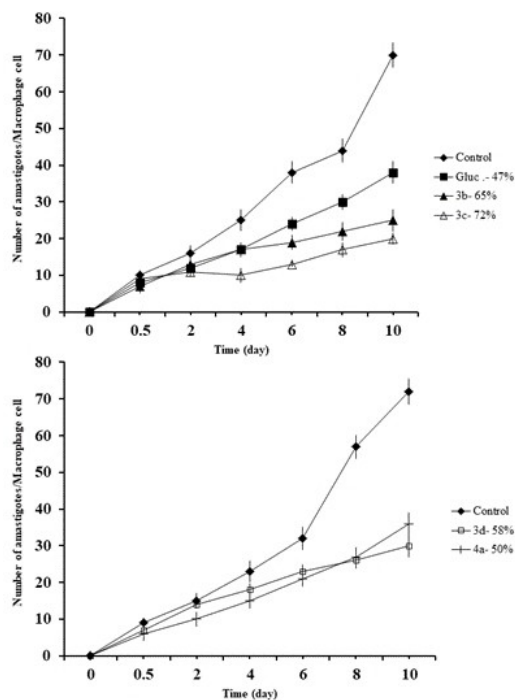


Figure 3. Effect of compounds 1a, 1f, 3c, 3d and 4b compared to reference drug Glucantime (G.) on the amastigote count per infected cell by *L. braziliensis*. Values are the means of three separate experiments.

cases, their inhibiting features were found to be related to the high complexing potentiality of the polyaminic structures assayed, since the environment of the active site could be modified by complexation.^[20–24,28]

Therefore, the effects of the compounds showing better selectivity index against each of the four parasites under study on the corresponding parasite Fe-SOD, were assayed at concentrations ranging from 1 μM to 100 μM. In each case, epimastigote (*Trypanosoma*) or promastigote (*Leishmania*)

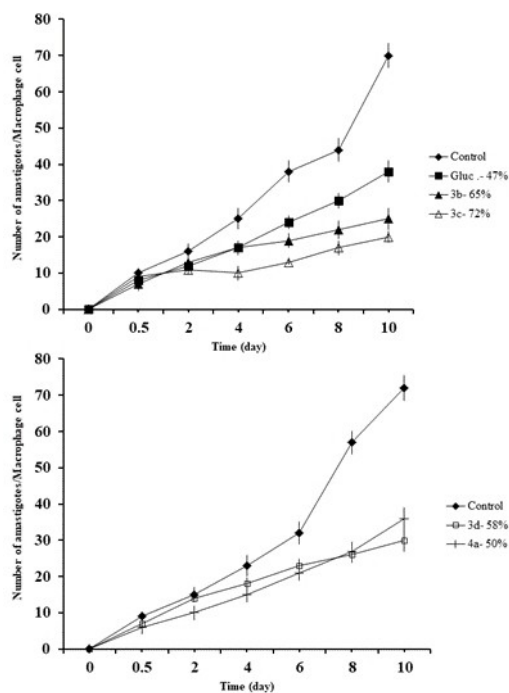


Figure 4. Effect of compounds **1a**, **1f**, **3c**, **3d** and **4d** compared to reference drug Glucantime (G.) on the amastigote count per infected cell by *L. donovani*. Values are the means of three separate experiments.

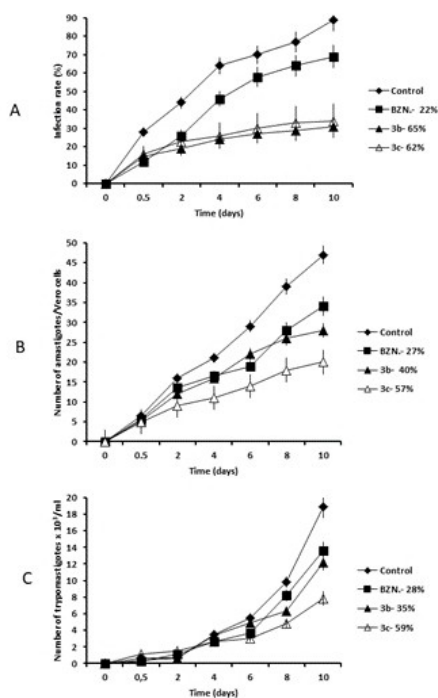


Figure 5. Effect of compounds **3b** and **3c** compared to reference drug Benznidazole (BZN) on the amastigote count per infected cell by *T. cruzi*. Values are the means of three separate experiments.

forms of the parasite, which excreted Fe-SOD when cultured in a medium lacking inactive fetal calf serum (FCS), were used.^[29]

The inhibition data obtained are shown in Figures 6–9, where the corresponding IC_{50} values are included in order to make the interpretation of results easier. For comparison, Figure 10 shows the effect of the same compounds on CuZn-SOD obtained from human erythrocytes.

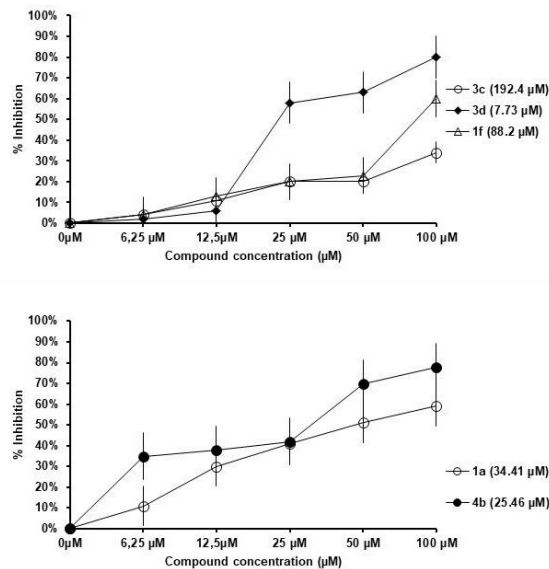


Figure 6. In vitro inhibition (%) of Fe-SOD in *L. infantum* promastigotes for compounds **1a**, **1f**, **3c**, **3d** and **4b**. Values are the average of five separate determinations. IC_{50} was calculated by linear regression analysis from the K_c values at concentrations used (6.25, 12.5, 25, 50 and 100 μ M).

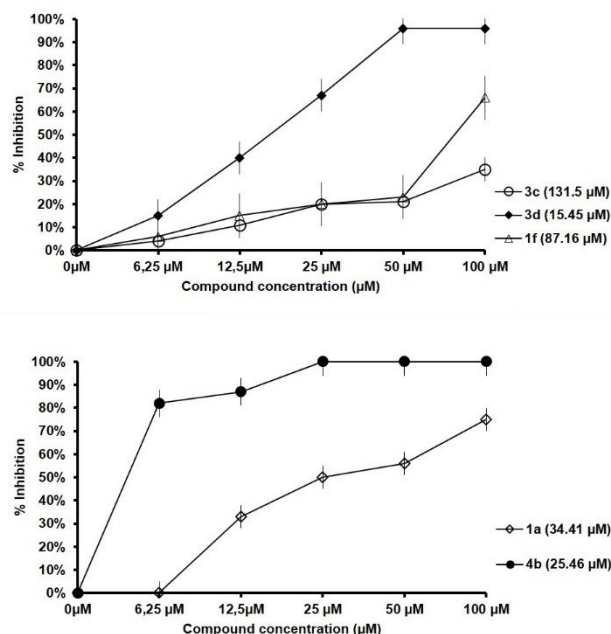


Figure 7. In vitro inhibition (%) of Fe-SOD in *L. braziliensis* promastigotes for compounds **1a**, **1f**, **3c**, **3d** and **4b**. Values are the average of five separate determinations. IC_{50} was calculated by linear regression analysis from the K_c values at concentrations used (6.25, 12.5, 25, 50 and 100 μ M).

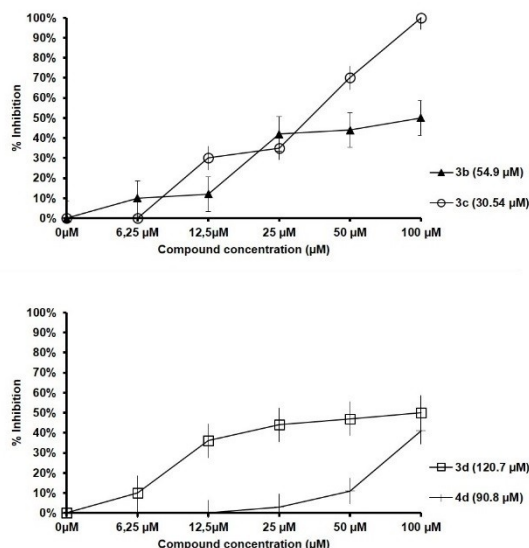


Figure 8. *In vitro* inhibition (%) of Fe-SOD in *L. donovani* promastigotes for compounds **3b**, **3c**, **3d** and **4d**. Values are the average of five separate determinations. IC₅₀ was calculated by linear regression analysis from the K_c values at concentrations used (6.25, 12.5, 25, 50 and 100 μM).

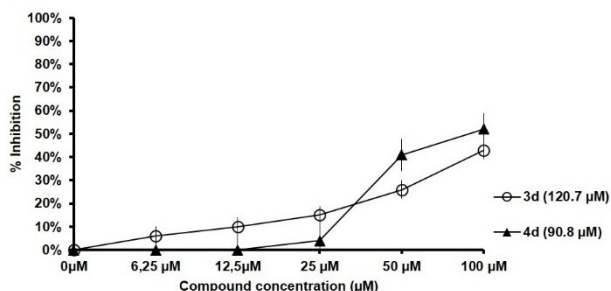


Figure 9. *In vitro* inhibition (%) of Fe-SOD in *T. cruzi* epimastigotes for compounds **3b** and **3c**. Values are the average of five separate determinations. IC₅₀ was calculated by linear regression analysis from the K_c values at concentrations used (6.25, 12.5, 25, 50 and 100 μM).

According to Figure 6, the most active compounds against *L. infantum*, **3d** and **4b**, inhibited the enzymatic activity to more than 80% extent, being especially noteworthy compound **3d** with a IC₅₀ value of 7,73 μM. Results shown in Figure 7 indicate that the same both compounds, **3d** and **4b**, inhibited the *L. braziliensis* enzyme activity at approximately 100% extent, being especially noteworthy in this case compound **4b** with a IC₅₀ value of 0,09 μM. Inhibition of *L. donovani* enzyme shown in Figure 8 shows some differences in relation to the preceding data. In this case, only compound **3c** reaches enzyme activity inhibition at 100% extent with a IC₅₀ value of 30,54 μM. Finally, data shown in Figure 9 show that, in this case, is **3b** the best compound although it reaches enzyme activity inhibition only at approximately 50% extent with a IC₅₀ value of 66,01 μM.

A simple visual comparison of graphs in Figures 6 and 9 and in Figure 10 shows that the parasitic enzyme is inhibited, while

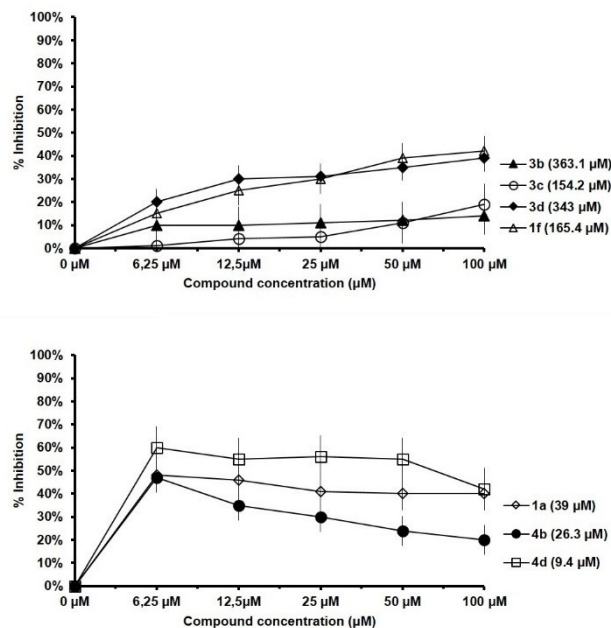


Figure 10. *In vitro* inhibition (%) of CuZn-SOD obtained from human erythrocytes for compounds **1a**, **1f**, **3b**, **3c**, **3d** and **4d**. Values are the average of five separate determinations. IC₅₀ was calculated by linear regression analysis from the K_c values at concentrations used (6.25, 12.5, 25, 50 and 100 μM).

the effect caused in CuZn-SOD is much lower in comparison. Compounds **3b** and **3d** caused the highest enzymatic inhibitions, which could explain their noteworthy *in vitro* results.

Molecular modeling

Fe-SOD enzymes are usually formed by two monomers, both of which contain a non-heme iron active site. This iron atom is coordinated by three histidine units, an aspartate group and an axial H₂O or HO⁻ ligand, in trigonal-bipyramidal structure. This structure is supported by an essential H-bonding network, comprised of amino acids and extending across the interface to the other monomer of the Fe-SOD dimer.^[27] In previous work with related phthalazine derivatives,^[22,28–32] tentative molecular modelling studies on their mode of interaction with the enzyme were performed. Some correlation between the experimental Fe-SOD inhibitory activity and theoretical perturbation of the geometry and H-bonding patterns at the enzyme core in the parasites was found, suggesting that these highly complexing polyaminic heterocycles could affect Fe-SOD performance by interacting with the H-bonding amino acid system of the iron-based moiety.^[24,28] On this basis we have modeled the interaction of compound **3d** with Leishmania Fe-SOD, as it is an especially effective compound in the inhibition of Fe-SOD.

The tertiary structures of superoxide dismutases are very similar, mainly among iron- and manganese-dependent ones. Moreover, the active site residues are conserved, suggesting a

common mechanism of action.^[29] Having this into account, the Fe-SOD enzyme structure used for our theoretical studies was obtained from the Brookhaven protein data bank (entry 4F2N corresponding to *L. major*, as it is the only published structure for a *Leishmania* species^[33]). The Amber force field implemented in Hyperchem 8,^[34] following the methodology specified in the Experimental Section, have been used as in our previous studies. The active site of the enzyme, with the H-bonding system outlined above displayed as dashed lines and the solvent ligand in the foreground linked to the iron atom, is shown in Figure 11A. The key interactions with Gln69 and Asp156 are also clearly seen. Each compound was then buried into one monomer of the Fe-SOD with the imidazole ring pointing towards the metal ion, in a slow motion to prevent the global structure distortion, and the energetically most favored disposition was identified in each case (Figure 11B for **3d** as an example). As a consequence of the approach of the molecule, the amino acids pattern surrounding the iron atom was distorted, being displaced apart from their initial positions and so the H-bonding pattern appeared remarkably changed. It is noteworthy to point out that a quite similar distortion could be obtained when the pyrimidine ring also present in **3d** pointed towards the metal ion (Figure 11C).

Experimental Section

Chemistry. The starting amines and chloro derivatives 2-(1*H*-imidazol-4-yl)ethylamine (histamine), 3-(1*H*-imidazol-1-yl)propylamine, *N*-(3-aminopropyl)imidazole, 2-(1*H*-benzo[*d*]

imidazol-2-yl)ethylamine, 2-(imidazo[1,2-*a*]pyridin-2-yl)ethylamine, 4-(1*H*-imidazol-1-yl)butylamine and 5-(1*H*-imidazol-1-yl)pentylamine, 3-chloropyridazine, 3,6-dichloropyridazine, 1-chlorophthalazine, 4-chloroquinazoline, 2-chloropyrimidine and 2-chloropyrazine were purchased from Sigma-Aldrich and used without purification. 1,4-Dichlorophthalazine was obtained from commercial phthalhydrazide (Sigma-Aldrich) following a standard method.^[35] Solvents were dried using standard techniques.^[36] All reactions were monitored using thin layer chromatography (TLC) on precoated aluminum sheets of silica gel 60PF₂₅₄ (Merck, layer thickness 0.2 mm). Compounds were detected with UV light (254 nm). Chromatographic separations were performed on columns in the indicated solvent system using flash chromatography on silica gel (particle size 0.040–0.063 mm). Melting points were determined in a Gallenkamp apparatus and were uncorrected. ¹H NMR spectra were recorded on a Bruker 300 and a Variant XL 300 a 300 MHz, and ¹³C NMR spectra were recorded at 75 MHz at room temperature employing DMSO-*d*₆ as the solvent. Chemical shifts were reported in ppm (δ scale) from tetramethylsilane (TMS). All assignments were performed on the basis of ¹H-¹³C heteronuclear multiple quantum coherence experiments (gHMQC). IR spectra were recorded on a Perkin-Elmer 257 spectrometer (4000–400 cm⁻¹ range). Electrospray mass spectra were recorded with a Hewlett-Packard 1100 MSD apparatus. Elemental analyses were performed in a Perkin-Elmer 2400-CHN instrument by CAI of Microanalysis, Universidad Complutense, Madrid, Spain. Elemental analysis was used to ascertain a purity higher than 95 % for all the biologically tested compounds.

Synthesis of 1a–b, 1e–f, 3a–d and 4a. A solution of the corresponding monochloroazine or monochlorobenzoazine, the corresponding aliphatic amine and trimethylamine in the adequate solvent was heated at 100–120 °C for several hours. The reaction mixture was cooled to room temperature and the solvent removed under reduced pressure. The residue was purified by column

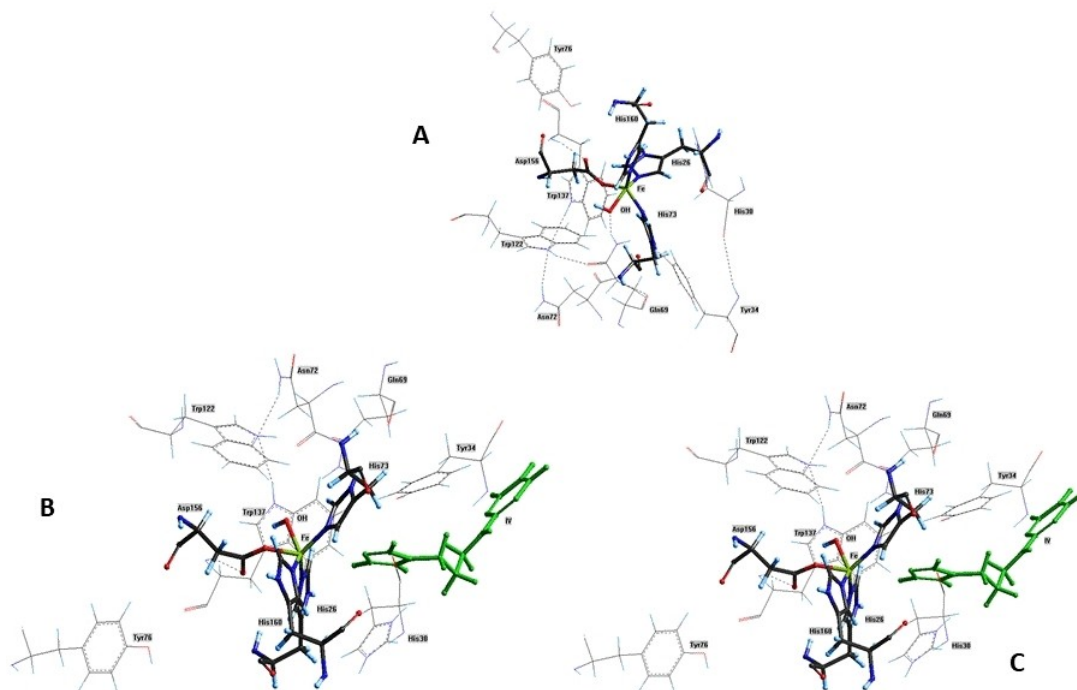


Figure 11. Molecular models of the free *Leishmania* Fe-SOD enzyme active site showing the supporting network of hydrogen bonds (A) and the same active site when compound **3d** is embedded in the proximity of the iron atom. Modification of the hydrogen-bonding network can be seen both when approaching through pyrazole ring (B) and through pyrimidine ring (C).

chromatography with the adequate eluent to obtain the desired product.

1-[2-(1*H*-imidazol-4-yl)ethylamino]pyridazine (1 a). A solution of 3-chloropyridazine (0.4 g, 3.49 mmol), 2-(1*H*-imidazol-4-yl)ethylamine (0.7 g, 6.98 mmol), and triethylamine (0.44 mL) in 1 mL of ethanol absolute was heated in a reactor during 25 h at 100–120 °C. After cooling to room temperature, the solvent removed under reduced pressure. The residue was purified by flash column chromatography (chloroform/ethanol 2:1). From the fraction with $R_f=0.11$, was isolated 160 mg (24%) of a yellow solid which and identified as **1 a**, mp 208–210 °C. IR (KBr) ν : 3259, 3100–2500, 1600–1500 cm^{-1} . $^1\text{H-NMR}$ (DMSO- d_6) δ : 8.41 (dd, 1H, H-6), 7.55 (s, 1H, H-2'), 7.22 (dd, 1H, H-5), 6.88 (t, 1H, NH), 6.88 (s, 1H, H-5'), 6.78 (dd, 1H, H-4), 3.56 (m, 2H, H α), 2.88 (t, 2H, H β) ppm. $^{13}\text{C-NMR}$ (DMSO- d_6) δ : 158.8 (C-3), 142.7 (C-6), 134.3 (C-2'), 131.1 (C-4'), 127.0 (C-5), 116.8 (C-5'), 114.1 (C-4), 40.5 (C α), 25.7 (C β) ppm. ESI-MS m/z (%): 190 (MH $^+$, 100). Anal. (C $_9$ H $_{11}$ N $_5$ ·0.25H $_2$ O) C, H, N.

1-[3-(imidazol-1-yl)propylamino]pyridazine (1 b). A solution of 3-chloropyridazine (0.2 g, 1.75 mmol), 3-(1*H*-imidazol-1-yl)propylamine (0.4 g, 3.35 mmol), and triethylamine (0.3 mL) in 0.4 mL of ethanol absolute was heated in a reactor during 24 h. at 100–120 °C. After cooling to room temperature, the solvent removed under reduced pressure. The residue was purified by flash column chromatography (dichloromethane/ethanol 2:1). From the fraction with $R_f=0.15$, was isolated 250 mg (70%) of a yellow solid which and identified as **1 b**, mp 79–83 °C. IR (KBr) ν : 3258, 2921, 1600–1500 cm^{-1} . $^1\text{H-NMR}$ (DMSO- d_6) δ : 8.42 (dd, 1H, H-6), 7.68 (s, 1H, H-2'), 7.26 (d, 1H, H-5), 7.23 (d, 1H, H-5'), 6.91 (s, 1H, H-4'), 6.79 (d, 1H, H-4), 4.07 (t, 2H, H γ), 3.28 (t, 2H, H α), 2.01 (m, 2H, H β) ppm. $^{13}\text{C-NMR}$ (DMSO- d_6) δ : 158.9 (C-3), 142.8 (C-6), 137.3 (C-2'), 128.3 (C-5), 127.1 (C-4'), 119.4 (C-5'), 114.2 (C-4), 43.4 (C γ), 37.5 (C α), 30.0 (C β) ppm. ESI-MS m/z (%): 226 (M $^+$ + Na, 17), 204 (MH $^+$, 94). Anal. (C $_{10}$ H $_{13}$ N $_5$ ·0.25H $_2$ O) C, H, N.

1-[2-(1*H*-imidazol-4-yl)ethylamino]phthalazine (1 e). A solution of 1-chlorophthalazine (0.5 g, 3.04 mmol), 2-(1*H*-imidazol-4-yl)ethylamine (0.67 g, 6.08 mmol), and triethylamine (1 mL) in 20 mL of xylene was heated at 120 °C during 20 h under atmosphere of argon. After cooling to room temperature, the solvent removed under reduced pressure. The solid obtained was purified by flash column chromatography (chloroform/ethanol 2:1). From the fraction with $R_f=0.13$, was isolated 280 mg (38%) of a white solid which and identified as **1 e**, mp 207–213 °C. IR (ATR) ν : 3205, 2927, 1600–1500 cm^{-1} . $^1\text{H-NMR}$ (DMSO- d_6) δ : 8.90 (s, 1H, H-4), 8.25 (m, 1H, H-8), 7.92 (m, 1H, H-5), 7.87 (m, 1H, H-6), 7.86 (m, 1H, H-7), 7.60 (t, 1H, NH), 7.59 (s, 1H, H-2'), 6.87 (s, 1H, H-5'), 3.78 (t, 2H, H α), 2.93 (t, 2H, H β) ppm. $^{13}\text{C-NMR}$ (DMSO- d_6) δ : 153.8 (C-1), 143.6 (C-4), 135.1 (C-2'), 132.1 (C-6), 132.0 (C-4'), 131.7 (C-7), 127.2 (C-4a), 126.7 (C-5), 122.2 (C-8), 118.1 (C-8a), 117.3 (C-5'), 41.7 (C α), 26.7 (C β) ppm. ESI-MS m/z (%): 262 (M $^+$ + Na, 4), 240 (MH $^+$, 100). Anal. (C $_{13}$ H $_{13}$ N $_5$ ·0.25H $_2$ O) C, H, N.

1-[3-(imidazol-1-yl)propylamino]phthalazine (1 f). A solution of 1-chlorophthalazine (0.5 g, 3.04 mmol), 3-(1*H*-imidazol-1-yl)propylamine (1 g, 6.08 mmol), and triethylamine (0.7 mL) in 20 mL of xylene was heated at 130 °C during 24 h. After cooling to room temperature, the solvent removed under reduced pressure. The yellow solid was purified by flash column chromatography (chloroform/ethanol 2:1). From the fraction with $R_f=0.16$, was isolated 510 mg (66%) of a white solid which and identified as **1 f**, mp 170–174 °C. IR (ATR) ν : 3265, 2927, 1600–1500 cm^{-1} . $^1\text{H-NMR}$ (DMSO- d_6) δ : 8.87 (s, 1H, H-4), 8.28 (m, 1H, H-8), 7.92 (m, 1H, H-5), 7.87 (m, 2H, H-6, H-7), 7.70 (s, 1H, H-2'), 7.47 (t, 1H, NH), 7.25 (s, 1H, H-5'), 6.91 (s, 1H, H-4'), 4.10 (t, 2H, H γ), 3.53 (m, 2H, H α), 2.15 (m, 2H, H β) ppm. $^{13}\text{C-NMR}$ (DMSO- d_6) δ : 154.1 (C-1), 143.7 (C-4), 137.2 (C-2'), 131.8 (C-6, C-7), 128.8 (C-4'), 127.3 (C-4a), 126.7 (C-5), 122.1 (C-8), 120.0 (C-5'),

118.2 (C-8a), 44.5 (C γ), 38.6 (C α), 31.3 (C β) ppm. ESI-MS (MeOH) m/z (%): 276 (M $^+$ + Na, 53), 254 (MH $^+$, 100), 186 (M $^+$ - 67, 22). Anal. (C $_{14}$ H $_{15}$ N $_5$) C, H, N.

N-[2-(1*H*-imidazol-4-yl)ethyl]-quinazolin-4-amine (3 a). A solution of 4-chloroquinazoline (0.5 g, 3.04 mmol), 2-(1*H*-imidazol-4-yl)ethylamine (0.68 g, 6.08 mmol), and triethylamine (0.4 mL) in 25 mL of xylene was heated at 110–120 °C during 20 h. After cooling to room temperature, the solvent removed under reduced pressure. The residue was purified by flash column chromatography (chloroform/ethanol 2:1). From the fraction with $R_f=0.19$ was isolated 595 mg (82%) of a white solid which was identified as **3 a**, mp 207–208 °C. IR (KBr) ν : 3267, 3108, 2877, 1575, 1524, 1366, 1082 cm^{-1} . $^1\text{H-NMR}$ (MeOD- d_4) δ : 8.47 (s, 1H, H-2), 8.10 (dd, 1H, H-8), 7.80 (m, 1H, H-7), 7.72 (dd, 1H, H-5), 7.63 (s, 1H, H-2'), 7.54 (m, 1H, H-6), 6.90 (s, 1H, H-5'), 3.88 (t, 2H, H α), 3.04 (t, 2H, H β) ppm. $^{13}\text{C-NMR}$ (MeOD- d_4) δ : 160.0 (C-4), 154.6 (C-2), 148.2 (C-8a), 134.9 (C-4'), 134.6 (C-2'), 132.8 (C-7), 126.2 (C-5), 126.0 (C-6), 122.0 (C-8), 116.5 (C-5'), 115.1 (C-4a), 40.9 (C α), 25.9 (C β) ppm. ESI-MS (MeOH) m/z (%): 262 (M $^+$ + Na, 100), 240 (M $^+$ + H, 56). Anal. (C $_{13}$ H $_{13}$ N $_5$ ·0.25 H $_2$ O) C, H, N.

N-[3-(1*H*-imidazol-1-yl)propyl]quinazolin-4-amine (3 b). A solution of 4-chloroquinazoline (0.5 g, 3.04 mmol), 3-(1*H*-imidazol-1-yl)propylamine (0.76 g, 6.08 mmol), and triethylamine (0.4 mL) in 25 mL of xylene was heated at 110–120 °C during 20 h. After cooling to room temperature, the solvent removed under reduced pressure. The residue was purified by flash column chromatography (chloroform/ethanol 2:1). From the fraction with $R_f=0.33$ was isolated as 469 mg (61%) of a yellow solid which was identified as **3 b**, mp 163–165 °C. IR (MeOH) ν : 3300–2900, 1618, 1584, 1542, 1445, 770 cm^{-1} . $^1\text{H-NMR}$ (MeOD- d_4) δ : 8.48 (s, 1H, H-2), 8.11 (dd, 1H, H-8), 7.81 (m, 1H, H-7), 7.74 (s, 1H, H-2'), 7.73 (dd, 1H, H-5), 7.56 (m, 1H, H-6), 7.21 (s, 1H, H-5'), 6.99 (s, 1H, H-4'), 4.19 (t, 2H, H γ), 3.67 (t, 2H, H α), 2.26 (m, 2H, H β) ppm. $^{13}\text{C-NMR}$ (MeOD- d_4) δ : 160.2 (C-4), 154.6 (C-2), 148.2 (C-8a), 137.1 (C-2'), 132.8 (C-7), 127.7 (C-4'), 126.3 (C-5), 126.1 (C-6), 122.0 (C-8), 119.2 (C-5'), 115.0 (C-4a), 44.4 (C γ), 38.0 (C α), 30.0 (C β) ppm. ESI-MS (negative mode, MeOH) m/z (%): 252 (M $^+$ - H, 100). Anal. (C $_{14}$ H $_{15}$ N $_5$) C, H, N.

2-[2-(1*H*-imidazol-4-yl)ethyl]pyrimidine (3 c). A solution the 2-chloropyrimidine (0.52 g, 4.54 mmol), 2-(1*H*-imidazol-4-yl)ethylamine (1.00 g, 9.08 mmol), and triethylamine (1.26 mL) in 30 mL of xylene was heated at 110 °C during 24 h. After cooling to room temperature, the solvent removed under reduced pressure. The residue was purified by flash column chromatography (chloroform/methanol 8:1). The fraction of $R_f=0.4$ afforded 0.52 g (60%) of a solid which was identified as **3 c**, mp 119–123 °C. IR (KBr) ν : 3266, 2925, 1591, 1534, 800 cm^{-1} . $^1\text{H-NMR}$ (DMSO- d_6) δ : 8.28 (d, 2H, H-4, H-6), 7.55 (d, 1H, H-2'), 7.19 (t, 1H, NH), 6.82 (s, 1H, H-5'), 6.56 (t, 1H, H-5), 3.49 (m, 2H, H α), 2.76 (t, 2H, H β) ppm. $^{13}\text{C-NMR}$ (DMSO- d_6) δ : 162.2 (C-2), 157.9 (C-4, C-6), 136.8 (C-2'), 134.5 (C-4'), 116.7 (C-5'), 109.9 (C-5), 40.9 (C α), 26.7 (C β) ppm. ESI-MS (MeOH) m/z (%): 401 (2M $^+$ + Na, 99), 212 (M $^+$ + Na, 100), 190 (M $^+$, 98). Anal. (C $_9$ H $_{11}$ N $_5$) C, H, N.

2-[3-(1*H*-imidazol-1-yl)propyl]pyrimidine (3 d). A solution the 2-chloropyrimidine (0.5 g, 4.36 mmol), *N*-(3-aminopropyl)imidazole (1.09 g, 8.72 mmol), and triethylamine (1.21 mL) in 30 mL of xylene was heated at 110 °C during 24 h. After cooling to room temperature, the solvent removed under reduced pressure. The residue was purified by flash column chromatography (chloroform/methanol 8:2). The fraction of $R_f=0.55$ afforded 0.64 g (72%) of a solid which was identified as **3 d**, mp 77–80 °C. IR (KBr) ν : 3272, 2945, 1586, 1532, 800 cm^{-1} . $^1\text{H-NMR}$ (DMSO- d_6) δ : 8.28 (d, 2H, H-4, H-6), 7.66 (d, 1H, H-2'), 7.21 (s, 1H, H-5'), 6.90 (s, 1H, H-4'), 6.57 (t, 1H, H-5), 4.04 (t, 2H, H γ), 3.22 (t, 2H, H α), 1.96 (m, 2H, H β) ppm. $^{13}\text{C-NMR}$ (DMSO- d_6) δ : 163.6 (C-2), 159.3 (C-4, C-6), 138.5 (C-2'), 129.1 (C-4'), 120.6 (C-5'), 111.5 (C-5), 45.6 (C γ), 39.2 (C α), 31.9 (C β) ppm. ESI-MS

(MeOH) m/z (%): 429 ($2M^+ + Na$, 100), 226 ($M^+ + Na$, 99), 204 (M^+ , 65). Anal. ($C_{10}H_{13}N_5$) C, H, N.

***N*-[2-(1*H*-imidazol-4-yl)ethylamino]pyrazine (4a).** A solution of 2-chloropyrazine (0.4 g, 3.4 mmol), 2-(1*H*-imidazol-4-yl)ethylamine (0.5 g, 4.5 mmol) and triethylamine (0.5 mL) in 0.5 mL of ethanol absolute was heated in a reactor during 24 h. at 115–120 °C. After cooling to room temperature, the solvent removed under reduced pressure. The residue was purified by flash column chromatography (chloroform/methanol 8:2). From the fraction with $R_f=0.17$, was isolated 280 mg (49%) of a solid which and identified as **4a**, mp 156–157 °C. IR (CHCl₃) ν : 3500–3000, 2924, 1593, 1523, 995, 819 cm⁻¹. ¹H-NMR (MeOD-*d*₄) δ : 7.96 (m, 1H, H-6), 7.87 (dd, 1H, H-5), 7.63 (dd, 1H, H-3), 7.61 (m, 1H, H-2'), 6.88 (bs, 1H, H-5'), 3.61 (t, 2H, H α), 2.90 (t, 2H, H β) ppm. ¹³C-NMR (MeOD-*d*₄) δ : 156.83 (C-2), 143.06 (C-6), 136.23 (C-2'), 136.05 (C-4'), 134.42 (C-3), 131.43 (C-5), 118.15 (C-5'), 41.85 (C α), 27.61 (C β) ppm. ESI-MS (MeOH) m/z (%): 188 ($M^+ - 1$) (100), 138 (30), 137 (74). Anal. ($C_9H_{11}N_5 \cdot 0.25CH_3OH$) C, H, N.

Synthesis of 4b–d. A solution of the corresponding monochloroazine and the corresponding aliphatic amine in absolute ethanol was heated at 120 °C for three hours in a reactor. The reaction mixture was cooled to room temperature and the solvent removed under reduced pressure. The residue was purified by column chromatography with chloroform/methanol 8:2 as eluent to obtain the desired product.

***N*-[3-(1*H*-imidazol-1-yl)propyl]pyrazin-2-amine (4b).** A solution of 2-chloropyrazine (0.5 g, 4.4 mmol), 3-(1*H*-imidazol-1-yl)propylamine (1.38 g, 11 mmol) in 0.7 mL of ethanol absolute was heated in a reactor during 3 h. at 120 °C. After cooling to room temperature, the solvent removed under reduced pressure. The residue was purified by flash column chromatography (chloroform/methanol 8:2). From the fraction with $R_f=0.61$, was isolated 674 mg (75%) of a dark brown oil which and identified as **4b**. IR (CHCl₃) ν : 3300–3100, 1671, 1594, 1515, 661 cm⁻¹. ¹H-NMR (MeOD-*d*₄) δ : 7.97 (m, 1H, H-6), 7.89 (dd, 1H, H-5), 7.70 (bs, 1H, H-2'), 7.66 (dd, 1H, H-3), 7.19 (bs, 1H, H-5'), 7.01 (bs, 1H, H-4'), 4.18 (t, 2H, H γ), 3.34 (t, 2H, H α), 2.13 (m, 2H, H β) ppm. ¹³C-NMR (MeOD-*d*₄) δ : 156.9 (C-2), 143.1 (C-6), 138.5 (C-2'), 134.5 (C-5), 131.7 (C-3), 129.1 (C-4'), 120.6 (C-5'), 45.6 (C γ), 38.7 (C α), 31.6 (C β) ppm. ESI-MS (MeOH) m/z (%): 204 ($M^+ + 1$) (100), 136 (42). Anal. ($C_{10}H_{13}N_5 \cdot 1H_2O$) C, H, N.

***N*-[4-(1*H*-imidazol-1-yl)butyl]pyrazin-2-amine (4c).** A solution of 2-chloropyrazine (0.17 g, 1.4 mmol), 4-(1*H*-imidazol-1-yl)butylamine (0.5 g, 3.6 mmol) in 0.21 mL of ethanol absolute was heated in a reactor during 3 h. at 120 °C. After cooling to room temperature, the solvent removed under reduced pressure. The residue was purified by flash column chromatography (chloroform/methanol 8:2). From the fraction with $R_f=0.39$, was isolated 192 mg (62%) of a light dense oil which and identified as **4c**. IR (CHCl₃) ν : 3300–3000, 1668, 1593, 1521, 663 cm⁻¹. ¹H-NMR (MeOD-*d*₄) δ : 7.81 (m, 1H, H-6), 7.73 (m, 1H, H-5), 7.56 (bs, 1H, H-2'), 7.50 (m, 1H, H-3), 7.03 (bs, 1H, H-5'), 6.86 (bs, 1H, H-4'), 3.97 (t, 2H, H δ), 3.25 (t, 2H, H α), 1.78 (m, 2H, H γ), 1.48 (m, 2H, H β) ppm. ¹³C-NMR (MeOD-*d*₄) δ : 156.9 (C-2), 143.0 (C-6), 140.2 (C-2'), 134.5 (C-5), 131.4 (C-3), 129.0 (C-4'), 120.4 (C-5'), 47.7 (C δ), 41.1 (C α), 29.6 (C γ), 27.1 (C β) ppm. ESI-MS (MeOH) m/z (%): 218 ($M^+ + 1$, 100), 168 (19). Anal. ($C_{11}H_{15}N_5 \cdot H_2O$) C, H, N.

***N*-[5-(1*H*-imidazol-1-yl)pentyl]pyrazin-2-amine (4d).** A solution of 2-chloropyrazine (0.25 g, 2.2 mmol), 5-(1*H*-imidazol-1-yl)pentylamine (0.5 g, 3.3 mmol) in 0.32 mL of ethanol absolute was heated in a reactor during 3 h. at 120 °C. After cooling to room temperature, the solvent removed under reduced pressure. The residue was purified by flash column chromatography (chloroform/methanol 8:2). From the fraction with $R_f=0.47$, was isolated

373 mg (74%) of a dark oil which and identified as **4d**. IR (CHCl₃) ν : 3200–3000, 2941, 1668, 1593, 1510, 1215, 663 cm⁻¹. ¹H-NMR (MeOD-*d*₄) δ : 7.94 (m, 1H, H-6), 7.86 (m, 1H, H-5), 7.67 (bs, 1H, H-2'), 7.63 (dd, 1H, H-3), 7.15 (bs, 1H, H-5'), 6.98 (bs, 1H, H-4'), 4.07 (t, 2H, H ϵ), 3.34 (t, 2H, H α), 1.87 (m, 2H, H δ), 1.67 (m, 2H, H β), 1.41 (m, 2H, H γ) ppm. ¹³C-NMR (MeOD-*d*₄) δ : 156.9 (C-2), 142.9 (C-6), 138.4 (C-2'), 134.4 (C-5), 131.2 (C-3), 128.9 (C-4'), 120.6 (C-5'), 47.9 (C ϵ), 41.5 (C α), 31.9 (C β), 29.7 (C δ), 24.9 (C γ) ppm. ESI-MS (MeOH) m/z (%): 254 ($M^+ + Na$, 32), 232 ($M^+ + 1$, 100). Anal. ($C_{12}H_{17}N_5 \cdot 0.25H_2O$) C, H, N.

Synthesis of compounds 1c–d. A solution of the dichloropyridazine, the corresponding aliphatic amine and trimethylamine in xylene was heated at 110 °C for several hours. The reaction mixture was cooled to room temperature and the solvent removed under reduced pressure. The residue was purified by column chromatography with the adequate eluent to obtain the desired product

***N*-[2-(1*H*-imidazol-4-yl)ethyl]-6-chloropyridazin-3-amine (1c).** A solution the 3,6-dichloropyridazine (0.3 g, 2.0 mmol), 2-(1*H*-imidazol-4-yl)ethylamine (0.48 g, 4.4 mmol), and triethylamine (0.55 mL) in 30 mL of xylene was heated at 110 °C during 24 h. After cooling to room temperature, the solvent removed under reduced pressure. The residue was purified by flash column chromatography (chloroform/methanol 8:2). The fraction of $R_f=0.55$ afforded 175 mg (38%) of a solid which was identified as **1c**, mp 191–193 °C. IR (KBr) ν : 3268–2930, 1664, 1603, 1462, 837 cm⁻¹. ¹H-NMR (DMSO-*d*₆) δ : 7.56 (s, 1H, H-2'), 7.36 (d, H, H-4), 7.19 (t, 1H, NH), 6.91 (d, 1H, H-5), 6.84 (s, 1H, H-5'), 3.55 (m, 2H, H α), 2.80 (t, 2H, H β) ppm. ¹³C NMR (DMSO-*d*₆) δ : 158.3 (C-3), 145.0 (C-6), 134.5 (C-2'), 128.9 (C-4'), 128.5 (C-4), 118.2 (C-5), 116.9 (C-5'), 40.9 (C α), 26.3 (C β) ppm. ESI-MS (MeOH) m/z (%): 246 ($M^+ + Na$, 3), 224 (M^+ , 100). Anal. ($C_9H_{11}N_5Cl$) C, H, N.

***N*-[3-(1*H*-imidazol-1-yl)propyl]-6-chloropyridazin-3-amine (1d).** A solution the 3,6-dichloropyridazine (0.5 g, 2.0 mmol), *N*-(3-amino-propyl)imidazole (1.27 g, 10.2 mmol), and triethylamine (1.0 mL) in 30 mL of xylene was heated at 110 °C during 3 h. After cooling to room temperature, the solvent removed under reduced pressure. The residue was purified by flash column chromatography (chloroform/methanol 8:2). The fraction of $R_f=0.37$ afforded 668 mg (83%) of a solid which was identified as **1d**, mp 109–111 °C. IR (KBr) ν : 3241–2928, 1600, 1504, 827 cm⁻¹. ¹H-NMR (DMSO-*d*₆) δ : 7.70 (s, 1H, H-2'), 7.37 (d, H, H-4), 7.25 (s, 1H, H-5'), 7.24 (s, 1H, H-4'), 6.95 (s, 1H, NH), 6.91 (d, 1H, H-5), 4.07 (t, 2H, H γ), 3.28 (t, 2H, H α), 2.02 (m, 2H, H β) ppm. ¹³C NMR (MeOD-*d*₄) δ : 158.3 (C-3), 145.7 (C-6), 137.1 (C-2'), 129.0 (C-4), 127.7 (C-5'), 119.3 (C-4'), 119.1 (C-5), 44.2 (C γ), 38.1 (C α), 29.9 (C β) ppm. ESI-MS (MeOH) m/z (%): 260 ($M^+ + Na$, 10), 238 (M^+ , 100). Anal. ($C_{10}H_{12}N_5Cl$) C, H, N.

Synthesis of compounds 2a–b. A solution of the dichlorophthalazine, the corresponding aliphatic amine and trimethylamine in absolute ethanol was heated at 100 °C for 24 hours in a reactor. The reaction mixture was cooled to room temperature and the solvent removed under reduced pressure. The residue was purified by column chromatography with the adequate eluent to obtain the desired products

***N*1,*N*4-bis[2-(1*H*-benzo[*d*]imidazol-2-yl)ethyl]phthalazine-1,4-diamine (2a) and *N*-[2-(1*H*-benzo[*d*]imidazol-2-yl)ethyl]-4-chlorophthalazin-1-amine (2b).** The reactive mixture 1,4-dichlorophthalazine (1.38 g, 6.94 mmol), 2-(1*H*-benzo[*d*]imidazol-2-yl)ethylamine (0.56 g, 3.47 mmol), triethylamine (1.44 mL) in 1.5 mL ethanol absolute was heated in a reactor during 24 h. at 100 °C. The residue was purified by flash column chromatography (chloroform/methanol 8:2). The fraction of $R_f=0.12$ afforded 78 mg (5%) of a solid which was identified as **2a**, mp 214–216 °C. IR (KBr) ν : 3324–2634, 2917, 2400, 1534, 767 cm⁻¹. ¹H NMR (MeOD-*d*₄) δ : 7.92 (m, 2H, H-5, H-8), 7.67 (m, 2H, H-6, H-7), 7.38 (m, 4H, H-4', H-7'), 7.07 (m, 4H, H-5',

H-6'), 3.85 (t, 4H, H α), 3.24 (m, 4H, H β) ppm. ¹³C-NMR (MeOD-*d*₄) δ : 155.1 (C-1, C-4), 150.8 (C-2'), 139.7 (C-3a', C-7a'), 132.8 (C-6, C-7), 123.7 (C-5', C-6'), 123.3 (C-5, C-8), 122.5 (C-4a, C-8a), 115.4 (C-4', C-7'), 41.7 (C α), 29.61 (C β) ppm. ESI-MS (MeOH) *m/z* (%): 449 (M⁺, 92), 471 (100). Anal. (C₂₆H₂₄N₈·2H₂O) C, H, N. The other fraction of R_f = 0.46 afforded 198 mg (16%) of a brownish oil which and identified as **2b**. IR (KBr) ν : 3019, 2400, 1580, 1400, 742, 667 cm⁻¹. ¹H-NMR (MeOD-*d*₄) δ : 9.50 (s, 1H, NH), 8.04 (m, 2H, H-5, H-8), 7.83 (m, 2H, H-6, H-7), 7.38 (m, 2H, H-4', H-7'), 7.09 (m, 2H, H-5', H-6'), 3.85 (t, 2H, H α), 3.24 (t, 2H, H β) ppm. ¹³C-NMR (MeOD-*d*₄) δ : 154.4 (C-1), 153.2 (C-2'), 145.2 (C-4), 137.9 (C-3a'), 137.9 (C-7a'), 132.7 (C-6), 132.7 (C-7), 127.9 (C-4a), 126.0 (C-8a), 122.1 (C-8), 120.7 (C-5', C-6'), 114.0 (C-4', C-7'), 40.1 (C α), 27.9 (C β) ppm. ESI-MS (MeOH) *m/z* (%): 323 (M⁺, 34), 321 (100). Anal. (C₁₇H₁₄N₅Cl·HCl·0.25H₂O) C, H, N.

Synthesis of compounds 2c–d. A solution of the dichlorophthalazine, the corresponding aliphatic amine and trimethylamine in xylene was heated at 100–130 °C for 48 hours in a reactor. The reaction mixture was cooled to room temperature and the solvent removed under reduced pressure. The residue was purified by column chromatography with the adequate eluent to obtain the desired products

N1,N4-bis(2-(imidazo[1,2-*a*]pyridin-2-yl)ethyl)phthalazine-1,4-diamine (2c) and 4-chloro-N-(2-(imidazo[1,2-*a*]pyridin-2-yl)ethyl)phthalazin-1-amine (2d). A solution of the 1,4-dichlorophthalazine (0.732 g, 3.68 mmol), 2-(imidazo[1,2-*a*]pyridin-2-yl)ethylamine (1.20 g, 7.36 mmol), in 50 mL of xylene was heated at 120–130 °C during 48 h. After cooling to room temperature, the solvent removed under reduced pressure. The residue was purified by flash column chromatography (chloroform/methanol 8:2). The fraction of R_f (Cl₃CH/MeOH/NH₄OH) = 0.52 afforded 145 mg (9%) of a solid which was identified as **2c**, mp 96–98 °C. IR (KBr) ν : 3019 1586, 1543, 745 cm⁻¹. ¹H-NMR (MeOD-*d*₄) δ : 8.40 (d, 2H, H-5'), 8.36 (m, 2H, H-5, H-8), 8.05 (m, 2H, H-6, H-7), 7.76 (s, 2H, H-3'), 7.53 (d, 4H, H-8'), 7.33 (t, 2H, H-7'), 6.92 (t, 2H, H-6'), 3.89 (t, 4H, H α), 3.25 (t, 4H, H β) ppm. ¹³C-NMR (MeOD-*d*₄) δ : 155.1 (C-1, C-4), 150.8 (C-2'), 139.6 (C-3a', C-7a'), 132.8 (C-6, C-7), 123.7 (C-5', C-6'), 123.3 (C-5, C-8), 122.5 (C-4a, C-8a), 115.4 (C-4', C-7'), 43.0 (C α), 28.1 (C β) ppm. MS-ESI (MeOH) *m/z* (%): 449 (M⁺, 100), 471 (10). Anal. (C₂₆H₂₄N₈·2HCl·4H₂O) C, H, N. The other fraction of R_f = 0.86 afforded 350 mg (43%) of **2d**, mp 144–146 °C. IR (KBr) ν : 3019, 1578, 1482, 744, 667 cm⁻¹. ¹H-NMR (MeOD-*d*₄) δ : 8.20 (d, 1H, H-5'), 8.06 (m, 1H, H-5), 8.00 (m, 1H, H-8), 7.81 (m, 1H, H-6), 7.79 (m, 1H, H-7), 7.56 (s, 1H, H-3'), 7.35 (d, 1H, H-8'), 7.14 (t, 1H, H-7'), 6.73 (t, 1H, H-6'), 3.84 (t, 2H, H α), 3.11 (t, 2H, H β) ppm. ¹³C-NMR (DMSO-*d*₆) δ : 154.0 (C-1), 145.8 (C-4), 143.9 (C-2'), 143.8 (C-8a'), 138.0 (C-4a), 132.9 (C-7), 132.6 (C-6), 126.6 (C-5'), 124.8 (C-7'), 124.5 (C-8), 122.6 (C-5), 119.8 (C-8a), 115.8 (C-8'), 111.9 (C-6'), 108.7 (C-3), 41.0 (C α), 27.4 (C β) ppm. ESI-MS (MeOH) *m/z* (%): 346 (M⁺ + Na, 100), 324 (M⁺, 14). Anal. (C₁₇H₁₄N₅Cl·H₂O) C, H, N.

Parasite strain and culture

Promastigote forms of *L. donovani* (MHOM/PE/84/LC26), *L. infantum* (MCAN/ES/2001/UCM-10) and *L. braziliensis* (MHOM/BR/1975/M2904) were cultured in vitro in medium trypanosomes liquid (MTL) supplemented with 10% inactive fetal calf serum (iFCS) in Roux flasks (Corning, USA), according to a methodology previously described.^[37] *T. cruzi* SN3 strain of IRHOD/CO/2008/SN3 was isolated from domestic *Rhodnius prolixus*, biological origin is Guajira (Colombia).^[38] Epimastigote forms were grown in axenic Grace's Insect Medium (Gibco) supplemented with 10% inactivated fetal bovine serum (FBS) at 28 °C in tissue-culture flasks.^[39] In order to obtain the parasite suspension for the trypanocidal assay, the epimastigote culture (in the exponential growth phase) was concentrated by centrifugation at 400 g for 10 min and the number of flagellates was counted in a hemocytometric chamber.

Transformation of epimastigotes to the metacyclic form. Metacyclogenesis was induced by culturing the parasites at 28 °C in modified Grace's medium (Gibco) for 12 days, as described previously.^[40] Twelve days after cultivation at 28 °C, metacyclic forms were counted in a Neubauer hemocytometric chamber. The proportion of metacyclic forms was around 40% at this stage.

Cell culture and cytotoxicity tests. Vero cells (Flow) were grown in RPMI (Gibco), supplemented with 10% inactivated fetal bovine serum, in a humidified 95% air, 5% CO₂ atmosphere at 37 °C for 2 days. For the cytotoxicity testing, cells were placed in 25 mL Colie-based bottles (Sterling) and centrifuged at 100 g for 5 min. The culture medium was removed, and fresh medium was added to a final concentration of 1 × 10⁵ cells/mL. This cell suspension was distributed in a culture tray (with 24 wells) at a rate of 100 μ L/well and incubated for two days at 37 °C in a humidified atmosphere enriched with 5% CO₂. The medium was removed, and fresh medium was added together with each test compound (at concentrations of 100, 50, 25, 10, and 1 μ M). After 72 h of treatment, cell viability was determined by flow cytometry according to a methodology described by us.^[40]

In vitro activity assays: extracellular forms

Epimastigote assay. Epimastigotes were collected in the exponential growth phase and distributed in culture trays (with 24 wells) at a final concentration of 5 × 10⁴ parasites/well. The compounds to be tested and benzimidazole or glucantime were dissolved in medium trypanosomes liquid (MTL) and were tested at 100, 50, 25, 12.5, and 6.25 μ M. The effects of the different concentrations of each compound against the epimastigotes were tested for 72 h using a Neubauer hemocytometric chamber. The trypanocidal effect is expressed as IC₅₀ values, i.e. the concentration required to result in 50% inhibition, as calculated by linear-regression analysis from the Kc values of the concentrations used.

In vitro activity assays: Intracellular forms

Axenic amastigotes assay for Leishmania species: Axenic amastigotes of *L. infantum*, *L. braziliensis*, *L. donovani* or *T. cruzi*, were obtained infecting J774.2 macrophages following the methodology described previously by Moreno *et al.*^[41] Thus, the promastigote transformation to amastigotes was achieved after three days of culture in M199 medium (Invitrogen, Leiden, Netherlands) supplemented with 10% heat-inactivated FCS, 1 g/L β -alanine, 100 mg/L L-asparagine, 200 mg/L saccharose, 50 mg/L sodium pyruvate, 320 mg/L malic acid, 40 mg/L fumaric acid, 70 mg/L succinic acid, 200 mg/L α -ketoglutaric acid, 300 mg/L citric acid, 1.1 g/L sodium bicarbonate, 5 g/L MES, 0.4 mg/L hemin, and 10 mg/L gentamicine, pH 5.4 at 37 °C. The effect of each compound against the axenic amastigotes was tested for 48 h using a Neubauer hemocytometric chamber. The trypanocidal effect is expressed as IC₅₀ values, i.e. the concentration required to result in 50% inhibition, as calculated by linear-regression analysis from the Kc values of the concentrations used.

Amastigotes assay for T. cruzi: Vero cells were cultured in RPMI medium in a humidified 95% air and 5% CO₂ atmosphere at 37 °C. The cells were seeded at a density of 1 × 10⁴ cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and then cultivated for 2 days. Afterwards, adhered Vero cells were infected *in vitro* with metacyclic forms of *L. infantum*, *L. braziliensis*, *L. donovani* or *T. cruzi*, at a ratio of 10:1 and maintained for 24 h at 37 °C in 5% CO₂ in air. The extracellular parasites were removed by washing, and the infected cultures were incubated with the compounds (6.25, 12.5, 25, 50 and 100 μ M concentrations) and

cultured for 72 h in RPMI and 10% inactivated foetal bovine serum. The activity of the compounds was determined from the percentage reduction in the number of amastigotes in the treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The values are the means of four separate determinations.^[30] The leishmanicidal or trypanocidal effect was expressed as IC₅₀ values.

Infectivity assay: Vero cells were cultured in RPMI medium as described above. Afterwards, the cells were infected *in vitro* with metacyclic forms of *L. infantum*, *L. braziliensis*, *L. donovani* or *T. cruzi* at a ratio of 10:1. The test compounds (IC₂₅ concentrations) were added immediately after infection and incubated for 12 h at 37 °C in a 5% CO₂ atmosphere. The extracellular parasites and the test compounds were removed by washing and the infected cultures were grown for 10 days in fresh medium. Fresh culture medium was added every 48 h. The activity of each compound tested was determined from the percentage of infected cells and the number of amastigotes per infected cell in treated and untreated cultures in the methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analyzing more than 100 host cells distributed throughout randomly chosen microscopic fields. The values are the means of four separate determinations. The number of trypomastigotes in the medium was determined microscopically (OLYMPUS CX41) for parasite counting using the Neubauer hemocytometric chamber (a dilution of 1:100 in PBS was necessary to get into the range of counting). The activity (% of parasites reduction) was compared with the control.^[30]

SOD enzymatic inhibition

Leishmania spp. promastigotes and *T. cruzi* epimastigotes were grown in tissue-culture flasks in axenic medium trypanosomes liquid (MTL) medium (Hank's Balanced Salt Solution – HBSS (Gibco®), supplemented with 10% heat-inactivated foetal bovine serum (FBS) at 26 °C until reaching a population of approximately 1 × 10⁷ parasites per mL. Cells were harvested at the logarithmic growth phase by centrifugation (1500 g for 10 min at room temperature). These conditions are stressful for parasites and lead to SODe excretion. These cultures were maintained at 27 °C for 24 h.

After 24 h, the promastigote culture was centrifuged (1500 × g for 10 min) and the supernatant was filtered (Minisart®, F 20 mm). The filtered supernatant was precipitated to ice-cold ammonium sulphate at 35% salt concentration.

Following centrifugation, the resultant supernatant was then treated with 85% ice-cold ammonium sulphate and the second precipitate collected. The resulting precipitate was finally dissolved in 2.5 mL of distilled water and desalted by chromatography in a Sephadex G-25 column (GE Healthcare Life Sciences®, PD 10 column), previously equilibrated with 25 mL of distilled water, bringing it to a final volume of 3.5 mL (P85e fraction).

Protein concentrations were determined using the Bradford method.^[42] Iron and copper-zinc superoxide dismutase (Fe-SOD and CuZn-SOD) activities were determined using the method described by Beyer and Fridovich,^[43] which measures the reduction in nitroblue tetrazolium (NBT) by superoxide ions. Into each bucket, 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, 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 tested. Five different concentrations were used for each product: 1, 2.5, 5, 12.5 and 25 µM (equivalent to 5 µL, 12.5 µL, 25 µL, 62 µL and 125 µL,

respectively, of the Stock solution). In the control experiment the volume was made up to 1000 µL with 50 mM potassium phosphate buffer (pH 7.8, 3 mL), whereas 30 µL of the parasite homogenate fraction was added to the mixtures containing the compounds. Then, the absorbance (A₀) was measured at 560 nm in a spectrophotometer. Following this, each bucket was illuminated with UV light for 10 min under constant stirring and the absorbance (A₁) was measured. The human CuZn-SOD, coenzymes and substrates used in these assays were obtained from Sigma Chemical Co. The data obtained were analyzed using the Newman-Keuls test.

Molecular modeling

Molecular modeling studies were carried out using the AMBER method implemented in Hyperchem 8.0 package,^[35] modified by the inclusion of appropriate parameters.^[44] Starting structure for compound **3d** were built by using Hyperchem capabilities. Its geometry was minimized to a maximum energy gradient of 0.1 kcal/mol with the AMBER force field, using the Polak-Ribiere (conjugate gradient) minimizer, and 'simulated annealing' procedure was used to cover all conformational space. The most stable extended geometry was used in the 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 distance dependent dielectric constant value. Charge assignments for all atoms was done by means of *ab initio* calculations using STO-3G basis set, as it is compatible with AMBER force field, prior to energy minimization using AMBER. The Fe-SOD enzyme structure was obtained from the Brookhaven protein data bank (4F2N for *Leishmania*) and its energy minimized in the same way. Interaction studies were performed starting from structures with the compound positioned in the border of the enzyme cavity. Entering the cavity was forced using a restraint to the N–Fe distance, slowly decreasing this distance, and letting the complex achieve the minimum energy conformation with no restraints, for all the small driving steps, using the same conditions mentioned above.

Abbreviations

SOD	superoxide dismutase
BZN	benznidazole
NADH	nicotinamide adenine dinucleotide
DNA	deoxyribonucleic acid
AIDS	acquired immunodeficiency syndrome
TEM	transmission electron microscopy
HMQC	heteronuclear single quantum coherence experiment
SI	selectivity index
Asp	aspartate
Gln	glutamine
Tyr	tyrosine
Asn	asparagine
Trp	tryptophan
dpi	days post-infection
IgG	immunoglobulin G
ELISA	enzyme-linked immunosorbent assay
OD	optical density
SD	standard deviation
PEP	phosphoenolpyruvate
TLC	thin-layer chromatography

HMBC	Heteronuclear multiple-bond correlation spectroscopy
TMS	tetramethylsilane
DMSO	dimethyl sulfoxide
MS-ESI	electrospray ionization mass spectra
FBS	fetal bovine serum
RPMI	Roswell Park Memorial Institute
MES	4-morpholinoethanesulphonic acid
PBS	phosphate-buffered saline solution
FCS	fetal calf serum
MTL	medium trypanosomes liquid
EDTA	ethylenediaminetetraacetic acid
STE	sodium chloride-tris-EDTA
NBT	nitroblue tetrazolium
BSA	bovine serum albumin
OPD	o-phenylenediamine dihydrochloride

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Conflict of Interest

The authors declare no conflicts of interest.

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