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Original article

In vitro activity of scorpiand-like azamacrocycle derivatives in promastigotes and intracellular amastigotes of *Leishmania infantum* and *Leishmania braziliensis*

Clotilde Marín [a], M. Paz Clares [b], Inmaculada Ramírez-Macías [a], Salvador Blasco [b], Francisco Olmo [a], Conxa Soriano [c], Begoña Verdejo [b], María José Rosales [a], David Gomez-Herrera [a], Enrique García-España [b,**], Manuel Sánchez-Moreno [a,*]

[a] Departamento de Parasitología, Facultad de Ciencias, Universidad de Granada, E-18071 Granada, Spain
[b] Instituto de Ciencia Molecular, Departamento de Química Inorgánica, Universidad de Valencia, E-46980 Paterna, Valencia, Spain
[c] Departamento de Química Orgánica, Universidad de Valencia, E-46100 Burjassot, Valencia, Spain

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ABSTRACT

The activity of a family scorpiand-like azamacrocycles against *Leishmania infantum* and *Leishmania braziliensis* was studied using promastigotes, axenic and intracellular amastigotes forms. All the compounds are more active and less toxic than meglumine antimoniate (Glucantime). Moreover, the data on infection rates and amastigotes showed that compounds **P2Py**, **PN** and **P3Py** are the most active against both species of *Leishmania*. On the other hand, studies on the inhibitory effect of these compounds on SOD enzymes showed that while the inhibition of the Fe-SOD enzyme of the promastigote forms of the parasites is remarkable, the inhibition of human CuZn-SOD and Mn-SOD from *Escherichia coli* is negligible. The ultrastructural alterations observed in treated promastigote forms confirmed that the compounds having the highest activity were those causing the largest cell damage. The modifications observed by ^{1}H NMR, and the amounts of catabolites excreted by the parasites after treatment with the compounds, suggested that the catabolic mechanism could depend on the structure of the side chains linked to the aza-scorpiand macrocycles.

1. Introduction

Leishmaniasis is still one of the world's most neglected diseases which occurs in 98 countries, affecting largely the poorest of the poor, mainly in developing countries. 350 Million people are considered at risk of contracting leishmaniasis, and over 2 million new cases occur yearly [1]. The etiological agents are different species of protozoa of the genus *Leishmania*, which are transmitted by Diptera of the genus *Phlebotomus* in the Old World, and *Lutzomyia* in the New World.

Leishmaniasis has a broad diversity of clinical forms: cutaneous (CL), mucosal or muco-cutaneous (MCL) ulcers that although can heal spontaneously produce disfiguring lesions on exposed body parts (arms, legs, face), or the visceral form (VL, also known as kala-azar) which is fatal if not treated in time.

Surprisingly, leishmaniasis is the only tropical disease which is being treated by nonleishmanial drugs [2]. The spectrum and efficacy of available antileishmanial drugs is limited. The first-line drugs used for treatment of leishmaniasis are pentavalent antimonial compounds (sodium stibogluconate or Pentostam and meglumine antimoniate or Glucantime), while amphotericin B, pentamidine and Paromomycin (aminosidine) are used as second-line chemotherapy. Other alternative antileishmanial medicines are miltefosine, azithromycin, allopurinol, dapsone, sitamaquine, rifampicin or azole medicines: ketoconazole, fluconazole, itraconazole. Combining drugs may bring significant advantages and better therapeutic effects than each of the substances alone [2,3]. Although in the past decade the number of treatments has increased, they have numerous drawbacks such as difficulty in administration, length of treatment, toxicity, cost, availability limited in disease endemic regions and increasing parasitic resistance [4,5]. In addition, the efficacy of the substances differs drastically depending on the clinical form of leishmaniasis, *Leishmania*

* Corresponding author. Tel.: +34 958 242 369; fax: +34 958 243 862.
** Corresponding author. Tel.: +34 963 544879; fax: +34 96 354 3274.
E-mail addresses: cmaris@ugr.es (C. Marín), enrique.garcia-es@uv.es (E. García-España), msanchem@ugr.es (M. Sánchez-Moreno).

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species and geographical region [6]. Thus, there is still a continuing need for new chemical substances with a leishmanicidal effect, for optimized delivery systems and newer cost effective drugs.

Thus, the development of new leishmanicidal drugs is an important goal. In this respect, several compounds including synthetic ones, or natural products extracted from plants or marine sources, have shown different degrees of efficacy in the treatment of experimental leishmaniasis [7–9].

In relation to this matter, our research group has designed in recent years a new family of polyamine compounds that consists of a macrocyclic pyridinophane core appended with lateral chains containing additional donor atoms; the structures are shown in Fig. 1. These macrocycles are termed scorpiand ligands since the lateral chain can fold towards the macrocyclic core, following the binding of target guest species such as protons, anions or metal ions [10].

The aim of the present work is to identify new candidates for designing potent, selective, and low toxicity anti-trypanosomatids. We have evaluated the antiproliferative *in vitro* activity against *Leishmania infantum* and *Leishmania braziliensis* (promastigote and amastigote forms). Unspecific mammal cytotoxicity of the most active compounds was evaluated *in vitro*, and the less toxic derivatives were submitted to a more thorough study of their possible action mechanisms. Furthermore, a nuclear magnetic resonance spectroscopy (^{1}H NMR) study was performed in order to gain details regarding its nature and the percentage of metabolites excreted causing inhibitory effects of the glycolic pathway, since it represents the primary source of energy for the parasite. Finally, the effects of the compounds on the ultra-structure of *Leishmania* spp. were considered on the basis of transmission electron microscopy studies (TEM).

2. Materials and methods

2.1. Synthesis

The synthesis of the compounds was performed as depicted in Scheme 1 [10,11].

2.1.1. 6-(2-Aminoethyl)-3,6,9-triaza-1-(2,6)-pyridinecyclodecaphane (***P***·3HBr)

Tris-2-(aminoethyl)amine (6 g, 41.7 mmol) was dissolved in 400 mL of THF adding 100 mL of an aqueous solution of K_2CO_3 (17 g, 125 mmol). TsCl (125 mmol) dissolved in 100 mL of THF was added dropwise for an hour and the stirring was kept for 24 h. The organic phase was taken to dryness and the residue was suspended in refluxing ethanol to give a white solid. The solid was filtered and washed with EtOH (yield 63%). mp 60–62 °C. Tris[2-(N-*p*-tolylsulfonylaminoethyl)]amine (**TrenTs**). ^{1}H NMR ($CDCl_3$, 300 MHz): δ_H (ppm): 2.41 (s, 9H), 2.47 (t, J = 5 Hz, 6H), 2.91 (t, J = 6 Hz, 3H), 2.92 (t, J = 6 Hz, 3H), 5.94 (s, 3H), 7.0 (d, J = 8 Hz, 6H), 7.80 (d, J = 8 Hz, 6H). ^{13}C NMR ($CDCl_3$, 75.43 MHz): δ_C (ppm): 21.9, 41.2, 54.5, 127.7, 130.2, 137.2, 143.8.

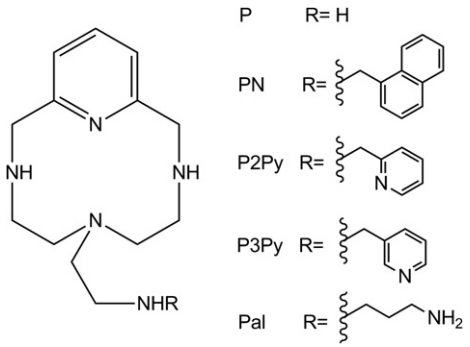
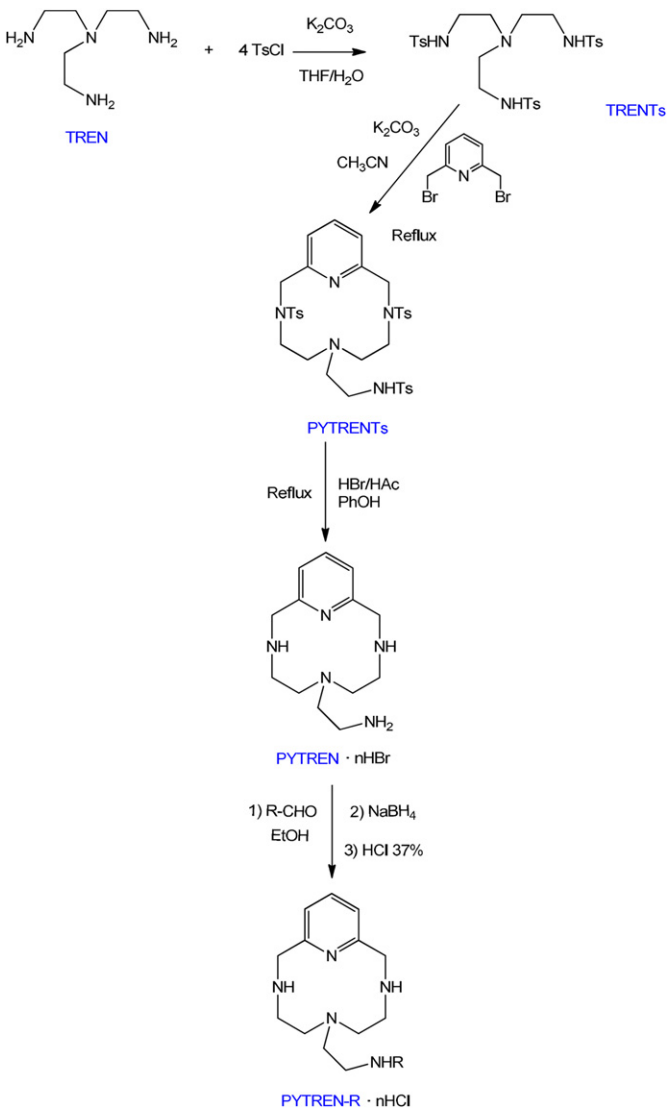


Fig. 1. Drawing of the compounds.



Scheme 1. General procedure for the synthesis of **P**, **PN**, **P2Py** and **P3Py.**

TrenTs (5.7 mmol) and K_2CO_3 (57.0 mmol) were dissolved in dry CH_3CN (50 mL). 2,6-Bis(bromomethyl)pyridine (5.7 mmol) dissolved in dry CH_3CN (30 mL) was slowly added. The mixture was refluxed under a nitrogen atmosphere for 20 h. The solid obtained was filtered, washed with dry CH_3CN, and then it was taken to dryness, getting an oil that was refluxed in ethanol to give a white solid (yield 51.4%). mp 151–153 °C. 6-[2-(*p*-Tolylsulfonylamino)ethyl]-3,6,9-triaza-3, 9-*p*-tolylsulfonyl-1-(2,6)-pyridinecyclodecaphane (**PytrenTs**). ^{1}H NMR ($CDCl_3$, 300 MHz): δ_H (ppm): 2.31 (t, J = 7 Hz, 4H), 2.36 (t, J = 7 Hz, 2H), 2.41 (s, 3H), 2.45 (s, 6H), 2.87–2.81 (m, 2H), 3.06 (t, J = 7 Hz, 4H), 4.31 (s, 4H), 5.05 (t, J = 6 Hz, 1H), 7.36–7.28 (m, 8H), 7.75–7.68 (m, 7H). ^{13}C NMR ($CDCl_3$, 75.43 MHz): δ_C (ppm): 21.9, 40.9, 45.9, 52.2, 54.2, 55.1, 124.1, 127.4, 130.2, 136.1, 137.1, 139.2, 143.9, 144.1, 155.5.

PytrenTs (6.75 mmol), HBr/HAc (250 mL) and PhOH (257 mmol), were refluxed for 24 h. The solid obtained was filtered washing with EtOH/CH_2Cl_2 (1:1) (yield 65.7%). mp 201–203 °C. ^{1}H NMR (D_2O, 300 MHz): δ_H (ppm): 7.95 (t, J = 8 Hz, 1H), 7.44 (d, J = 8 Hz, 2H), 4.63 (s, 4H), 3.29–3.21 (m, 6H), 3.02 (t, J = 7 Hz, 2H), 2.92 (t, J = 5 Hz, 4H). ^{13}C NMR (D_2O, 75.43 MHz): δ_C (ppm): 30.5, 35.6, 46.2, 49.8, 50.9, 52.3, 122.5, 140.1, 149.2. Anal. Calcd. for $C_{13}H_{23}N_5 \cdot 4HBr$: C, 27.25; H, 4.74; N, 12.22. Found: C, 27.94; H, 5.11; N, 11.86.

*2.1.2. 6-[4-(1-Naphthyl)-3-azabutyl]-3,6,9-triaza-1-(2,6)-pyridinecyclodecaphane (**PN**·3HCl)*

6-(2-Aminoethyl)-3,6,9-triaza-1-(2,6)-pyridinecyclodecaphane (**P**) (1.43 g, 5.73 mmol) and 1-naphthaldehyde (0.90 g, 5.73 mmol) were dissolved in 100 mL of dry ethanol and stirred at room temperature for 2 h. Then, sodium borohydride (2.2 g, 58 mmol) was added, and the bulk was stirred for 2 h more. The solution was vacuum evaporated and extracted with $CHCl_3/H_2O$ (6 × 100). The organic phase was taken to dryness and redissolved in dry ethanol. The hydrochloride salt of the product was precipitated by adding a concentrated hydrochloride acid solution (yield 65%). mp 207–209 °C. ^{1}H NMR (D_2O, 300 MHz): δ_H (ppm): 2.91 (t, J = 5 Hz, 4H), 3.09 (t, J = 6 Hz, 2H), 3.25 (t, J = 6 Hz, 4H), 3.43 (t, J = 8 Hz, 2H), 4.62 (s, 4H), 4.82 (s, 2H), 7.45 (d, J = 8 Hz, 2H), 7.58–7.75 (m, 4H), 7.96 (t, J = 8 Hz, 1H), 8.07 (t, J = 7 Hz, 2H), 8.14 (d, J = 8 Hz, 1H). ^{13}C NMR (D_2O, 75.43 MHz): δ_C (ppm): 43.1, 46.2, 48.8, 49.7, 50.8, 51.2, 122.5, 122.8, 125.9, 127.1, 127.8, 129.5, 129.9, 131.0, 132.3, 140.1. Anal. Calcd. for $C_{24}H_{31}N_5 \cdot 3HCl \cdot 1.5H_2O$: C, 54.81; H, 7.09; N, 13.31. Found: C, 55.10; H, 7.42; N, 13.38.

*2.1.3. 6-[4-(2-Pyridyl)-3-azabutyl]-3,6,9-triaza-1-(2,6)-pyridinecyclodecaphane (**P2Py**·3HCl)*

The same procedure as for the synthesis of **PN** was applied, but using 2-pyridinecarbaldehyde instead of 1-naphthaldehyde (yield 65.7%). mp 208–210 °C. ^{1}H NMR (D_2O, 300 MHz): δ_H (ppm): 2.93 (t, J = 5 Hz, 4H), 3.13 (t, J = 8 Hz, 2H), 3.28 (t, J = 5 Hz, 4H), 3.45 (t, J = 8 Hz, 2H) 4.57 (s, 2H), 4.64 (s, 4H), 7.45 (d, J = 8 Hz, 2H), 7.75 (ddd, $J_1 = J_2$ = 8 Hz, J_3 = 1 Hz, 1H), 7.83 (d, J = 8 Hz, 1H), 7.96 (t, J = 8 Hz, 1H), 8.25 (ddd, $J_1 = J_2$ = 8 Hz, J_3 = 2 Hz, 1H), 8.72 (dd, J_1 = 5 Hz, J_2 = 1 Hz, 1H). ^{13}C NMR (D_2O, 75.43 MHz): δ_C (ppm): 43.9, 46.2, 49.8, 50.0, 50.8, 51.2, 122.5, 126.5, 126.6, 140.1, 143.3, 146.8, 147.6, 149.2. Anal. Calcd. for $C_{19}H_{28}N_6 \cdot 3HCl$: C, 50.68; H, 6.94; N, 18.75. Found: C, 50.3; H, 7.1; N, 18.5.

*2.1.4. 6-[4-(3-Pyridyl)-3-azabutyl]-3,6,9-triaza-1-(2,6)-pyridinecyclodecaphane (**P3Py**·3HCl)*

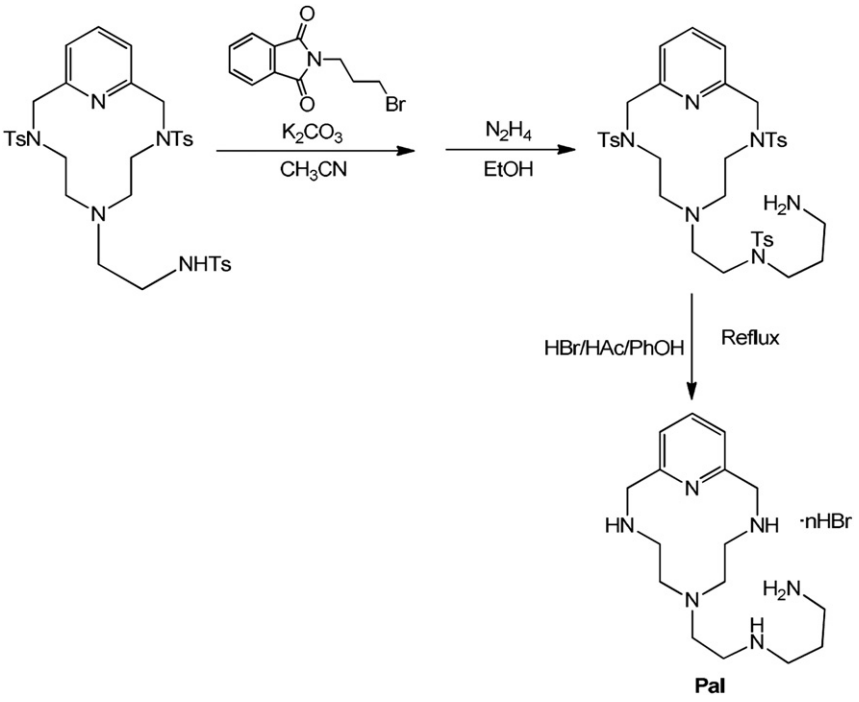
The same procedure as for the synthesis of **P2Py** was applied, but using 3-pyridinecarbaldehyde instead of 2-pyridinecarbaldehyde (yield 75.8%). mp 210–215 °C. ^{1}H NMR (D_2O, 300 MHz): δ_H (ppm): 2.94 (t, J = 5 Hz, 4H), 3.11 (t, J = 7 Hz, 2H), 3.30 (t, J = 5 Hz, 4H), 3.48 (t, J = 7 Hz, 2H), 4.64 (s, 4H), 4.62 (s, 2H), 7.45 (d, J = 8 Hz, 2H), 7.96 (t, J = 8 Hz, 1H), 8.20 (dd, J_1 = 8 Hz, J_2 = 6 Hz, 1H), 8.83 (d, J = 8 Hz, 1H), 8.91 (d, J = 6 Hz, 1H), 9.05 (d, J = 2 Hz, 1H). ^{13}C NMR (D_2O, 75.43 MHz): δ_C (ppm): 43.9, 45.8, 47.7, 49.5, 50.4, 50.8, 122.1, 128.0, 129.5, 131.1, 142.5, 148.7, 148.8. Anal. Calcd. for $C_{19}H_{28}N_6 \cdot 3HCl$: C, 50.68; H, 6.94; N, 18.75. Found: C, 50.8; H, 7.0; N, 18.8.

The synthesis of **Pal**·5HBr was accomplished as illustrated in Scheme 2 following Ref. [12].

*2.1.5. 6-(3,7-Diazaheptyl)-3,6,9-triaza-1-(2,6)-pyridinecyclodecaphane (**Pal**·**5**HBr)*

6-[2-(*p*-Tolylsulfonylamine)ethyl]-3,6,9-triaza-3, 9-*p*-tolylsulfonyl-1-(2,6)-pyridinecyclodecaphane (**PytrenTs**) (2.59 g, 3.64 mmol), and K_2CO_3 (1.51 g, 10.93 mmol) were suspended with 300 mL of CH_3CN. To this mixture N-(3-bromopropyl)phthalimide (1.19 g, 4.14 mmol) in 150 mL of CH_3CN was added, then it was refluxed for 48 h and filtered off. The solution was vacuum evaporated to dryness and the residue was suspended in refluxing ethanol to give a white solid (yield 98%). ^{1}H NMR ($CDCl_3$, 300.13 MHz) δ_H (ppm): 1.74–1.84 (m, 2H), 2.26–2.39 (m, 11H), 2.55 (t, 2H, J = 7 Hz), 2.91 (t, 2H, J = 7 Hz), 3.03–3.10 (m, 8H), 3.60–3.67 (m, 2H), 4.26 (s, 4H), 7.19–7.29 (m, 10H), 7.61–7.80 (m, 9H). This solid (2.75 g, 3.57 mmol) was treated with hydrazine hydrate 85% (3 mL) and ethanol (500 mL) and the mixture was refluxed for 24 h, then the resulting solid was filtered off. The solution was vacuum evaporated to give an oil (yield 90%). ^{1}H NMR ($CDCl_3$, 300.13 MHz): δ_H (ppm): 1.50–1.61 (m, 2H), 2.33–2.42 (m, 12H), 2.52 (t, 2H, J = 7 Hz), 2.70 (t, 2H, J = 7 Hz), 2.96–3.10 (m, 8H), 4.30 (s, 4H), 7.18–7.30 (m, 8H), 7.60–7.70 (m, 7H). ^{13}C NMR ($CDCl_3$, 75.43 MHz): δ_C (ppm): 21.7, 21.8, 32.2, 39.0, 44.7, 47.2, 47.3, 51.9, 54.7, 124.1, 127.4, 127.4, 130.0, 130.1, 136.0, 136.4, 138.9, 143.5, 143.8, 155.2.

Pytren-alTs (6.75 mmol) was suspended with PhOH (257 mmol) and HBr/HAc 33% (250 mL) and the mixture was subjected to reflux for 24 h. The solid obtained was filtered washing with EtOH/CH_2Cl_2 (1:1) to give **Pal**·5HBr (yield 63%). mp 207–209 °C. ^{1}H NMR (D_2O, 300 MHz): δ_H (ppm): 1.99–2.10 (m, 2H), 2.80–2.90 (m, 4H), 2.95–3.06 (m, 4H),



Scheme 2. General procedure for the synthesis of **Pal**.

3.08–3.30 (m, 8H), 4.55(s, 4H), 7.36 (d, $J = 7.8$ Hz, 2H), 8.01 (t, $J = 7.8$ Hz, 1H). ^{13}C NMR (D_2O, 75.43 MHz): δ_C (ppm): 24.2, 36.8, 43.6, 45.4, 46.2, 49.8, 50.8, 51.2, 122.5, 140.1, 149.2. Anal. Calcd. for $C_{16}H_{32}N_6 \cdot 5HBr \cdot 2H_2O$: C, 25.68; H, 5.03; N, 10.86. Found: C, 25.66; H, 5.52; N, 11.22.

2.2. Parasite strain and culture

L. infantum (MCAN/ES/2001/UCM-10) and *L. braziliensis* (MHOM/BR/1975/M2904) were cultured *in vitro* in medium trypanosomes liquid (MTL) together with 10% inactive foetal 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 in Ref. [13].

2.3. Superoxide dismutase enzymatic inhibition

Parasites cultured as described above were suspended (0.5–0.6 g/mL) in 3 mL of STE buffer 1 (0.25 M sucrose, 25 mM Tris–HCl, 1 M EDTA, pH 7.8) and disrupted by three cycles of sonic disintegration, for 30 s each at 60 W. The sonicated homogenate was centrifuged at 1500*g* for 10 min at 4 °C, and the pellet was washed three times with ice-cold STE buffer 1, giving a total supernatant fraction of 9 mL. This fraction was centrifuged (2500*g* for 10 min at 4 °C), the supernatant was collected and solid ammonium sulphate was added. The protein fraction, which precipitated between 35% and 85% salt concentration, was centrifuged (9000*g* for 20 min at 4 °C), dissolved in 2.5 mL of 20 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA (buffer 2) and dialysed on a Sephadex G-25 column (GE Healthcare, PD 10), previously balanced with buffer 2, bringing it to a final volume of 3.5 mL (fraction of the homogenate). The protein concentrations were determined by the Bradford method (Sigma–Aldrich, St. Louis, M.O.), using albumen from bovine serum as standard.

Iron superoxide dismutase (Fe-SOD) activity was performed by spectrometric measurement of NBT-u.v. light oxidation according to the method described by Beyer and Fridovich [14] which measures the reduction of NBT by superoxide ions. One unit was the amount of enzyme required to inhibit the rate of NBT reduction by 50%. The manganese superoxide dismutase (Mn-SOD) and copper–zinc superoxide dismutase (CuZn-SOD) from *Escherichia coli* and human erythrocytes, respectively, used in these assays were obtained from Boehringer (Mannheim), while all the coenzymes and substrates came from Sigma–Aldrich. Data obtained were analyzed according to the Newman–Keuls test.

2.4. In vitro activity assays: extracellular forms

2.4.1. Promastigote assay

The compounds were dissolved in DMSO (Panreac, Barcelona, Spain) at a concentration of 0.1% and were assayed as non-toxic and without inhibitory effects on parasite growth, according to Ref. [13]. The compounds were dissolved in the culture medium at concentrations of 100, 50, 25, 10 and 1 μM. The effects of each compound against the promastigote forms and their 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.

2.4.2. Infectivity assay

J774.2 macrophage cells were grown in minimal essential medium (MEM) plus glutamine (2 mM) and 20% inactive FCS, in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were seeded at a density of 1×10^4 cells/well in 24-well micro plates (Nunc) with rounded cover slips on the bottom and cultured for 2 days. The cells were then infected *in vitro* with promastigote forms of *L. infantum* or *L. braziliensis* at a ratio of 10:1. The drugs (IC_{25} concentrations) were added immediately after infection and incubation was performed for 12 h at 37 °C in 5% CO_2. Non-phagocytised parasites and drugs 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. Drug 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 analyzing more than 200 host cells distributed in randomly chosen microscopic fields. Values are the means of three separate determinations.

2.5. Cell culture and cytotoxicity tests

The macrophage line J774.2 [European collection of cell cultures (ECACC) number 91051511] was derived in 1968 from a tumour in a female BALB/c mouse. The cytotoxicity test on macrophages was performed according to the method described by Ref. [15]. After 72 h of treatment with 100, 50, 25, l0, and 1 μM of product, cell viability was determined by flow cytometry. Thus, 100 mL/well of propidium iodide solution (100 mg/mL) was added and incubated for 10 min at 28 °C in darkness. Afterwards, 100 mL/well of fluorescein diacetate (100 ng/mL) was added and incubation was performed under the conditions described above. Finally, the cells were recovered by centrifugation at 400*g* for 10 min and the precipitate was washed with PBS. Flow cytometric analysis was performed with a FACS Vantage TM flow cytometer (Becton Dickinson). The percentage viability 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.

2.6. In vitro activity assays: intracellular forms

2.6.1. Axenic amastigote assay

Axenic amastigote forms of *Leishmania* spp. were cultured following the methodology described previously in Ref. [16]. Thus, promastigote transformation to amastigotes was achieved after 3 days of culture in M199 medium (Invitrogen, Leiden, The Netherlands) supplemented with 10% heat-inactivated FCS, 1 g/L β-alanine, 100 mg/L L-asparagine, 200 mg/L sucrose, 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 2-(N-morpholino) ethanesulfonic acid (MES), 0.4 mg/L haemin and 10 mg/L gentamicin, pH 5.4, at 37 °C. The effect of each compound against axenic amastigote forms was tested at 48 h using a Neubauer haemocytometer chamber.

2.6.2. Amastigote assay

Adherent macrophage cells were infected with promastigotes in the stationary growth phase of *Leishmania* spp. at a ratio of 10:1 and maintained for 24 h at 37 °C in air 5% CO_2. Non-phagocytosed parasites were removed by washing, and the infected cultures were incubated with the compounds (1, 10, 25, 50 and 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 numbers in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. Values are the averages of three separate determinations [13].

2.7. Metabolite excretion

Cultures of *L. infantum* and *L. braziliensis* promastigotes (initial concentration 5×10^5 cells/mL) received the IC_{25} of the compounds (except for control cultures). After incubation for 96 h at 28 °C the cells were centrifuged at 400*g* for l0 min. The supernatants were collected to determine the excreted metabolites by ^1H NMR, and chemical shifts were expressed in parts per million (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 in Ref. [17].

2.8. Ultrastructural alterations

The parasites were cultured at a density of 5×10^5 cells/mL in MTL medium, each of which contained the compounds tested at the IC_{25} concentration. After 96 h, the cultures were centrifuged at 400*g* for 10 min, and the pellets produced were washed in PBS and then incubated with 2% (v/v) *p*-formaldehyde/glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 2 h at 48 °C. The pellets were then prepared for TEM employing the technique of Ref. [13].

3. Results

3.1. Chemistry

The polyamine macrocycles **P**, **PN**, **P2Py** and **P3Py** (Fig. 1) were prepared as shown in Scheme 1 by a procedure previously reported by some of us [10,11]. The synthesis of the ligands was achieved following a modification of the Richman–Atkins procedure by reaction of the pertosylated polyamine tren with 2,6-bis(bromomethyl)pyridine in 1:1 molar ratio using K_2CO_3 as a base in refluxing CH_3CN. Detosylation is carried out with HBr/HAc, and the final product (**P**) is obtained as the hydrobromide salt. Compounds **PN**, **P2Py** and **P3Py** are obtained by reacting **P** in its free amine form with 1-naphthaldehyde, 2 or 3-pyridinecarbaldehyde, respectively, in dry ethanol followed by in situ reaction with sodium borohydride and precipitation as hydrochloride salts. The polyamine **Pal** was obtained by reaction of the pertosylated polyamine **P** with N-(3-bromopropyl)phthalimide in 1:1 molar ratio using K_2CO_3 as a base in refluxing CH_3CN (Scheme 2) [12]. Deprotection and detosylation were carried out with hydrazine and HBr/HAc respectively, and the final product (**Pal**) was obtained as its hydrobromide salt.

A property that could be affecting the in vivo activity of these compounds is their actual protonation degree at the physiological pH of 7.4. We have determined by pH-metric titration the basicity constants of the polyamines used in this study [10,11].

Among the compounds studied, **Pal** is the most basic since it possesses an additional secondary nitrogen atom in the tail with respect to **P**, while **PN** is the less basic likely due to the hydrophobic environment generated by the naphthalene spacer. The basicity of **P2Py** and **P3Py** is close basicity although slightly higher for the former.

These constants allow for deriving the average protonation degree at any pH (see bottom file of Table 1) shows such a representation for pH 7.4. While, as expected, **Pal** is the compound having the highest charge, **P3Py** is however the one with a lower net charge at pH 7.4. As below discussed **P3Py** is also the compound showing the most promising leishmanicidal activity.

3.2. In vitro antileishmanial evaluation

The IC_{50} values obtained for the promastigote, axenic amastigote and amastigote forms of the *L. infantum* and *L. braziliensis* for the tested compounds at concentrations ranging from 1 to 100 μM, shown in the first three columns of Tables 2 and 3, respectively, which also include the results obtained for the reference drug meglumine antimoniate (Glucantime). Toxicity values against J774.2 macrophages cells were also calculated, and the selectivity indexes (SI, IC_{50} macrophage cells toxicity/IC_{50} activity of extracellular or intracellular forms of the parasite), also shown in the last three columns of Tables 2 and 3. The number of times that the compound SI exceeded the Glucantime SI, is shown in brackets, since this value is very illustrative of the *in vitro* potentiality of the compounds tested with respect to the reference drug.

Tables 2 and 3 show the IC_{50} values obtained after 72 h of exposure, when compounds were assayed against the different parasite forms studied. Values for the reference drug, Glucantime, are included in all cases for comparison.

If we consider now the results displayed in Table 2 for activity against *L. infantum*, the leishmanicidal activity in both extra- and intracellular forms was similar or, in most cases, higher than founded for Glucantime, the best results were achieved by compounds **P**, **P2Py**, **PN** and **P3Py**. However, when we have checked the Mn^{II} complexes of these compounds (**P***, **P2Py***, **PN***, **P3Py*** and **Pal***) an opposite effect was observed. Such an effect can be ascribed to the protective effect that has been described for Mn^{II} complexes of aza-scorpiand ligands [18]. More interesting are the toxicity data, since all compounds tested were found to be much less toxic for macrophages than the reference drug. In general, all the compounds were from 14 to 30-fold less toxic than Glucantime. Toxicity values substantially influence the more informative selectivity index (SI) data, and best values were again obtained for the compounds **P2Py**, **PN** and **P3Py**, with SI exceeding those of the reference drugs by 37-, 32- and 30-fold in the case of **P2Py**, by 49-, 29, -27-fold for **PN**, and by 34-, 39-, 57-fold for **P3Py**.

Very similar conclusions are extracted from the *L. braziliensis* results shown in Table 3. The compounds **P2Py**, **PN** and **P3Py**, and in the same way the **P** gave the best SI results in the three assays performed, with values exceeding those of the reference drugs 37-, 32-, 30-fold in the case of **P2Py**, 49-, 29- and 27-fold for **PN**, 34-, 39- and 57-fold for the compound **P3Py** and for the **P** was 30-, 29- and 29-fold, respectively. It was also observable that the compounds **P2Py**, **PN**, **P3Py** and **P** had a better SI than the rest of compound assayed. The aza-scorpiand like macrocyclic derivatives **P3Py** had the lowest toxicity and highest SI values in both *L. infantum* and *L. braziliensis*.

Different authors have claimed that compounds having a SI values 20-fold higher than the reference are to be considered in possession of leishmanicidal properties [19]. This requirement is satisfied by compounds **P2Py**, **PN** and **P3Py**, in the case of *L. infantum* and also the compound **P** in the case of *L. braziliensis*.

The parasites invaded the cell and were converted to amastigotes within one day after infection and the rate of host-cell infection reached its maximum on day 10 (control experiment). The propagation of the parasite in J774.2 macrophages was studied by

Table 1
Stepwise basicity constants for compounds **P**, **PN**, **P2Py**, **P3Py** and **Pal** determined in 0.15 M $NaClO_4$ at 298.1 K.

Reaction	P[a]	PN[a]	P2Py[b]	P3Py[b]	Pal[c]
$L + H \leftrightarrows LH$[d]	10.19 (6)[e]	10.01 (1)	10.21 (1)	9.84 (5)	10.13 (1)
$HL + H \leftrightarrows LH_2$	9.19 (3)	8.71 (1)	8.84 (1)	8.52 (8)	9.40 (1)
$H_2L + H \leftrightarrows LH_3$	7.94 (4)	7.27 (1)	7.39 (2)	6.8 (1)	8.27 (1)
$H_3L + H \leftrightarrows LH_4$			3.12 (2)	3.4 (1)	7.02 (1)
log β[c]	27.32	25.99	29.56 (2)	28.6 (1)	34.84 (1)
Mean n° protons at pH = 7.4	2.7	2.4	2.5	2.0	3.2

[a] Taken from Ref. [7].
[b] Taken from Ref. [8].
[c] This work.
[d] Charges omitted.
[e] Values in parenthesis are standard deviations in the last significant figures.

Table 2
In vitro activity, toxicity and selectivity index found for the scorpiand-like azamacrocycle derivatives on extracellular and intracellular forms of *Leishmania infantum*.

Compounds	Activity IC_{50} (μM)[a]			Toxicity J774.2 macrophages IC50[b]	SI[c]		
	Extracellular promastigote forms	Axenic amastigote forms	Intracellular amastigote forms		Extracellular promastigote forms	Axenic amastigote forms	Intracellular amastigote forms
Glucantime	18.0	31.0	30.8	15.2	0.8	0.5	0.5
P	18.9	29.2	25.6	231.6	12.2 (15)	8.0 (16)	9.0 (18)
P*	40.7	40.4	30.7	218.6	5.4 (7)	5.4 (11)	7.1 (14)
P2py	15.5	25.4	19.6	254.0	16.4 (20)	10.0 (20)	13.0 (26)
P2py*	21.2	77.8	50.3	275.7	13.0 (16)	3.5 (7)	5.5 (11)
PN	21.7	28.1	17.4	365.3	18.8 (23)	13.0 (26)	21.0 (42)
PN*	38.2	45.0	60.1	387.9	10.2 (13)	8.6 (17)	6.5 (13)
P3py	18.8	15.9	6.3	467.8	24.9 (31)	29.4 (59)	74.3 (149)
P3py*	40.8	33.7	50.1	223.9	5.5 (7)	6.6 (13)	4.5 (9)
Pal	24.2	29.8	44.9	220.4	9.1 (11)	7.4 (15)	4.9 (10)
Pal*	27.0	33.6	61.6	258.1	9.6 (12)	7.7 (15.4)	4.2 (8)

Results are averages of four separate determinations.
[a] IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at concentrations employed (1, 10, 25, 50 and 100 μM).
[b] Towards J774.2 macrophage after 72 h of culture.
[c] Selectivity index = IC_{50} macrophage/IC_{50} extracellular and intracellular form of parasite. In brackets: number of times that compound SI exceeds the reference drug SI.

measuring the infection rates and the average number of amastigotes presented during a 10-day treatment period in *L. infantum*, where the rate of host-cell infection gradually increased: values close to 62% percent cells were infected on day 10 (Fig. 2A). The IC_{25} of each of the products was used as the test dosage in these assays and Glucantime was the reference drugs. The decrease in the infection rate for the compounds **P2Py**, **PN** and **P3Py** was always greater than 62%, making them much more effective than Glucantime (24%). Compound **P3Py** showed an especially remarkable behaviour by dropping the infection rate an 84%, close but minor is the effectiveness of compounds **P2Py** and **PN** with a reduction of 77% and 74% respectively, when were compared against the control. The average number of amastigotes increased to a peak on the 10th day of culture (Fig. 2B); at the end of treatment, the three compounds tested had significantly decreased the number of amastigotes present in the cells by 67%, 71%, and 77% respectively for **P2Py**, **PN** and **P3Py**, whereas Glucantime only showed a reduction of 30%. The same experiment was performed with *L. braziliensis*, and the results obtained concerning infection rates (Fig. 3A) and amastigote numbers (Fig. 3B) are shown in Fig. 3. Those results were similar to the ones obtained by *L. infantum*, with the exception of the inclusion of **P** compound. Nevertheless, the best result was provided by the compound **P3Py** which was the most effective reducing the inhibition capacity and intracellular replication, against both *Leishmania* spp.

3.3. Studies on the mechanism of action

We performed several experiments to elucidate a possible mechanism of action for the aza-scorpiand like macrocyclic derivatives, which showed a SI number 20 times higher. Thus, the compounds selected in the case of *L. infantum* were **P2Py**, **PN** and **P3Py**, while in the case of *L. braziliensis* (**P**, **P2Py**, **PN** and **P3Py**).

3.3.1. SOD enzymatic inhibition in parasites and human erythrocytes

These results prompted us to evaluate the inhibitory effect of the **P**, **P2Py**, **PN** and **P3Py** compounds on SOD activity to test their potential as competitors for the metallic ions of the enzyme. We used promastigote forms of *Leishmania* spp., which were shown to excrete Fe-SOD when cultured in a medium lacking inactive foetal bovine serum [20], and a range of drug concentrations from 0.1 to 50 μM was applied in each case.

The results obtained shown in Fig. 4, and the corresponding IC_{50} values were calculated and are included in the same figure. Significant inhibitory values of Fe-SOD activity were found for all compounds tested (Fig. 4C and D). In general, all the compounds significantly inhibited the *Leishmania* spp. Fe-SOD activity. Distinguishing, the compound **P3Py** which showed a 100% inhibition at the 5 μM and 12.5 μM dose for *L. infantum* and *L. braziliensis*, respectively (Fig. 4C and D). The design of an effective drug able to

Table 3
In vitro activity, toxicity and selectivity index found for the scorpiand-like azamacrocycle derivatives on extracellular and intracellular forms of *Leishmania braziliensis*.

Compounds	Activity IC_{50} (μM)[a]			Toxicity J774.2 macrophages IC50[b]	SI[c]		
	Extracellular promastigote forms	Axenic amastigote forms	Intracellular amastigote forms		Extracellular promastigote forms	Axenic amastigote forms	Intracellular amastigote forms
Glucantime	25.6	30.9	19.7	15.20	0.6	0.5	0.8
P	11.8	16.0	10.0	231.6	19.6 (30)	14.5 (29)	23.2 (29)
P*	32.8	54.3	30.4	218.6	6.8 (11)	4.0 (8)	7.3 (9)
P2py	11.3	16.0	10.4	254.0	22.5 (37)	15.9 (32)	24.4 (30)
P2py*	12.4	26.7	16.4	275.7	22.2 (37)	10.3 (20)	16.8 (21)
PN	12.3	24.9	16.6	365.3	29.7 (49)	14.7 (29)	22.0 (27)
PN*	29.5	59.2	28.9	387.9	13.1 (22)	6.6 (13)	13.4 (17)
P3py	23.0	23.9	10.3	467.8	20.3 (34)	19.6 (39)	45.4 (57)
P3py*	21.2	41.6	47.0	223.9	10.6 (18)	5.4 (11)	4.8 (6)
Pal	15.5	31.4	19.1	220.4	14.2 (24)	7.0 (14)	11.5 (14)
Pal*	25.5	42.6	77.8	258.1	10.1 (17)	6.1 (12)	3.3 (4)

Results are averages of four separate determinations.
[a] IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at concentrations employed (1, 10, 25, 50 and 100 μM).
[b] Towards J774.2 macrophage after 72 h of culture.
[c] Selectivity index = IC_{50} macrophage/IC_{50} extracellular and intracellular form of parasite. In brackets: number of times that compound SI exceeds the reference drug SI.

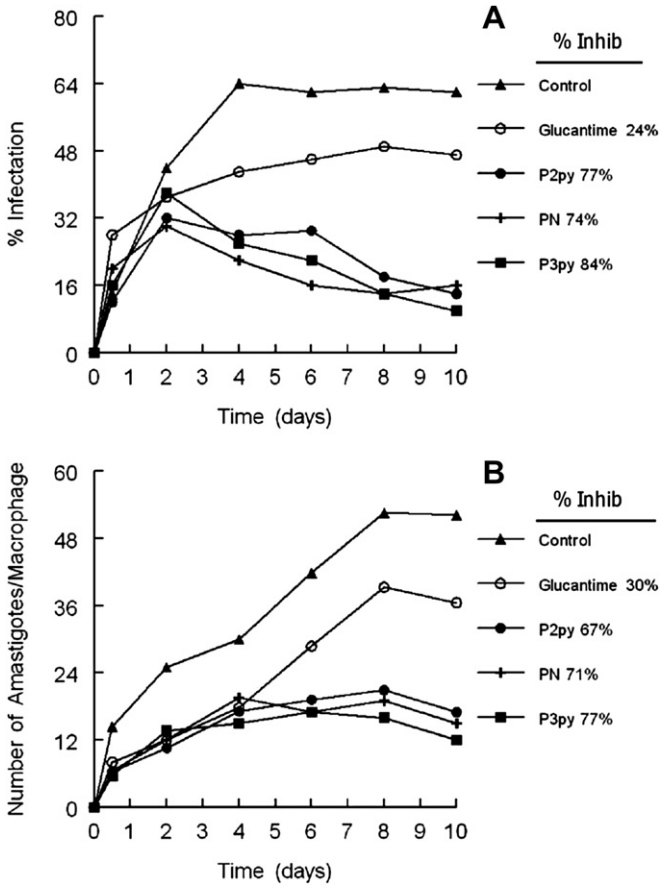


Fig. 2. Effect of scorpiand-like azamacrocycle derivatives on the infection rate and growth of *L. infantum*: (A) rate of infection; (B) mean number of amastigotes per infected macrophage cell: (▲) control; (○) Glucantime; (●) **P2Py**; (+) **PN**; (■) **P3Py** measured at IC_{25}. Values are the means of three separate experiments.

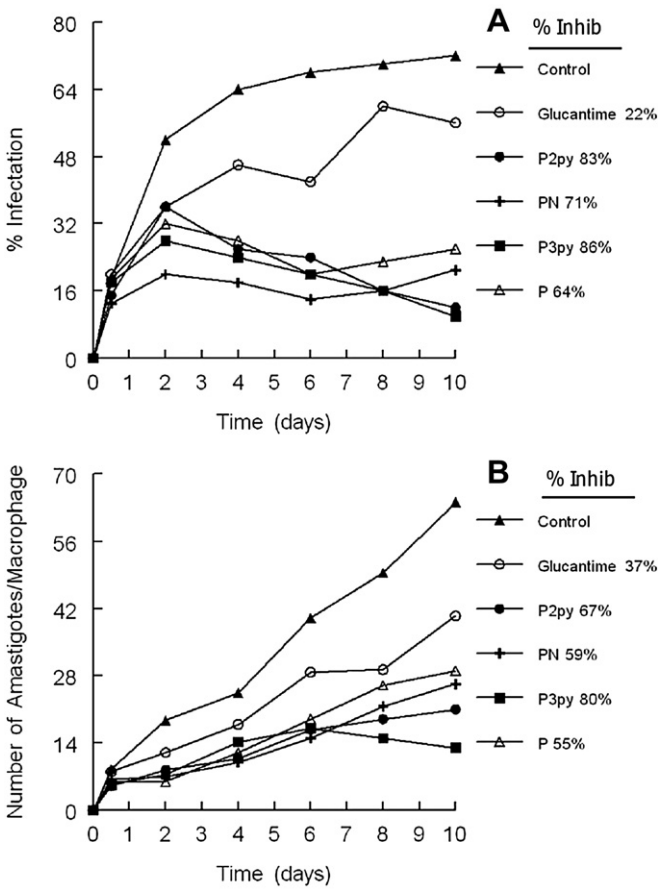


Fig. 3. Effect of scorpiand-like azamacrocycle derivatives on the infection rate and growth of *L. braziliensis*: (A) rate of infection; (B) mean number of amastigotes per infected macrophage cell: (▲) control; (○) Glucantime; (●) **P2Py**; (+) **PN**; (■) **P3Py** measured at IC25. Values are the means of three separate experiments.

inhibit the parasite Fe-SOD requires the inhibition of human SOD to occur to a lower extent; therefore, we have also assayed the effect of compounds on Mn-SOD and Cu/Zn-SOD of human erythrocytes (Fig. 4A and B). The results obtained showed that the inhibition percentages for human CuZn-SOD and Mn-SOD were very small for both the higher and the lower dosages, IC_{50} values 381.1, 178.5, 408.0 and 641.0 μM were reached on Mn-SOD from *E. coli* and for compounds **P**, **P2Py**, **PN** and **P3Py** against *L. infantum* (Fig. 4A) values 111.4, 40.1, 58.3 and 63.6 μM were obtained on CuZn-SOD from human, for the tested compounds against *L. braziliensis* (Fig. 4B).

3.3.2. Metabolite excretion effect

Leishmania species have a high rate of glucose consumption, which results in the acidification of the culture medium due to the incomplete oxidation of glucose, they excrete into the medium a considerable part of the hexose skeleton as partially oxidized fragments in the form of fermented metabolites, although their nature and percentage depend on the pathway used for glucose metabolism by each of the species considered [21,22]. ^{1}H NMR spectroscopy enables the identification of the fermented metabolites excreted by the parasites during *in vitro* culture. Fig. 5A shows the spectrum produced by cell-free medium four days after inoculation with *L. infantum*. The peaks that correspond to the major metabolites produced and excreted during growth were apparent when these spectrums were compared versus fresh medium (spectra not shown). *L. infantum* excretes mainly succinate and acetate as its major metabolites and in lower amounts D-lactate and L-alanine (Fig. 6A). The ^{1}H NMR spectra of the medium from drug-treated cells showed that the three compounds: **P2Py**, **PN** and **P3Py** significantly altered the metabolites excreted by *L. infantum*. When the promastigote forms of *L. infantum* were treated with compound **P2Py** at IC_{25} doses, the excretion of the catabolites succinate was clearly altered (Fig. 5B and Table 4) and a new peak, which was subsequently identified as ethanol, appeared in the spectrum. All excreted metabolites presented a reduction when **PN** product was added to the culture medium (Fig. 5C and Table 4) although significant ethanol excretion increase was observed. After addition of product **P3Py**, succinate, D-lactate and L-alanine excretions were reduced (Fig. 5D and Table 4), an increase on ethanol production could also be observed. When the test was ran against *L. braziliensis* in fresh medium succinate, acetate and both D-lactate and L-alanine were excreted, also a significant amount of ethanol (Fig. 6A). After the treatment of cultures with products: **P**, **P2Py**, **PN**, and **P3Py**; all compounds exhibited similar behaviour comparing with *L. infantum* but with some exceptions, such as, the ethanol outputs were clearly decreased (**P2Py** > **P3Py** ≫ **PN**). Regarding the D-lactate, the only compound that showed an increase effect was **P3Py** (Fig. 6 and Table 4).

3.3.3. Ultrastructural alterations

TEM revealed substantial morphological alterations in *Leishmania* spp. promastigotes after treatment with the newly aza-scorpiand like macrocyclic derivatives compared with the control sample (Figs. 7 and 8). All of the tested compounds (**P2Py**, **PN**, and **P3Py**) induced alterations in *L. infantum* promastigotes, but the product

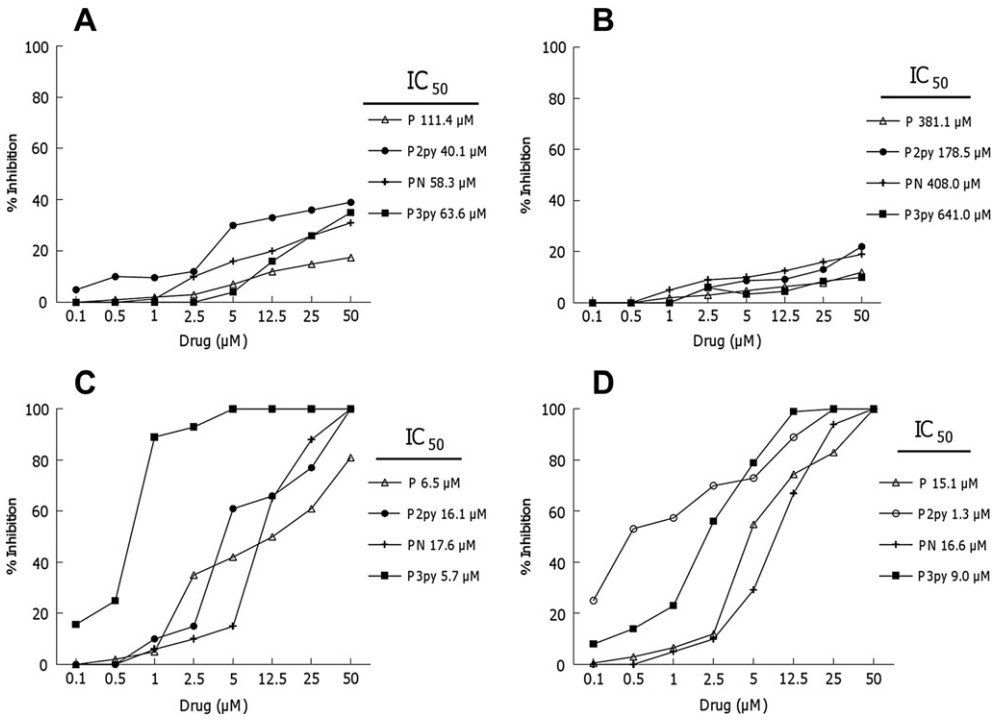


Fig. 4. (A) *In vitro* inhibition (%) of Mn-SOD from *E. coli* for the scorpiand-like azamacrocycle derivatives. (B) *In vitro* inhibition (%) of CuZn-SOD from human erythrocytes for the scorpiand-like azamacrocycle derivatives. *In vitro* inhibition (%) of Fe-SOD in (C) *L. infantum* and (D) *L. braziliensis* promastigotes for the scorpiand-like azamacrocycle derivatives. Values are means of three separate determinations. Differences between the activities of the control homogenate and those incubated with compounds were analyzed with the Newman–Keuls test. An IC_{50} was calculated by linear regression analysis from the Kc values at the concentrations employed (0.1–50 μM).

P2Py was one of the most effective products inducing the parasites to death (Fig. 7-2), those that were not dead were high electro density, suffering from size reduction and corrugated citoplasmatic membrane surface. In all treated parasites an increase on the number of glycosomes was observed in comparison with the control cultures. On parasites treated with **PN** product (Fig. 7-3) a high number of strongly electro-dense vesicles was observed. Promastigote forms treated contained a higher number of vesicles showing death-like aspect. Majority of the parasites treated with those products presented large vacuoles on their cytoplasm, most of those were empty and in some others the kinetoplast or the mitochondria were swollen. Equally very effective was the **P3Py** compound as can be observed on Fig. 7-4, were the massive alterations on that promastigote sample were produced and are extendable to all parasites treated. As could be observed, the cytoplasm was disorganized, both mitochondria and the kinetoplast were swollen and some areas of the citoplasmatic membrane were disrupted. When the effects of the products **P**, **PN**, **P2Py** and **P3Py** were tested against *L. braziliensis* using MET microscopy many structural alterations were observed shown on Fig. 8. Only **PN** product induced a very few alterations (photos not shown), while the remaining products were very effective when compared with the control. On Fig. 8-2, the full activity of **Py** products is shown were promastigotes treated were all dead, presenting all altered and almost empty cytoplasm. Some electro-dense vesicles were observed in both dead and living parasites. On cultures treated using **P2Py** product (Fig. 8-3) the supernatant was full with cellular rests from the disruption of parasites, although product **P2Py** was not as effective as product **P**, severe alterations on *L. braziliensis* promastigotes were induced, therefore most of the parasites were swollen and with very few of the cytoplasm content, presenting high numbers of empty vacuoles.

Also very effective was **P3Py** compound (Fig. 8, plate 4) that produced great alterations either on the cytoplasm at its ultrastructural level neither on the nuclear structure. Parasites treated with **P3Py** product presented the cytoplasm full of lipid nature small size vacuoles and some other empty ones, both the flagellum and the flagellum pocket seem to be disorganized, many nature free flagellum were observed on the supernatant.

4. Discussion

The present results document the leishmanicidal effect of new synthetic aza-scorpiand like macrocycles. Most studies on the *in vitro* biological activity of new compounds against *Leishmania* spp. are performed on promastigote forms because it is much easier to work with these forms *in vitro*. However, since extracellular forms are not the developed forms of the parasite in vertebrate hosts, evaluations made with extracellular forms are merely indicative of the potential leishmanicidal activity of the compounds tested. Consequently, a preliminary test using extracellular promastigote forms should always be complemented by a subsequent evaluation using intracellular forms (amastigotes in vertebrate host cells) for a better understanding of the activity results obtained [13]. Besides extracellular promastigotes, we also prepared extracellular axenic amastigote forms of both parasites according to the procedure described by Ref. [16]. Intracellular assays were performed by infecting macrophage cells with promastigotes, which transformed into amastigotes within 1 day after infection.

After taking in consideration the IC_{50} data shown in Tables 2 and 3, it can be seen that the **P**, **P2Py**, **PN** and **P3Py** were the most active of the 10 compounds tested in the three assays performed and against two *Leishmania* spp. studied (*L. infantum* and *L. braziliensis*). Furthermore, they were much more active than Glucantime against both the extra- and intracellular forms of the parasite. Although the rest of compounds even showed some interesting activity, they were less active in all cases. Regarding the cytotoxicity evaluation against macrophage cells, it was shown that almost all the compounds were substantially less toxic than the reference drug with

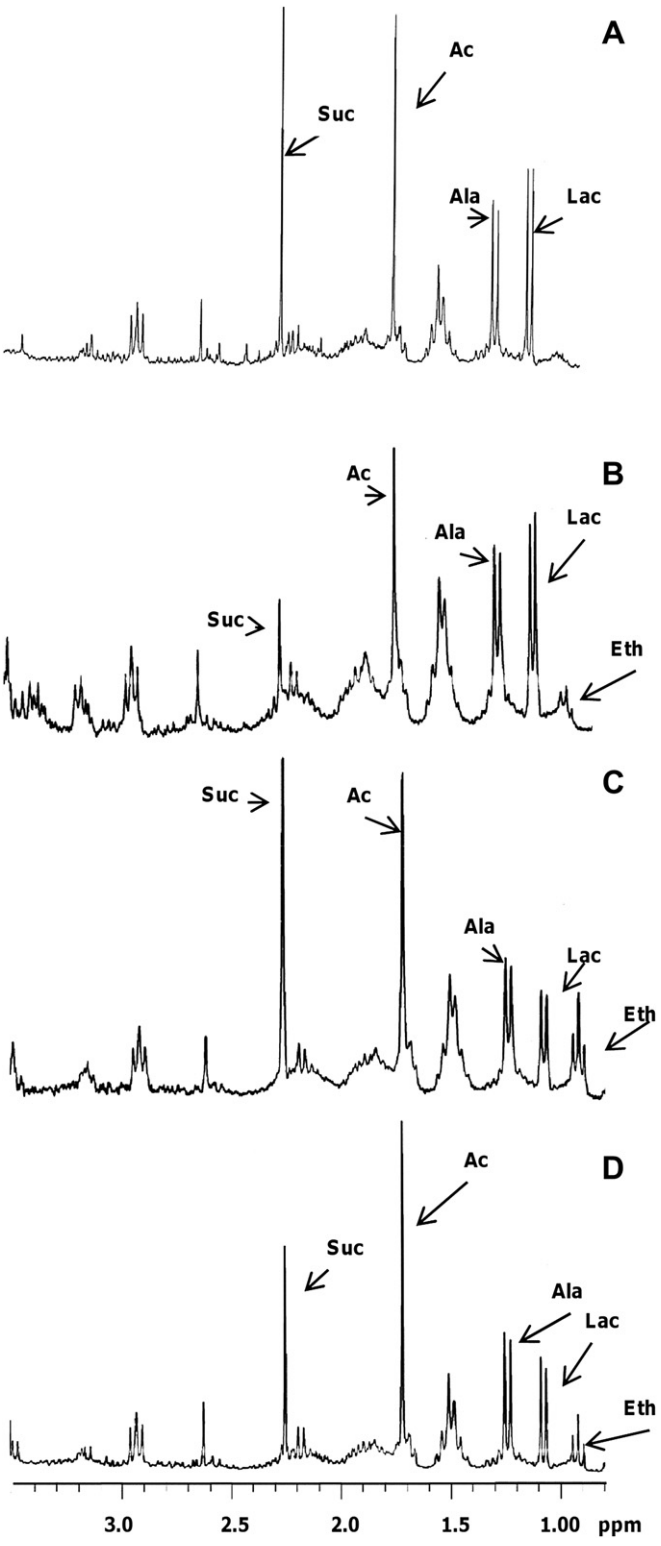


Fig. 5. ^{1}H NMR spectra showing metabolites excrete by promastigote forms of *L. infantum* untreated (A), *L. infantum* treated with compounds **P2Py** (B), *L. infantum* treated with compounds **PN** (C) and *L. infantum* treated with **P3Py** (D), IC_{25} dosage. Lac, D-lactate; Ala. L-alanine; Ac, acetate; Suc, succinate; Eth, ethanol; Pyr, pyruvate; Gly, glycerol and DMSO, dimethyl sulfoxide.

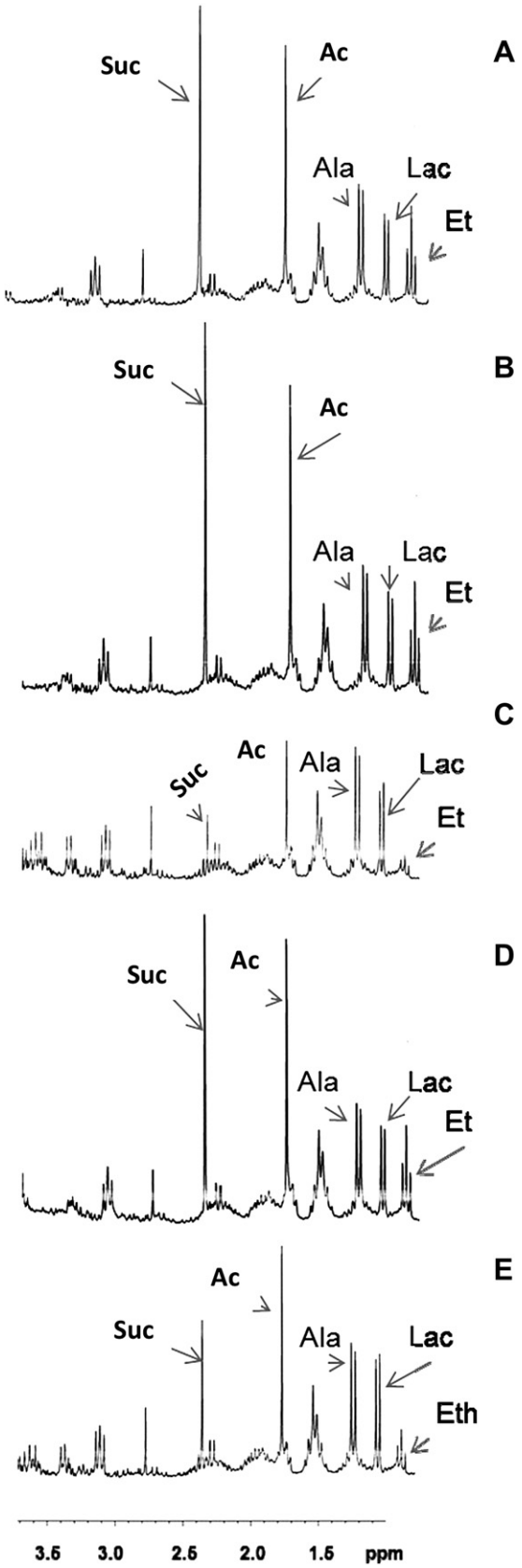


Fig. 6. ^{1}H NMR spectra showing metabolites excrete by promastigote forms of *L. braziliensis* untreated (A), *L. braziliensis* treated with **P** (B), *L. braziliensis* treated with **P2Py** (C) and *L. braziliensis* treated with **PN** (D), *L. braziliensis* treated with **P3Py** (E) IC_{25} dosage. Lac, D-lactate; Ala. L-alanine; Ac, acetate; Suc, succinate; Eth, ethanol; Pyr, pyruvate; Gly, glycerol and DMSO, dimethyl sulfoxide.

Table 4
Variation in the height of the peaks corresponding to catabolites excreted by *L. infantum* and *L. braziliensis* promastigote forms in the presence of scorpiand-like azamacrocycle derivatives with respect to the control test.

Compounds	*Leishmania infantum*					*Leishmania braziliensis*				
	Ac	Suc	Lac	Ala	Et	Ac	Suc	Lac	Ala	Et
P	nd	nd	nd	nd	nd	18%	22%	6%	5%	6%
P2py	−19%	−59%	13%	12%	10%	−46%	−78%	0%	8%	−71%
PN	−6%	−3%	−44%	−18%	110%	8%	2%	0%	0%	−6%
P3py	1%	−36%	−38%	−12%	28%	−13%	−47%	19%	10%	−59%

Ac, acetate; Suc, succinate; Lac, D-lactate; Et, ethanol; Ala, L-alanine. (−) peak inhibition; (+) peak increasing; (=) no difference detected; (nd) no determined.

IC_{50} values over 200 μM, while against Glucantime it was 15.2 μM. Considering now the more illustrative selectivity index values, compounds **P**, **P2Py** and **P3Py** were again the most potentially interesting, since their SI values were >20 times higher than that one of Glucantime, both for *L. infantum* as for *L. braziliensis*, and according to some authors [16], only those which the SI value is over 20 are good enough to be consider interesting as antiparasitic.

The propagation in macrophage J774.2 cells data showed results that are in accordance with those described for intra- and extracellular forms of *Leishmania* spp. Attending to other studies to elucidate the possible mechanisms of action, it should be noted that these compounds not only showed greater alterations in glucose catabolism, but also that it led to greater levels of Fe-SOD inhibition. Since the Fe-SOD is a specific chemotherapy target also present in mitochondrial localization, this fact would agree with the hypothesis above that this less bulky compound might have a greater ability to pass through the mitochondrial membrane.

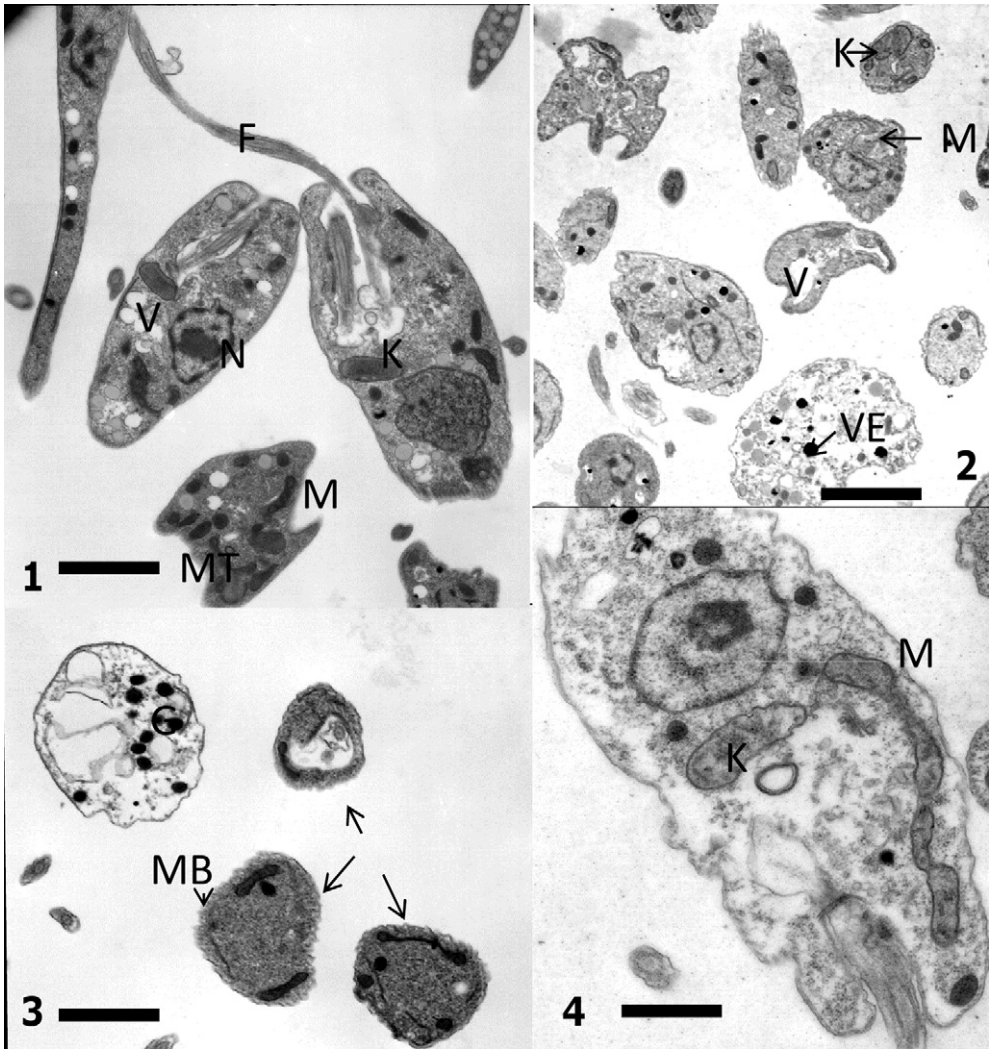


Fig. 7. Ultrastructural alterations by TEM in promastigotes of *L. infantum* treated with **PN**, **P2Py** and **P3Py** products. Plate 1. Control parasite of *L. infantum* showing organelles with their characteristic aspect, such as nucleus (N), mitochondrion (M), glycosomes (G), microtubules (MT), flagellum (F), kinetoplast (K) and vacuoles (V) (bar = 1.000 μm). Plate 2. Promastigotes of *L. infantum* treated with **PN** showed high-density vesicles (VE) and kinetoplast (K) and swollen mitochondria (M) (bar = 1.590 μm). Plate 3. *L. infantum* treated with **P2Py** with glycosomes (G), high electro-dense (arrow) and corrugated citoplasmatic membrane (MB) (bar = 1.590 μm). Plate 4. Parasites treated with **P3Py** with mitochondrion (M) and swollen kinetoplast (K) (bar = 0.350 μm).

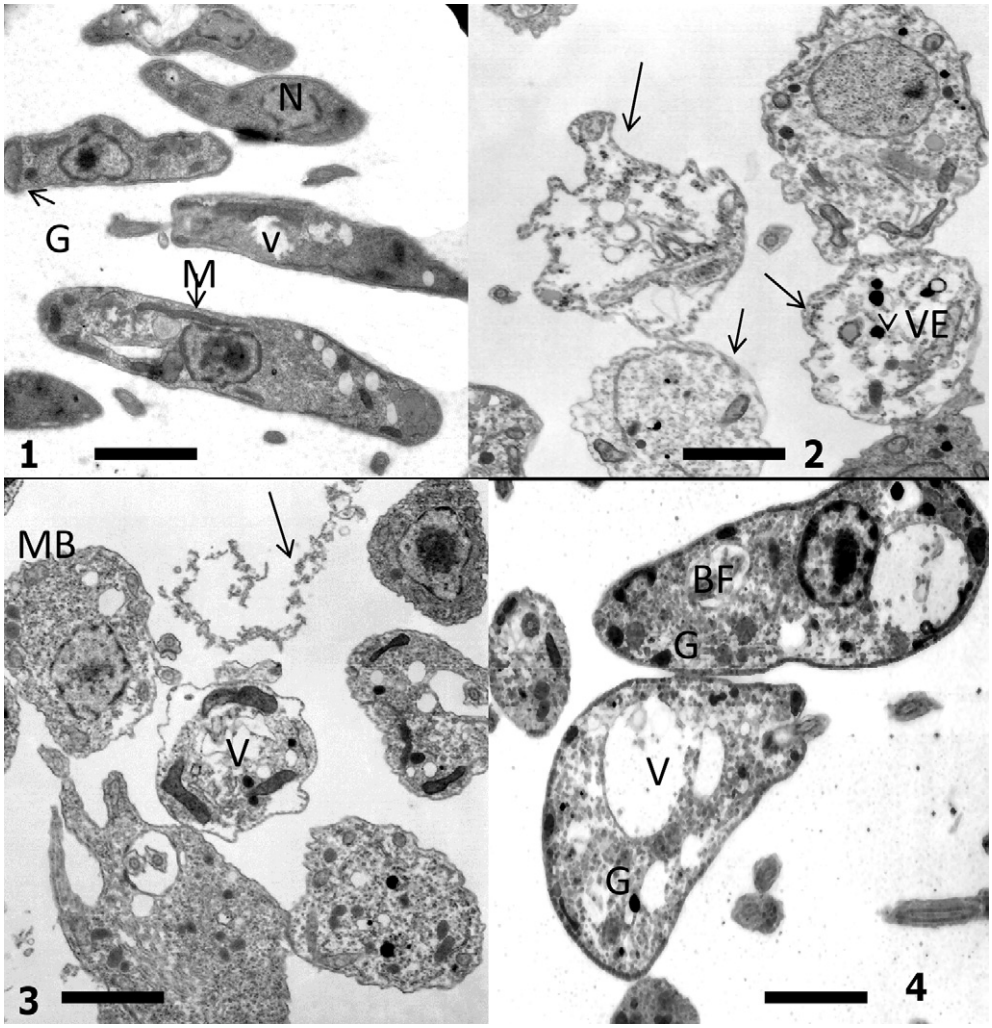


Fig. 8. Ultrastructural alterations by TEM in promastigotes of *L. braziliensis* treated with **P**, **P2Py** and **P3Py** products. Plate 1. Control parasite of *L. braziliensis* showing organelles with their characteristic aspect, such as nucleus (N), mitochondrion (M), glycosomes (G) and vacuoles (V) (bar = 1.000 μm). Plate 2. Promastigotes of *L. braziliensis* treated with **P** dead (arrow) with high-density vesicles (VE) (bar = 1.590 μm). Plate 3. *L. braziliensis* treated with **P2Py** with remaining fragments of destroyed parasites (arrow), empty vacuoles (V) and corrugated citoplasmatic membrane (MB) (bar = 1.590 μm). Plate 4. Parasites treated with **P3Py** with large empty vacuoles (V), large number of granulites (G) and altered flagellum pocket (BF) (bar = 0.583 μm).

It is well known that trypanosomatids are unable to completely degrade glucose to CO_2 under aerobic conditions. As a consequence, they excrete into the medium a considerable part of the hexose skeleton as partially oxidized fragments in the form of fermented metabolites, although their nature and percentage depend on the pathway used for glucose metabolism by each of the species considered [21,22]. The final products of glucose catabolism in *Leishmania* are usually CO_2, succinate, acetate, D-lactate, L-alanine, and ethanol [23]. Among them, succinate specially is relevant since its main role is in maintaining the glycosomal redox balance, allowing the reoxidation of NADH produced in the glycolytic pathway. Succinic fermentation has the advantage of requiring only half of the phosphoenolpyruvate produced to maintain the NAD^+/NADH 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 each species [24].

In order to obtain some information concerning the effect of the tested compounds on metabolism of glucose in the parasites, we registered the ^{1}H NMR spectra of promastigotes from *L. infantum* and *L. braziliensis* after treatment with compounds studied and the final excretion products were identified qualitatively and quantitatively. The results were compared with those found for promastigotes maintained in a cell-free medium (control) for four days after the inoculation with the parasite. The characteristic presence of acetate, D-lactate, and succinate was confirmed in the control experiments performed on all two species. However, noteworthy differences between them were reduction in the excretion of ethanol by *L. braziliensis*, whereas ethanol was not detected in *L. infantum* promastigotes. When parasites (*L. braziliensis*) were treated with the compound **P**, no significant effect was found. The other compound tested, **P2py**, showed a significant decrease of acetate in *L. braziliensis*, whereas no variations were found in the case of *L. infantum*. The cause of this metabolic change appears to be an action on enzymes acetate: succinate CoA transferase and succinyl-CoA synthetase directly involved in mitochondrial production of acetate from acetyl-CoA [25]. Another metabolite, which appears significantly reduced in both species is the succinate, so it seems that the glycosomal enzymes that are involved in the succinate production must be disrupted. Finally in *L. braziliensis* a remarkable decrease in the output of ethanol was found also, which means that the only pathway properly working that keep the parasite in optimum level of energy to grow, is the way to use alanine in the cytosol, being both glycosomal and mitochondrial pathways altered [26]. Those

parasites, which were grown in the presence of compound **PN** (*L. infantum*), showed a disruption at glycosomal level, doing the pathway to obtain ethanol the main way to obtain the energy, rather than the D-lactate via (levels of excretion drastically decreased, potentially inhibition at trypanothione reductase/glyoxilase levels) [26]. Regarding to the compound **P3py**, both species were inhibited in somehow in their glycosomal enzymatic clusters, so the subsistence of the parasites is allowed by the energy obtained at mitochondrial level. The way to affect one of the most significant metabolite excretion, succinate, was also shared by the two species, decreasing its excretion to the medium with respect to control. On the other hand, the end metabolites of anaerobic oxidation, such as the D-lactate (in *L. braziliensis*) and ethanol (*L. infantum*) appeared slightly increased in the culture medium, this suggests that despite being available glucose in the medium compound **P3Py** interferes with some step of tricarboxylic cycle (TCA) preventing the parasite uses this fundamental way and having to resort to means of compensation for energy production such as those used in absence of glucose. Another mechanism of action could be postulated for this compound is inhibition of transaminases, so that pyruvate cannot be transformed into alanine in an amount sufficient for it to appear in the culture medium [27].

They also showed a wide range of ultrastructural alterations to the promastigotes forms of *Leishmania* spp. treated with compounds were found. These alterations, which mainly took place at the mitochondrial and cytoplasmic levels, could be related to the metabolic changes mentioned above concerning the production of succinate and acetate, which might originate from a disturbance in the enzymes involved in pyruvate metabolism inside the cells.

Thus, these *in vitro* results show that the synthetic aza-scorpiand like macrocyclic derivatives are potentially promising agents for the treatment of *Leishmania* infection. Further in vivo studies are warranted to further evaluate this potential, actually a patent about this family of compound has been already filled (P201132035).

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