UNIVERSIDAD DE GRANADA

DEPARTAMENTO DE PARASITOLOGÍA

Memoria de Tesis presentada por D. Álvaro Miguel Martín Montes para aspirar al título de Doctor con Mención Internacional por la Universidad de Granada, Programa de Doctorado en Medicina Clínica y Salud Pública (B12.56.1)

Study of new synthetic compounds against *Leishmania* species

Álvaro Miguel Martín Montes

Granada, septiembre de 2019

Editor: Universidad de Granada. Tesis Doctorales
Autor: Álvaro Miguel Martín Montes
ISBN: 978-84-1117-324-7
URI: http://hdl.handle.net/10481/74723

1. Summary

Leishmaniasis is a widespread vector-borne neglected tropical disease (NTD) caused by a protozoan parasite of the Genus *Leishmania* spp. There are 21 known species that belong to this genus, all of then pathogenic. Depending on the species infecting the host, different clinical features may appear. These clinical forms can be classified depending on the affected organ or organs, these forms are: Visceral Leishmaniasis (VL) when the liver and spleen among other organs are compomised.

Cutaneous Leishmaniasis (CL) takes place when a cutaneous ulcer or ulcers appear as a result of the parasite activity in the host skin.

The last clinical form is Mucocutaneous Leishmaniasis (ML) that causes facial deformations as a result of connective tissue destruction.

Although similar, these three species present many variations between themselves, which reduces the chance of finding a single drug that could be useful against them three.

Leishmaniasis is endemic in 98 countries, it is estimated that a million of new cases take place annually and it is responsible of 20,000-30,000 deaths each year. The most concerning facts of the disease are its recent worldwide expansion and the lack of vaccines and effective treatments, due to the fact that they're based and outdated drugs with many side effects, such as an elevated toxicity and cost, along the increase of resistances in the recent years. This work is oriented towards the search of new drugs that could suit as candidates for new treatments that overcome the present drawbacks that current ones have.

SUMMARY

Current treatments are mainly based on pentavalent antimonials being the most used sodium stibogluconate (Glucantime®) and meglumine antimoniate (Pentostam®). Those drugs are nephrotoxic and hepatotoxic and also present cardiac effects and cause pancreatitis. Being the most used drugs, in the recent years they had triggered resistances, failing in 60% of cases in India.

These drugs are also quite unstable, and require special storage conditions which end rising its cost, for that reason, stability is a desirable feature for a new drug.

Another drawback that present treatments have it's the difficulty of compliance, as all of them require to be administered mainly via intravenous injection that has to be performed by qualified personnel and this makes compulsory travelling to a health center, what can imply long distances in developing countries.

The aim of this work and the one of our research group is searching for new compounds that could serve as candidates for new medicines against Leishmaniasis, due to the WHO considers that its study should be prioritary [1] and its treatments updated [2].

These compounds should have the following features in order to be considered better than the current ones: lower toxicity, affordable price, stability, and easy administration regime.

For many years we have been collaborating with several chemistry groups that synthetize these new compounds for us, and we test them in our laboratory.

Chemistry groups who provided compounds for being studied in our laboratory and are present in this thesis research come from University of Valencia, who provided the [1,2,3]triazolo[1,5-a]pyridine salts and alkylated ethylenediamine units, University of Pamplona provided the arylamine Mannich base derivatives and the seleno-

compounds and University of Chile and CSIC (Spain) synthetized the 3-alkoxy-5-nitroindazole-derived ethylamines

1. Resumen

La leishmaniasis es una enfermedad muy extendida, de transmisión vectorial, y carácter tropical y está considerada una enfermedad olvidada (Neglected Tropical Disease) causada por un protozoo parásito del género *Leishmania* spp. A este género pertenecen 21 especies y todas ellas son patogénicas. Según la especie que infecte al hospedador aparecen diferentes manifestaciones clínicas. Estas formas clínicas se pueden clasificar dependiendo del órgano o los órganos afectados, estas formas son: Leishmaniasis Visceral (LV) cuando se ven afectados el hígado y el páncreas entre otros órganos.

La Leishmaniasis cutánea (LC) toma lugar cuando una úlcera o varias aparecen como resultado de la actividad parasitaria en la piel del hospedador.

La última forma clínica es la Leishmaniasis Mucocutánea (LM) que causa deformaciones faciales como resultado de la destrucción del tejido conectivo de la cara.

A pesar de sus similitudes, estas tres especies presentan muchas variaciones entre ellas, lo que reduce las posibilidades de encontrar una única droga que pueda ser usada contra todas ellas.

La leishmaniasis es endémica en 98 países, y se estima que un millón de nuevos casos se suceden anualmente y que es responsable de entre 20000 y 30000 muertes al año. Los hechos más preocupantes de la enfermedad son su reciente expansión a nivel global y la ausencia tanto de vacunas como de tratamientos eficaces, dado el hecho de que los tratamientos actuales están basados en drogas desfasadas con muchos efectos secundarios, como una elevada toxicidad y alto coste, además del aumento de resistencia en los últimos años. El presente trabajo está orientado hacia la

búsqueda de nuevos compuestos que puedan servir como candidatos para nuevos tratamientos que superen los inconvenientes que los actuales tienen.

Los tratamientos actuales están basados principalmente en antimoniales pentavalentes, siendo los más usados el estibogluconato de sodio (Glucantime®) y el antimoniato de meglumina (Pentostam®). Estas drogas son nefrotóxicas y hepatotóxicas y además presentan efectos cardíacos y pueden ocasionar pancreatitis. Al ser las drogas más usadas, en los últimos años han aparecido resistencias, llegando al fallo en un 60% en la India.

Además, estos medicamentos son inestables y requieren de condiciones de almacenamiento especiales que acaban incrementando el coste, por esa razón, la estabilidad es una característica deseable en una nueva droga.

Otro inconveniente que presentan los tratamientos actuales es la dificultad de cumplimiento del calendario de administración, pues todos ellos requieren ser administrados principalmente por vía intravenosa y esta requiere que sea administrada por personal cualificado, lo que obliga a los pacientes a trasladarse a un centro médico, lo que puede significar largas distancias en aquellos países en desarrollo.

El objetivo de este trabajo y el de nuestro grupo de investigación es buscar nuevos compuestos que puedan servir como candidatos para nuevos tratamientos contra la leishmaniasis, ya que la OMS la considera una enfermedad cuyo estudio debe ser prioritario [1] y sus tratamientos deben de actualizarse [2].

Estos compuestos deberían presentar las siguientes características para ser considerados mejores que los actuales: baja toxicidad, precio asequible, estabilidad y facilidad de administración.

RESUMEN

Durante muchos años hemos estado colaborando con diferentes grupos de Química para que ellos sinteticen los compuestos que nosotros ensayamos en nuestro laboratorio.

Los grupos de química que nos han provisto de compuestos para ser estudiados en nuestro laboratorio y que se encuentran presentes en este trabajo de tesis son: la Universidad de Valencia, que proporcionó las sales [1,2,3]triazolo[1,5-a]piridinas y las unidades alquiladas de etilendiamina, la Universidad de Pamplona, que proporcionó los derivados de arilaminas de bases de Mannich y los compuestos de selenio y la Universidad de Chile y el CSIC (España) sintetizó las etilaminas derivadas de 3-alcoxi-5-nitroindazol.

2. Objectives:

1- Test new synthesis compounds against three species of genus *Leishmania*, each one producer of a clinical form of the disease (*L. infantum*, *L. braziliensis* and *L. donovani*)

2- Reveal the compound's citotoxicity by testing them against macrophage cells from the J774.2 cell line.

3- Find out for every compound their leishmanicidal activity against both forms (extracellular and intracellular) for each parasite.

4- Compare citotoxicity and leishmanicidal activity with that of the reference drug Glucantime ®

5- For those compounds whose features result better than the reference drug, test how they affect the parasite ability to infect and reproduce within the host cells.

6- Make a first approach to the drug mechanism of action, determining if their leishmanicidal activity is due to their ability to inhibite the parasite FeSOD activity, the alteration of glucose catabolism or of the mitochondrial membrane potential.

2. Objetivos:

1- Probar nuevos compuestos de síntesis contra tres especies del género *Leishmania*, cada uno productor de una forma clínica de la enfermedad (*L. infantum*, *L. braziliensis* y *L. donovani*)

2- Poner de manifiesto la citotoxicidad de los compuestos probándolos contra células de macrófago de la línea J774.2.

3- Calcular la actividad leishmanicida de cada compuesto para ambas formas (extracelular e intracelular) de cada parásito.

4- Comparar citotoxicidad y actividad leishmanicida con la de la droga de referencia Glucantime ®

5- Para aquellos compuestos cuyas características superen a las de la droga de referencia, examinar cómo afectan a la capacidad de infección y de división celular dentro de las células del hospedador.

6- Realizar una primera aproximación al posible mecanismo de acción de las drogas, determinando si su actividad leishmanicida es debida a que pueden inhibir la actividad FeSOD, alterar el catabolismo de la glucosa o el potencial de membrana mitocondrial.

3. Introduction

3.1. *Leishmania spp*. and Leishmaniasis

Genus *Leishmania* belongs to the protozoan parasite family of Trypanosomatids, being phyllogeneticaly linked to other flagellated parasites such as the ones of genus *Trypanosoma, Chritidia* and *Phytomonas* according to Molyneux and Ashford's 1983 classification. There are 21 species of genus *Leishmania* and all of them are pathogenic and parasitic. For this work, three species were selected, each of them produces one of the three possible clinical forms of the disease: *L. infantum* as producer of Cutaneous Leishmaniasis (CL), *L. braziliensis* as a representative for Mucocutaneous Leishmaniasis (ML) and *L. donovani* as the most common etiologic agent of Visceral Leishmaniasis (VL).

The illness is endemic in 98 countries and causes 20,000-30,000 reported deaths annually, with a million of new cases each year. Areas in which Leishmaniasis dwells tend to be developing countries whose conditions complicate prevention, detection and treatment of the disease, so probably, statistics are underreported.

Along all these facts, there's another one that is even more concerning, and is the fact that the illness is spreading worldwide, as new cases has been reported in countries that were free of the disease such as Paraguay, where 19 cases of visceral leishmaniasis were reported in 2018 [3] Other examples of expansion are found in Brazil, Argentina and Uruguay, where several human cases of visceral leishmaniasis have been reported [4].

also, in Spain, hares and rabbits have been found as new reservoirs that enable a new life cycle to take place in the country [5].

Leishmaniasis is not only a disease, is more likely disease complex, as there are different clinical forms that can be caused depending of the infecting species. Symptoms may also vary depending on the host immunocompetence and number of

parasites invading the host, so it is not easy to find to patients that present exactly the same symptoms.

Cutaneous leishmaniasis is the most common clinical form and causes one or several ulcers (disseminated cutaneous leishmaniasis) in areas close to the sandfly bite. When the lesion is cured, a lifelong scar remains. In occasions, when the treatment is not administered on time or in an inappropriate quantity, this form can evolve to visceral leishmaniasis. This one is the mildest form of the disease, but sometimes it can evolve to VL when the treatment is not delivered on time or if it is ineffective. The studied species that causes this form is *L. infantum*. A particular feat of this species is that there are dermotropic and viscerotropic strains that cause CL or VL respectively.

Visceral leishmaniasis is the most life-threatening form, as it means a parasite attack to internal organs such as liver and spleen, where the main symptom, hepato and splenomegaly is caused (which can be seen at first glance as a swollen abdomen) along with irregular fever and anemia. If there is no treatment, its lethality can reach a 95%, making it the most life-threatening clinical form of the disease. Two species that cause this clinical form are *Leishmania donovani* and *L. infantum*.

Mucocutaneous leishmaniasis is a clinical form that affects mainly face and throat and causes deformations due to destruction of connective tissue. In the most severe cases, it can cause holes in nose and palate. These deformations often cause social rejection.

A species causing each of the clinical forms has been selected for this research work. As a representative of CL, *L. infantum* has been studied, for VL, *L. donovani* has been selected, as it is the only known species that causes this form, along some *L. infantum* viscerotropic strains. Finally, *L. braziliensis* is the species that has been studied for ML. The most remarkable difference is their geographical distribution, VL is located in India and East Africa, but in the recent years it has spread to Spain and Brazil, where the

traditional forms were CL and ML, respectively. It also has recently expanded to Paraguay, a country that was free of the disease until now, with 19 cases reported in 2018 and 560 cases reported since 2009 till the present day in Madrid, Spain, with a 70% of these cases taking place in immunocompetent hosts.

CL is located in America, the Mediterranean basin, Africa and Central Asia and ML is only present in South America (mainly in Brazil, Bolivia and Peru).

Parasites are transmitted to the human host by the bite of hematophagus sandflies. In Asia and Europe those vectors belong to genus Phlebotomus spp. and in America to genus Lutzomya spp. Due to their need of the proteins present in vertebrate's blood, female sandflies bite the host, and inoculate the parasite extracellular form known as promastigote. This form is flagellated and can move freely in the bloodstream, and uses this ability to reach macrophage cells. Those cells proceed to engulf the parasite and try to fuse lysosomes to the vacuole where the parasites are. Trypanosomatids are able to resist the enzymes present in the lysosomes, and they proceed to morph into the intracellular form know as amastigotes. This is the reproductive form of trypanosomatid parasites, and they multiply within the vacuole that now receives the name of parasitophorous vacuole. Amastigotes divide in such large numbers that end lysing the cell, and again in the bloodstream they morph into promastigotes to continue the cycle. Another sandfly can come and take another bloodmeal, this time absorbing infected macrophages, that will underwent another transformation to promastigotes in the midgut and will move to the salivary glands in order to be ready for a new infection.

Current treatments are mainly based on pentavalent antimonials being the most used sodium stibogluconate (Glucantime®) and meglumine antimoniate (Pentostam®). Those drugs are nephrotoxic and hepatotoxic and also present cardiac effects.

Amphotericin B and Miltefosine are other alternatives, but are also toxic specially Miltefosine, that possess teratogenic effects. The most effective drug is Liposomal

amphotericin B, but it is the most expensive one. Bearing in mind that Leishmaniasis tends to appear in developing countries, most of the times it is unaffordable.

3. 2. Taxonomic classification

When classifying *Leishmania* species, two main criteria are the geographic location and the clinical traits they cause. On the other hand, subgenus is defined by the development location in the vector's midgut, according to [6]. There are two subgenus, *Leishmania* and *Viannia. Leishmania* subgenus presents its development in the midgut upper areas, above the pylorus, in the thoracic and abdominal midgut, being called suprapylarian development, typical of the most evolved species. *Viannia's* development however, is not restricted to these areas, and requires part of this development in the bottom regions of the midgut before migrating to the upper regions, being this called in consequence peripylarian development, as takes places in all the midgut.

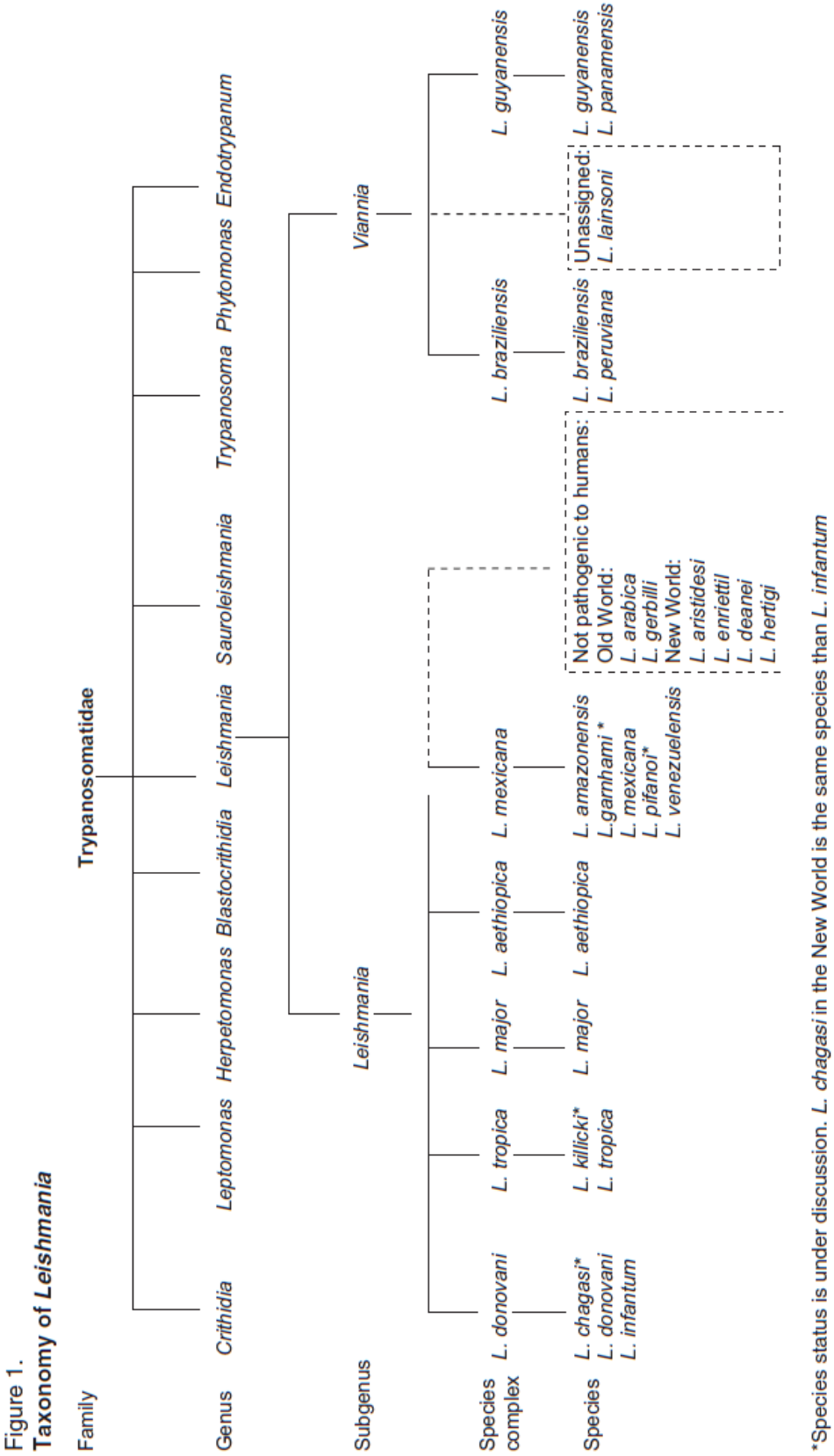


Figure 1.
Taxonomy of *Leishmania*

Control of leishmaniasis. WHO TRS Series, #949, 2010 [7]

3. 3 Morphology

Leishmania parasites possess, as other species belonging to *Trypanosomatidae* family, different forms due to the different environments in which the parasite lives along its life cycle. Roughly, it can be simplified to two different forms, an extracellular one that can be found in the sandfly vector's digestive tract and serves as the infective stage is known as promastigote, and the intracellular one that is the one in charge of parasite division that can be found in the host cells is known as amastigote. [8].

Promastigotes main feature is a flagellum that the parasite uses to move freely inside the vector and inside the host. In order to reach the macrophages in which the infection and division take place. This structure can be as long as the parasite's body or even twice as long. Promastigotes are long and fusiform and tend to measure 15-20 µm. Along the flagellum, the most important structure is the kinetoplast, a heap of mitochondrial DNA located inside in one of the extremes of the only but large mitochondria trypanosomatids have [9].

Amastigotes, on the other hand, lack of flagellum and can be found only in the host monocyte system cells, mainly macrophages, where the parasite division takes place. Once the macrophage phagocytes the parasites, it tries to destroy them by fusing a lysosome to the phagosome, but *Leishmania* parasites can resist the lytic enzymes and then underwent conversion to amastigotes and start dividing. The cell compartment where they are is now known as parasitophorous vacuole.

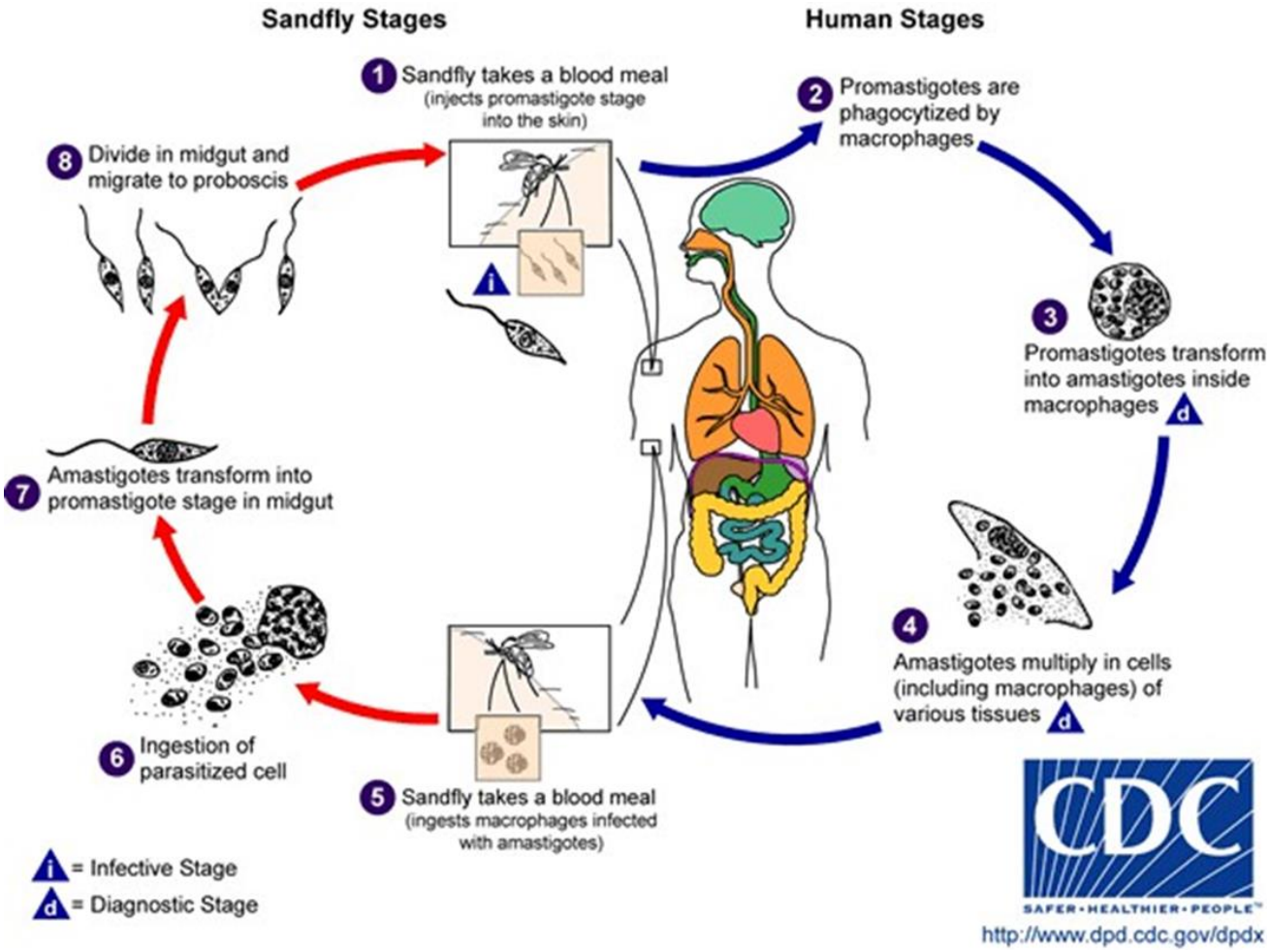
Amastigotes are small (2-5 µm diameter) and round. The flagellum in this form is vestigial [10].

3. 4. Life cycle

Leishmania life cycle starts when a phlebotomine sandfly takes a bloodmeal in a vertebrate host. Diptera inoculate their saliva with the purpose to fluidify blood and ease their intake. In this point promastigotes are inoculated in the host. Promastigotes use their flagellum to reach macrophages that will phagocytose them. Once inside the macrophage, the host cell will fuse a lysosome to the phagosome, but this will not work

as *Leishmania* resists the lytic enzymes, and then will turn into amastigotes, that will start dividing inside the parasitophorous vacuole until the host cell is lysed. Once outside the cell, amastigotes will turn again in promastigotes and so the cycle inside the host will be closed.

Another sandfly that bites an infected host will take infected macrophages. Amastigotes will underwent conversion to promastigotes and will migrate to the salivary glands once their development is complete, being ready for a new infection. These promastigotes are called metacyclic promastigotes, they take from 6 to 9 days to reach this stage through a process called metacyclogenesis, making these forms are responsible of human infection and disease [11].



https://www.cdc.gov/parasites/leishmaniasis/biology.html [12]

3. 5 Diagnosis

As leishmaniasis has different clinical forms, several diagnosis methods can be used. They can be roughly divided in parasitologic methods and molecular methods.

Parasitologic methods are mainly based in direct observation of the parasite in any of it forms. Those methods comprise biopsies, lesion scraps, impregnations, aspirations, punctions and other techniques that involve collecting samples of places where parasites are suspected to be such as ulcers in CL or hepatosplenomegaly in VL. Those techniques are quick and affordable, and are used worldwide for these reasons. However, they can often lead to false positives due to their low sensibility [13].

Molecular techniques are a better choice in areas where the technology and materials are available, as they can detect parasite DNA in very low quantities, making the PCR the best choice for its high sensitivity and specificity values [14]. Another advantage is that the sample collection is non-invasive.

3.6 Current treatments and associated problems

Treatment for leishmaniasis is based in outdated drugs, as it is a group of diseases that mainly threats developing countries, so pharmaceutical companies are not interested in its research as they won't take profit from it. The most used drugs were developed at the beginning of the 20th century.

Pentavalent antimonials are the main group of drugs that is used nowadays. They selectively inhibit glycolysis and fatty acid oxidation enzymes. There are two presentations of this group, meglumine antimoniate (known by the commercial name of Glucantime ® Aventis, France) and sodium stibogluconate, whose commercial name is Pentostam ® (Glaxo SmithKline, UK). These drugs are administered via the parenteral route, which requires trained staff to perform it, so patients must travel to health centers to take their medicines, and in a developing country this means vast distances in many cases. This fact and the administration schedule (from 10 to 30 days) complicate the

treatment, and this is thought to have triggered resistances over the last years elevating the treatment failure to 60% in Bihar, India [15].

These drugs also have other drawbacks, since the painful injections for the patient to toxic effects for the heart mainly, and also toxic for liver, kidneys and pancreas to a minor extent. They also produce mortality in 3-6% of cases.

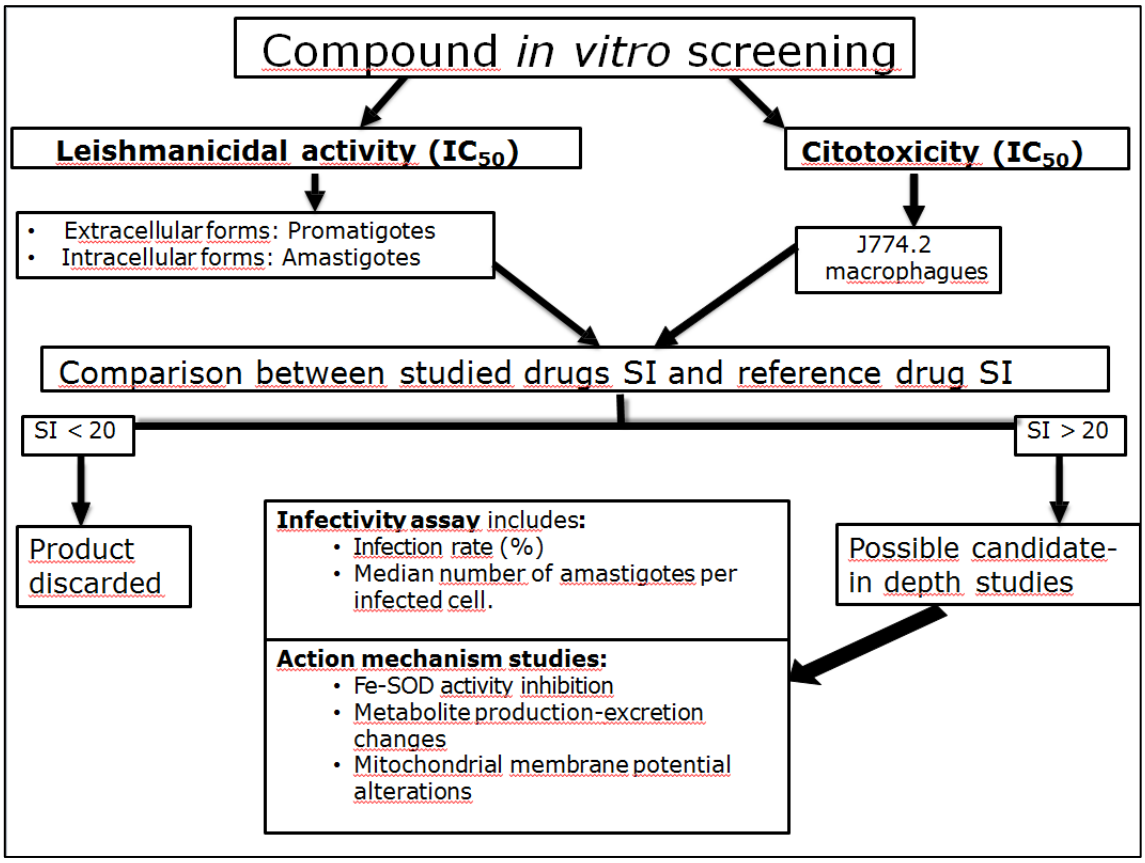
Pentamidine is another extended drug in human and canine leishmaniasis [16]. *In vivo* effects of this drug are yet unclear, but its many side effects are well known, such as nephrotoxicity and hypoglycemia and even eventual diabetes, along cardiac and respiratory effects [17].

Amphotericin B is another commonly used drug, but despite its efficacy it shows nephrotoxicity and can force the patient to be monitored in order to prevent further damage. It joins to the ergosterol in *Leishmania's* membrane and increases its permeability, which eases the ionic intake and so it leads to cell death. Among the 4 formulations this drug is produced, liposomal amphotericin is the safer one, but its price is too high for its worldwide implementation [18], and the renal toxicity cannot be ignored [2].

Miltefosine constitutes another good alternative for treatment, being the main reason the possibility of oral administration, which eases treatment greatly. However, along its gastrointestinal effects, less dangerous than the ones other drugs cause, it has been found that this drug can lead to teratogenicity, making this drug contraindicated for pregnant women [19].

4. Materials and methods

Our research group uses a screening strategy in order to deem which compounds are candidates to new treatments. Overall, it can be described as whether if compounds are suitable to be administered to a vertebrate host in the first place (citotoxicity assay), second, if they present anti-leishmanial activity for both intracellular and extracellular forms (*in vitro* antileishmanial evaluation). In the case that they damage parasite cells but leave host cells unharmed, further studies are carried out.



To determine which compounds are not cytotoxic but show leishmanicidal activity, we follow the criteria published by Nwaka and col. [20] that state that compounds showing a selectivity index 20 times higher than the one of the reference drug are candidates for treatments. Selectivity index is calculated using the following formula: J774.2 Macrophage IC_{50}/parasite cell IC_{50}. Both forms of the parasite can be used for this purpose, however, the most interesting results are those obtained for amastigotes.

Those compounds that show leishmanicidal activity are studied in terms of cell infection and parasite division (infectivity assay).

Finally, in order to get a first approach on the way selected drugs work, studies on the mechanism of action are performed, such as: antioxidant defense reduction (FeSOD inhibition assay), alterations in glucose metabolism (H^1 MNR experiment) and the newest experiment, mitochondrial membrane alteration study (rhodamine assay).

4. 1. Compound dissolution

Compounds were dissolved in MTL (Medium Trypanosomes Liquid) culture medium to reach the required concentrations (100, 50, 25 and 12.5 µM).

4. 2. Citotoxicity assay

To determine drug citotoxicity, macrophage line J774.2 (ECACC 85011428) was used as model for host cell. Macrophages were kept in the laboratory by cryopreservation at -80ºC and then by successive subcultures in RPMI medium. For the cytotoxicity test, macrophages were placed in 25 mL cone-based bottles (Sterling), and centrifuged at 750 rpm for 5 min. The culture medium was removed and Hank's solution was added to a final concentration of 10^4 cells/mL according to the method described in [21]. This cell suspension was distributed in a culture tray (with 24 wells) at a rate of 100 mL per well and incubated for 24 h at 37 ◦C in a humid atmosphere enriched with 5% CO_2. The medium was removed, and the fresh medium was added together with the product to be studied (at concentrations of 100, 50, 25 and 12.5 µM). Cultures were incubated for 72 h. The vital stain trypan blue (0.1% P/B in phosphate buffer) was used to determine cell viability.

The number of dead cells was recorded, and the percentage of viability was calculated in comparison to that of the control culture, and the IC_{50} was calculated by linear regression analysis from the Kc values at the concentrations used.

4. 3. Promastigote and amastigote assay

Promastigotes of studied *Leishmania* species were grown *in vitro* in medium trypanosomes liquid (MTL) supplemented with 10% inactive fetal calf serum and kept in an air atmosphere at 27°C in Roux flasks (Corning, New York, USA) with a surface area of 75 cm^2. References for parasite species are: *L. infantum* (MCAN/ES/2001/UCM-10), *L. braziliensis* (MHOM/BR/1975/M2904) and *L. donovani* (LCR-L 133 LRC), Jerusalem (Israel).

Promastigotes were harvested in a 24-well microplate and compounds were added in order to reach concentrations of 100, 50, 25, 12.5 µM and were maintained at 27ºC for 48 hours. Later, parasites were counted using a Neubauer haemocytometric chamber.

For amastigote inhibitory effect, J774.2 macrophages were harvested in 24-well sterile microplates (Nunc ©) with a round coverslip in the bottom of each well at a concentration of 10^4 cells per well as described in [22]. Once cells were fixated to the coverslip, they were infected with promastigote (being 10:1 promastigotes per macrophage) of each studied species and at the same time drugs were added in order to reach final concentrations of 12.5, 25, 50 and 100 µM. Promastigotes underwent conversion to amastigotes one day after infection. Those microplates were maintained for 2 days, and then coverslips were collected, fixed to a microscope slide and stained with Giemsa for their later examination at an optical microscope.

4. 4. Infectivity and amastigote quantity assay

Adherent macrophage cells grown as described above were infected in vitro with promastigote forms of *L. infantum*, *L. braziliensis* and *L. donovani* at a ratio of 10 : 1. The tested compounds (IC_{25} concentration) were added immediately after infection, and incubated for 12 h at 37 °C in 5% CO_2 [23]. Compounds and non-phagocytozed parasites were removed by washing, and then the infected cultures were cultured for

10 days in fresh medium. Cultures were washed every 48 h and fresh culture medium was added.

Compound activity was determined on the basis of both the percentage of infected cells and the number of amastigotes per infected cell in treated and untreated cultures in methanol-field and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analysing more than 200 host cells distributed in randomly chosen microscopic fields.

4. 5. Superoxide dismutase (SOD) inhibition studies

Promastigotes of Leishmania spp. were grown in tissue-culture flasks and an axenic medium, as described above, until reaching a population of approximately 1×10^7 parasites/mL. Cells were harvested at the logarithmic growth phase by centrifugation (1500 g for 10 min at room temperature). The pellet of cells was washed twice in the MTL medium without serum, and the cells were counted, distributed into aliquots of 5×10^9 parasites/mL in MTL medium without serum, and allowed to grow for 24 h.

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

Following centrifugation, the resultant supernatant was then treated with 85% ice-cold ammonium sulphate and the second precipitate was 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 (Fraction P85e). The protein content was quantified using the Sigma Bradford test [24], which uses bovine serum albumin as a standard (Bradford, 1976). Iron and copper–zinc Fe-SOD activities were determined using the method that measures the reduction in nitroblue tetrazolium (NBT) by superoxide ions [25].

According to the protocol, 845 µL of stock solution [3 mL of L-methionine (300 mg, 10 mL−l), 2 mL of NBT (1.41 mg, 10 mL−1) and 1.5 mL of Triton X-100 1% (v/v)] were added to each well, along with 30 µL of the parasite homogenate fraction, 10 µL of riboflavin (0.44 mg, 10 mL−l) and an equivalent volume of the different concentrations of the compounds being tested. Four different concentrations were used for each agent, from 6.25 to 100 µM.

In the control experiment, the volume was made up to 1000 µL with 50 mM potassium phosphate buffer (pH 7·8, 3 mL), and 30 µL of the parasite homogenate fraction were added to the mixtures containing the compounds. Next, the absorbance (A0) was measured at 560 nm in a UV spectrophotometer.

Afterwards, each well was illuminated with UV light for 10 min under constant stirring and the absorbance (A1) was measured again. The human CuZn-SOD and substrates used in these assays were obtained from Sigma-Aldrich®. The resulting data were analysed using the Newman–Keuls test.

4. 6. Metabolite production and excretion

Another approach in order to gain insight into the mechanism of action of our drugs is to assess their effect on the parasite metabolism. Trypanosomatids are not able to oxidize glucose to carbon dioxide and water as vertebrates do; instead of that they excrete part of the carbon skeleton in the form of different organic acids [26]. There is an enzyme responsible of the production of each excreted compound of the glycolitic metabolic pathway so it can be inferred which enzyme is having its activity altered because of the drug effect just by observing which metabolite is seeing its production enhanced or reduced compared to the control.

Parasite cultures containing $5x10^6$ parasites/mL were treated with an IC_{25} dosage of the compounds and let react for 72 hours in 27ºC. Once that time elapsed, cultures

were centrifuged at 2500 rpm for 10 minutes and the supernatant was collected and analyzed by H^1 MNR.

4. 7. Mitochondrial membrane potential alteration study

Other experiment used to try to understand the possible mechanism of action is to state if the leishmanicidal effect of the compounds can be caused by an alteration of the mitochondrial membrane potential, as a big alteration in it can lead to Programmed Cell Death. Other interesting aspect of this experiment is that it can support results of the previously described one, as some metabolic alterations can be also explained by changes on the mitochondrial structure.

Cultures were treated with IC_{25} of each drug, similarly as we did before for metabolite excretion test and were incubated for 72h at 27ºC. After that, a centrifugation (1500 rpm during 10 minutes) took place and pellets were collected and stained with Rho 123 (Sigma-Aldrich®) and were analyzed by flow cytometry. Changes in fluorescence intensity were calculated using the equation VI= (TM-CM)/ CM, meaning: VI (variation index, TM (treated parasites median fluorescence) and CM (untreated parasites median fluorescence) [27].

5. Results

In this section the different scientific publications that compound this thesis are displayed.

The article *Heterocyclic Diamines with Leishmanicidal Activity* has been submitted to the patent office and it has been accepted. At the time this thesis has been submitted to the university office the aforementioned publication is undertaking the process to be patented

Title	Journal	Impact Factor
Synthesis and *in vitro* Leishmanicidal activity of novel[1,2,3] Triazolo [1,5-*a*] pyridine salts	RSC Advances	3.049
In vitro antileishmanial activity and iron superoxide dismutase inhibition of arylamine Mannich base derivatives	Parasitology	2.456
Library of Seleno-Compounds as Novel agents against *Leishmania* species	Antimicrobial Agents Chemotherapy	4,715
In vitro assessment of 3-alkoxy-5-nitroindazole-derived ethylamines and related compounds as potential antileishmanial drugs	Bioorganic Chemistry	3.926
Heterocyclic Diamines with Leishmanicidal Activity	[Patent Pending]	----------

Synthesis and *in vitro* Leishmanicidal activity of novel [1,2,3]Triazolo[1,5-*a*]pyridine salts

Álvaro Martín-Montes,[a] Rafael Ballesteros-Garrido,[b,c] Rubén Martín-Escolano,[a] Clotilde Marín,[a] Ramón Guitiérrez-Sánchez,[d] Belén Abarca,[b*] Rafael Ballesteros,[b*] Manuel Sanchez-Moreno[a*]

[a]Departamento de Parasitología, Instituto de Investigación Biosanitaria (ibs.GRANADA), Hospitales Universitarios de Granada/Universidad de Granada, (Granada), Spain.

[b]Departamento de Química Orgánica, Facultad de Farmacia, Universidad de Valencia, Avda. Vicente Andrés Estellés s/n, 46100 Burjassot (Valencia), Spain

[c]ICMol Instituto de Ciencia Molecular, Universidad de Valencia, C. Catedrático José Beltrán 2, 46100 Paterna (Valencia) Spain.

[d]Department of Statistics, University of Granada, Severo Ochoa s/n, E-18071 Granada, Spain

Supporting Information

ABSTRACT

Leishmaniasis disease remain a significant worldwide problem, it is of great interest to develop new drugs to fight this illness. Recently we described some [1,2,3]triazolo[1,5-*a*]pyridine compounds with significant leishmanicidal activity. The importance of water solubility in drug action made us think to develop tirazolopyridine analogues that could increase the degree of water solubility. With this objective we report here the synthesis of novel [1,2,3]triazolo[1,5-*a*]pyridinium salts **2-7** from triazolopiridines **1**, and the study of their *in vitro* leishmanicidal activity. The activity was tested on *Leishmania infantum*, *Leishmania braziliensis* and *Leishmania donovani* parasites, using promastigotes and intracellular amastigotes forms. The cytotoxicity of the tested compounds on J774.2 macrophage cells was also measured. Five of the tested compounds (**2b**, **4a, 4c**, **6**, **7d**) showed selectivity indexes higher than those of the reference drug Glucantime for the three Leishmania species. Moreover, the data on infection rates and on amastigotes showed that these compounds are the most active against the three Leishmania species. The changes in the excretion products profile of parasites treated with the compounds were also consistent with substantial cytoplasmic alterations. On the other hand, the most active compounds were potent inhibitors of Fe-SOD in the three parasite species considered whereas their impact on human CuZn-SOD was low.

INTRODUCTION

Leishmaniases are a group of parasitic diseases caused by different protozoan species of genus Leishmania, transmitted by phlebotomine sand flies. In humans, the disease presents as three main clinical forms, depending on the involved species of Leishmania: cutaneous, mucocutaneous and visceral leishmaniasis, with the latter being the most severe and life-threatening form. Leishmaniases are prevalent in tropical and subtropical areas; they currently affect 98 countries with 12 million cases, and a further 350 million people are presently at risk; the annual incidence is estimated at 1-1.5 million cases of cutaneous disease and 500,000 cases of visceral disease.[1-4]

Treatment of leishmaniases has been based for many years on pentavalent antimonials, which are still the first-line drugs. The second-line drugs include pentamidine, amphotericin B, miltefosine and paromomycin, and more recently, sitamaquine has shown very promising properties.[2,3,5,6]

As in the case of other neglected tropical diseases, most of the current therapies are inadequate essentially due to several factors such as the low therapeutic indexes leading to high toxicities and unacceptable side-effects, the emergence of resistant parasites, high prices that are unaffordable for the affected countries, etc. These drawbacks of the current therapy, together with the fact that a vaccine is an unachieved goal, make the search for new drugs urgently needed.[3,1,5] In the last few years, many compounds showing leishmanicidal properties have been reported in the literature, and furthermore, several potential drug targets have been proposed and validated.[3,5,6] Nevertheless, owing to the low income of the affected population, investment in the development of new drugs against leishmaniases has not been financially attractive for pharmaceutical companies, and the interest of academic institutions is rather limited.[3,5]

1,2,3-Triazole compounds do not exist in natural substances but they have been widely investigated because they are considered privileged scaffolds in medicinal chemistry.[7,8] This structure is present in molecules described to have antibiotic,[9,10] antineoplasic,[11,12] anti-HIV,[13] or antifungal activities.[14] Furthermore, some molecules containing 1,2,3-triazole structure have been recently described as antileishmanial agents.[15-19] In the last years, several compounds containing 1,2,3-triazole scaffold, such as quinolone triazoles,[20] triazole naphtalimides,[21] or triazoloacridones,[22] able to interact with DNA have been described. The anticancer activity of the latter family of compounds is remarkable.[23]

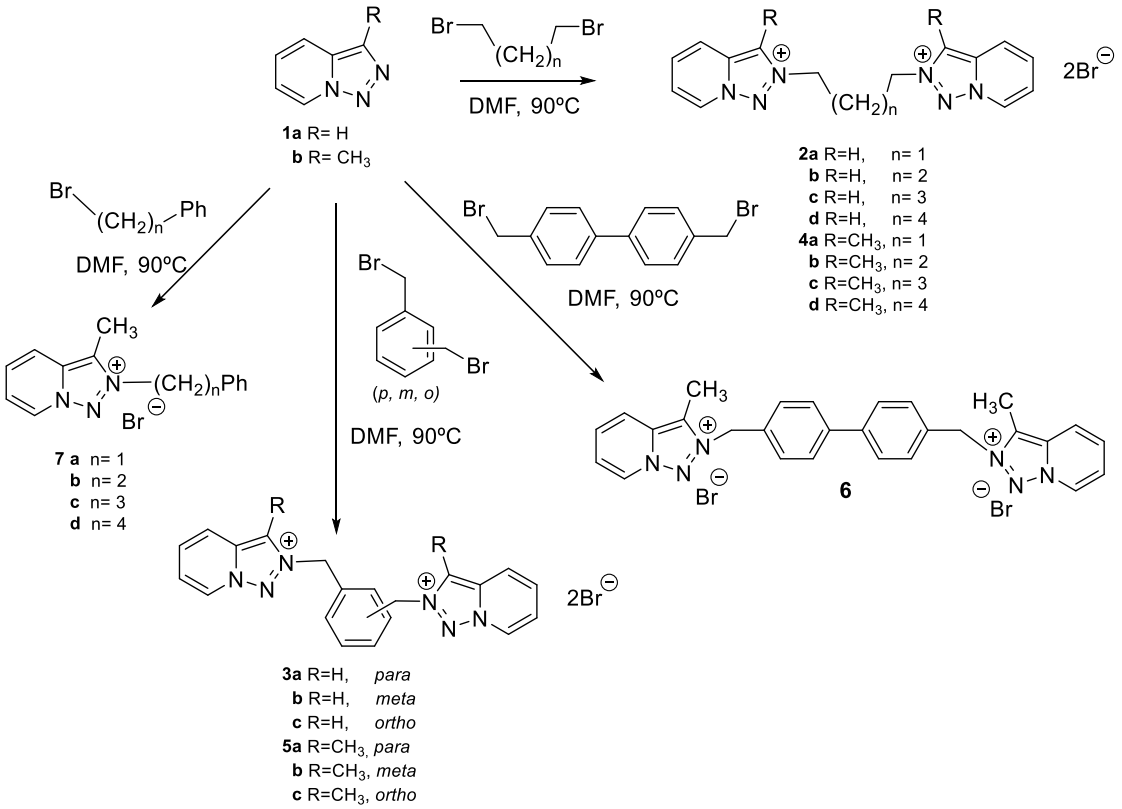
RESULTS

During last years, the research of some of us has been focused on the functionalization of [1,2,3]triazolo[1,5-*a*] pyridines **1** and on the study of the potential applications of the derivatives obtained.[24] Related of its possible biological activity, recently we have reported the first [1,2,3]triazolo[1,5-*a*]pyridine compounds with leishmanicidal activity, their interaction with DNA could be a mechanism to explain the activity found.[25] We have also studied some triazolopyridopyrimidines, a fluorescent family of compounds, that have demonstrated to present photoinduced DNA cleavage, and antiprotozoal activity against different types of *Leishmania* spp.[26] Moreover, in our group we have synthesized a series of triazoles known as [1,2,3]triazolo[1,5-*a*]pyridyl pyridyl ketones, that by the presence of the carbonyl group, being able to be reduced and form a free radical that could interact with molecular oxygen generating ROS. As Leishmania species as vulnerable to the effects of ROS we considered interesting to study the leishmania activity of these compounds. The results showed that the series are reduced electrochemically at radical species that were observed inside the parasite by ESR. Furthermore, the compounds are able to affect the metabolic pathway of ergosterol indicating mainly in CYP51 inhibition where enzyme lanosterol increased metabolite was observed. The antiproliferative effect would be also related to the inhibition of this pathway.[27]

Supported by these hopeful results we decided to design a new series of compounds containing the triazolopyridine scaffold to explore its potential activity as anti Leishmania drugs. The importance of water solubility in drug action made us think to develop tirazolopyridine analogues that they could increase the degree of water solubility. A general method to improve this property is the formation of salts. It is known that the triazolopyridines can made quaternary salts.[28] We report here the synthesis of new triazolopyrine disalts **2-6** and monosalts **7** (**Scheme 1**), and the study of their Leishmanicidal activity in vitro against *L. infantum*, *L. donovani* and *L. braziliensis* (promastigote and amastigote forms) as representative species causing visceral and cutaneous leishmaniasis, respectively; unspecific cytotoxicity against mammalian cells of all compounds, as well as infectivity assays for compounds, were carried out using macrophages. Furthermore, a ^{1}H NMR study has been conducted in order to observe changes in the nature and percentage of metabolites excretion directed to obtain information about the effect of our compounds on the glycolytic pathway of parasites; finally, we have also studied inhibition of the parasitic Fe-SOD, and human CuZn-SOD enzymes was tested and compared.

RESULTS AND DISCUSSION

Chemistry. The synthesis of triazolopyridines salts **2-7** has been carried out from [1,2,3]triazolo[1,5-*a*]pyridine **1a** and 3-methyl-[1,2,3]triazolo[1,5-*a*]pyridine **1b**, that are easily prepared from commercial products.[29] The disalts **2-6** were prepared by a reaction between the corresponding triazolopyridine (**1a, 1b**) (4 eq) and a dibromo compound (1 eq). Similar procedure was applied for the preparation of compounds **7**, in this case an excess of a monobromo derivative was employed. The alkylation of triazolopyridines **1**, in all the cases studied have been regioselective in N2,[28] as shown in compound **4a** by NOEDIF studies (see supplementary material) (**Scheme 1**).



Biological evaluation. We report now on the results obtained concerning the toxic activity of the triazolopyridines salts **2-7** against three species of Leishmania (*L. infantum*, *L. braziliensis* and *L. donovani*).

In vitro antileishmanial evaluation. We measured the in vitro biological activity of 21 triazolopyridine derivatives on both extra- and intracellular forms of the parasites. Extracellular forms are more commonly used due to ease of working with them, but are less

indicative of leishmanicidal activity. Use of intracellular forms use is more cumbersome but gives more accurate results, as they are converted to amastigotes in vertebrate host cells.[30] Intracellular assays were performed by infecting macrophage cells with promastigotes, which transformed into amastigotes within 1 day after infection. **Table 1** collects the IC_{50} values obtained after 72 h of exposure to *L. infantum*, *L. braziliensis* and *L. donovani species*.

Toxicity values against J774.2 macrophage after 72 h of culture were also calculated, and the selectivity indices (SI = IC_{50} macrophages toxicity/IC_{50} activity of extracellular or

Compounds	IC_{50} µM[a]						Toxicity IC_{50} Macrophage (µM)
	Leishmania infantum		*Leishmania braziliensis*		*Leishmania donovani*		
	Promastigote forms	Amastigote forms	Promastigote forms	Amastigote forms	Promastigote forms	Amastigote forms	
Glucantime	18.0±1.3	24.2±2.6	25.6±1.7	30.4±6.1	22.1±2.3	18.4±2.8	15.2± 1.0
1a	209.7±8.9	Nd	176.2±7.9	Nd	263.1±16.8	Nd	812.4±70.2
1b	56.8±5.6	Nd	63.2±2.5	Nd	64.7±0.7	Nd	916.7±37.5
2a	245.0±17.3	Nd	122.8±7.3	Nd	429.7±17.4	Nd	843.6±66.8
2b	10.6±0.9	15.8±1.7	15.7±0.8	19.7±0.8	19.3±0.9	13.2±1.8	693.9±54.7
2c	230.4±11.5	Nd	227.5±4.1	Nd	233.7±18.2	Nd	804.7±63.8
2d	263.8±28.6	Nd	89.6±5.5	Nd	84.4±1.1	Nd	1538.3±100.2
3a	315.9±17.2	Nd	261.7±12.3	Nd	302.5±15.3	Nd	774.3±36.9
3b	275.1±15.3	Nd	115.9±6.9	Nd	215.8±7.8	Nd	806.3±22.8
3c	200.1±8.7	Nd	207.5±10.0	Nd	159.5±10.0	Nd	636.3±40.9
4a	15.2±1.2	20.7±3.6	16.8±0.6	14.6±2.6	67.2±3.0	Nd	942.9±33.7
4b	215.3±8.8	Nd	253.7±13.5	Nd	157.8±4.7	Nd	408.5±15.8
4c	12.3±3.2	7.8±3.8	54.8±2.1	Nd	67.5±2.4	Nd	683.1±37.5
4d	109.3±5.6	Nd	179.3±6.8	Nd	227.9±5.9	Nd	1445.6±77.4
5a	85.1±7.3	Nd	72.0±4.9	Nd	155.4±1.9	Nd	1236.7±88.5
5b	64.6±4.4	Nd	58.4±3.6	Nd	50.4±1.6	Nd	743.6±23.4
5c	115.4±7.1	Nd	118.7±7.9	Nd	162.9±12.4	Nd	993.5±47.3
6	12.5±3.1	8.7±2.0	5.3±0.8	6.3±1.1	13.4±2.5	9.1±0.7	755.3±56.9
7a	186.5±8.5	Nd	180.4±8.4	Nd	215,2±35.8	Nd	1724.6±43.8
7b	66.6±7.6	Nd	62.9±1.5	Nd	86,8±4.1	Nd	1223.0±118.5
7c	71.5±2.5	Nd	63.9±0.3	Nd	57,6±3.6	Nd	1023.6±66.4
7d	17.3±2.7	11.6±1.6	6.3±1.7	9.5±2.5	204,4±12.6	Nd	1435.7±81.1

Table 1. In vitro activity and toxicity for the triazole pyridine salts on extracellular and intracellular forms of *Leishmania* spp.

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 to 2000 µM). [b)] Against J774.2 macrophages after 72 h of culture.

intracellular forms of the parasite) are shown in the **Table 2.** Results obtained for the reference drug glucantime were included for comparison.

It was shown that the leishmanicidal activities against both the extra- and intracellular forms of the parasites by the some triazolopyridines salts were higher than those seen with glucantime.

However, more interesting are the toxicity data in mammalian cells, since all compounds tested were found to be much less toxic for macrophages than the reference drug. Their values range from 101-fold for compound **2d** to 27-fold for compound **4b** respectively, less toxic than the parasites by the some triazolopyridines salts were higher than those seen with glucantime. Toxicity values substantially influence the more informative selectivity index (SI) **Table 2** data, so the best values were again obtained by compounds **2b, 4a, 4c, 6,** and **7d**, in tests performed on *L. infantum* species.

Table 2. Selectivity index for the triazolo-pyridine salts on extra- and intracellular forms of *Leishmania spp*.

Compounds	SI_{50} μM[a]					
	Leishmania infantum		*Leishmania braziliensis*		*Leishmania donovani*	
	Promastigote forms	Amastigote forms	Promastigote forms	Amastigote forms	Promastigote forms	Amastigote forms
Glucantime	0.8	0.6	0.6	0.6	0.7	0.8
1a	4 (5)	Nd	5 (8)	Nd	3 (4)	Nd
1b	16 (20)	Nd	14 (23)	Nd	14 (20)	Nd
2a	3 (4)	Nd	7 (11)	Nd	3 (5)	Nd
2b	65 (82)	44 (73)	44 (74)	35 (59)	36 (51)	53 (66)
2c	3 (4)	Nd	3 (6)	Nd	3 (5)	Nd
2d	6 (7)	Nd	17 (28)	Nd	18 (26)	Nd
3a	2 (3)	Nd	3 (5)	Nd	2 (4)	Nd
3b	3 (4)	Nd	7 (12)	Nd	4 (5)	Nd
3c	3 (4)	Nd	3 (5)	Nd	4 (6)	Nd
4a	62 (77)	45 (76)	56 (93)	65 (108)	14 (20)	Nd
4b	2 (2)	Nd	2 (3)	Nd	3 (4)	Nd
4c	55 (69)	88 (146)	12(21)	Nd	10 (14)	Nd
4d	13 (16)	Nd	8 (13)	Nd	6 (9)	Nd
5a	14 (18)	Nd	17 (29)	Nd	8 (11)	Nd
5b	11 (14)	Nd	13 (21)	Nd	15 (21)	Nd
5c	9 (11)	Nd	8 (14)	Nd	6 (9)	Nd
6	60 (75)	87 (145)	143 (237)	120 (200)	56 (80)	83 (104)
7a	9 (12)	Nd	9.6 (16)	Nd	8 (11)	Nd
7b	18 (23)	Nd	19 (32)	Nd	14 (20)	Nd
7c	14 (18)	Nd	16 (27)	Nd	18 (26)	Nd
7d	83 (104)	124 (206)	228 (380)	42 (71)	7 (10)	Nd

[a]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.

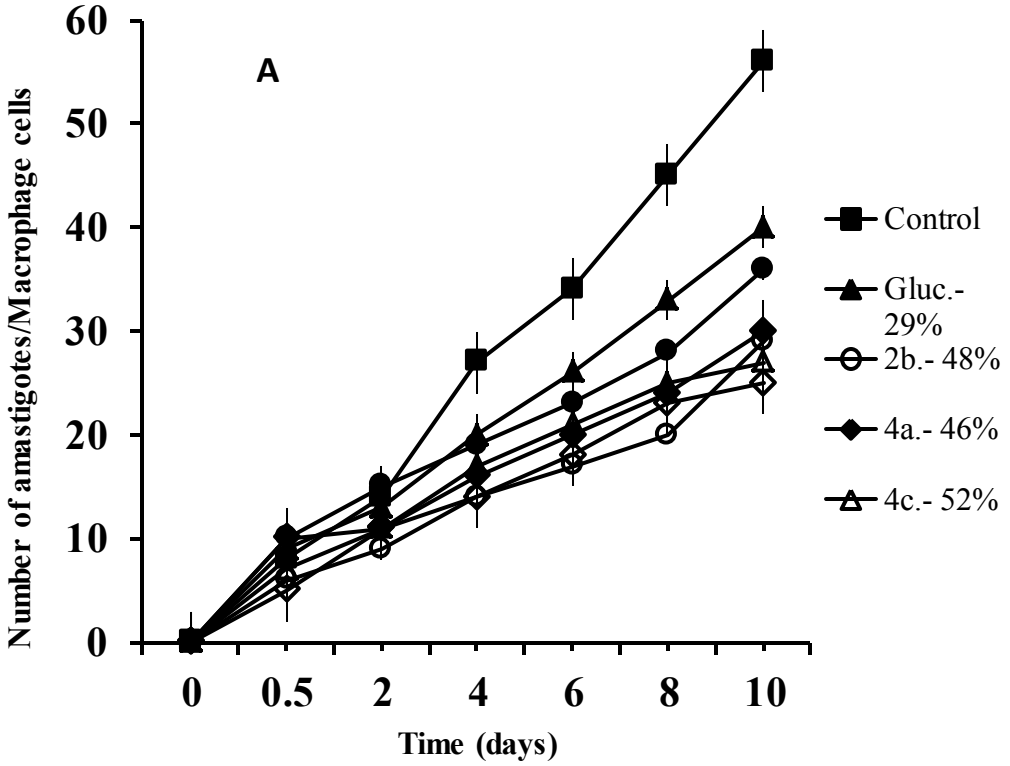
SI exceeded that of the reference drug by 82- and 73-fold for the extra- and intracellular forms in the case of **2b**, by 77- and 76-fold with **4a**, 69-fold and 146-fold with **4c,** 75-fold and 145-fold with **6**, 104-fold and 206-fold with **7d** for the extra- and intracellular forms, respectively, while on *L. braziliensis*, the respective values obtained were 74- and 59-fold with **2b,** 93- and 108-fold with **4a,** 237- and 200-fold with **6,** 380- 71-fold with **7d** for the extra- and intracellular forms, respectively**,** and in the case of *L. donovani* similar results were obtained: 51- and 66-fold with **2b**, 80- and 104-fold with **6**, respectively. It should be noted that a SI value more than 20-fold that of the reference drug is one of the usual basic criteria for considering primarily screened compounds as candidates for more advanced testing in vitro and in animal models.[31]

The tests described above represent only a first crude approach to the leishmanicidal properties of the compounds assayed. In order to gain better insight into the activity of compounds, their effect on the infectivity and intracellular replication of amastigotes was subsequently determined. In accordance with the usual working procedure, compounds without an IC_{50} value lesser or circa 10 µM and with a SI smaller than 20-fold of the reference drug index, were not included in this second stage, due to the poor **SI** results obtained. Other compounds with not so high **SI** values were also excluded. In this sense the following compounds were selected for the case of *L. infantum* **2b**, **4a**, **4c**, **6** and **7d,** for *L. braziliensis* **2b**, **4a**, **6** and **7d**, and in the case of *L. donovani* **2b**, and **6**.

Infectivity assays. Macrophage cells were grown and infected with promastigotes in the stationary phase. The parasites invaded the cells and underwent morphological conversion to amastigotes within 1 day after infection. On day 10, the rate of host cell infection reached its maximum (control experiment). We used the IC_{25} of each product as the test dosage.

As shown in **Figure 1A**, when triazolopyridine salts were added to macrophages infected with *L. infantum* promastigotes, the infection rate decreased significantly with respect to the control and, furthermore, the six compounds were also more effective in decreasing infectivity than glucantime (74%, 73%, 72%, 59% and 51% for **2b**, **6**, **4c**, **4a** and **7d** respectively, versus only 29% for the reference drug). A measure of the average number of

amastigotes per infected macrophage (**Figure 1B**) led to similar conclusions: all compounds were more effective than glucantime (with only a 29% decrease), and their order of effectiveness followed the same pattern as that seen in the infectivity measures, although the differences between them were less pronounced (48%, 46%, 52%, 39% and 36%) for **2b, 6, 4c, 4a** and **7d** respectively.



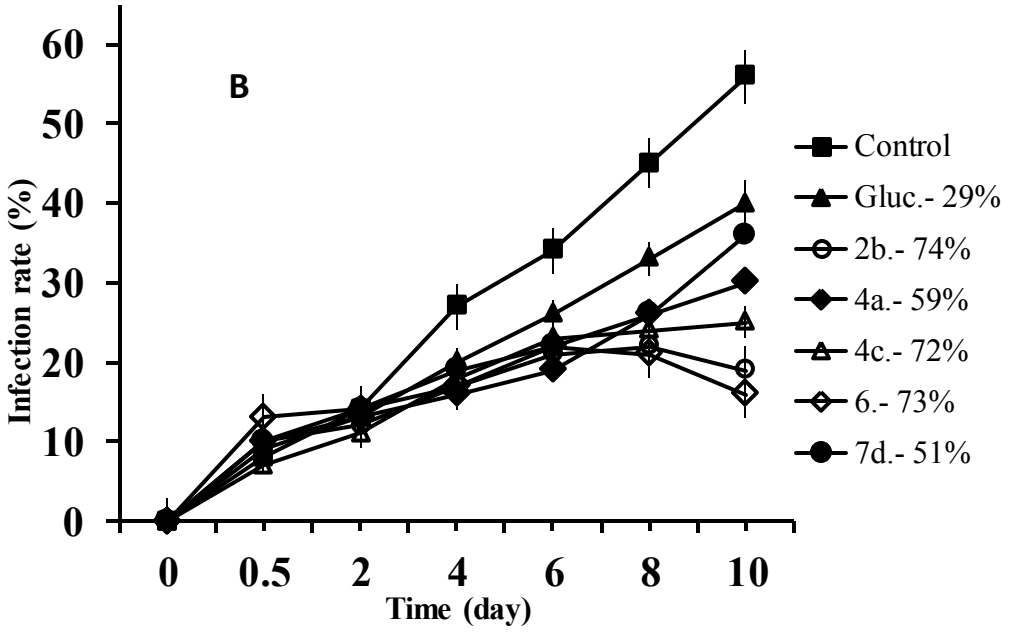
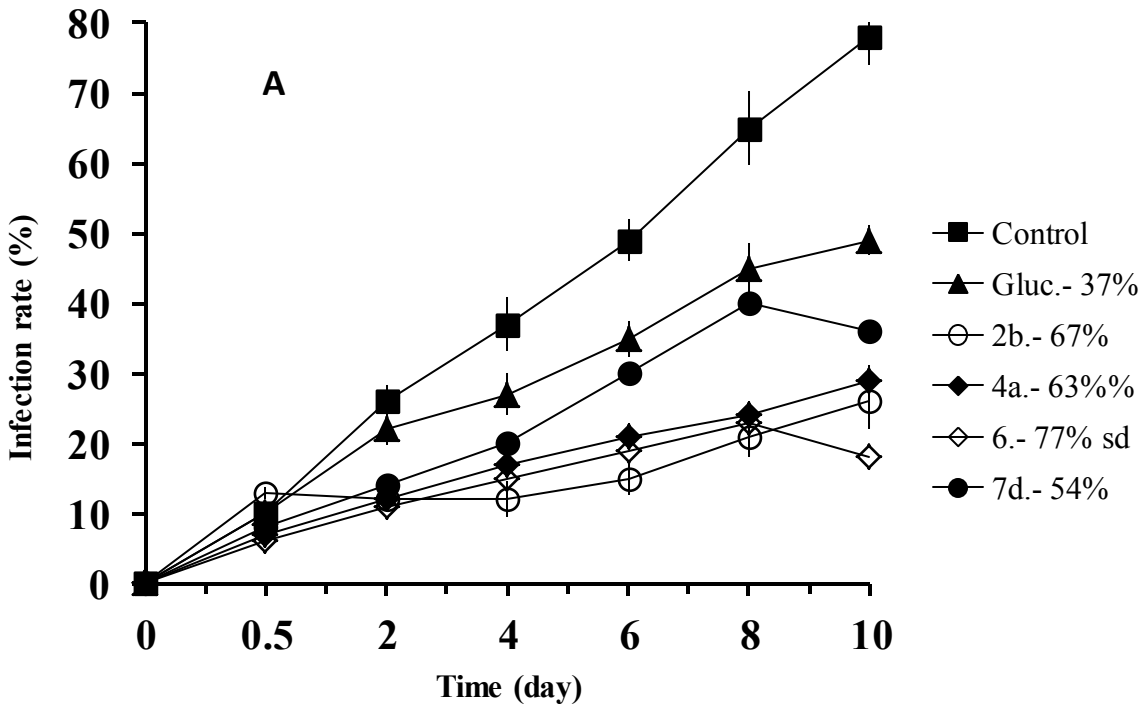


Figure 1. Reduction of the infection of *L. infantum* in macrophage cells treated with Glucantime and triazolepyridine salts. (**A**) Rate of infection, (**B**) means number of amastigotes per infected macrophage cells. Measured at IC_{25} .Values are the means of the three separate experiments.

The same experiment was performed with *L. braziliensis*, and the results obtained concerning infection rates (a) and amastigote numbers (b) can be observed in **Figure 2**. In both cases, the compounds were also more effective than glucantime, and also in both cases the order of effectiveness was now **6** > **2b** > **4a** > **7d**, and since the infectivity rates calculated from **Figure 2A** were: 77%, 67%,63%, 54% and 37% for glucantime; and the decrease in amastigote numbers was: 69%, 52%, 39%, 21% and 11% for **6**, **7d**, **2b**, **4a**, and glucantime respectively (**figure 2B**).



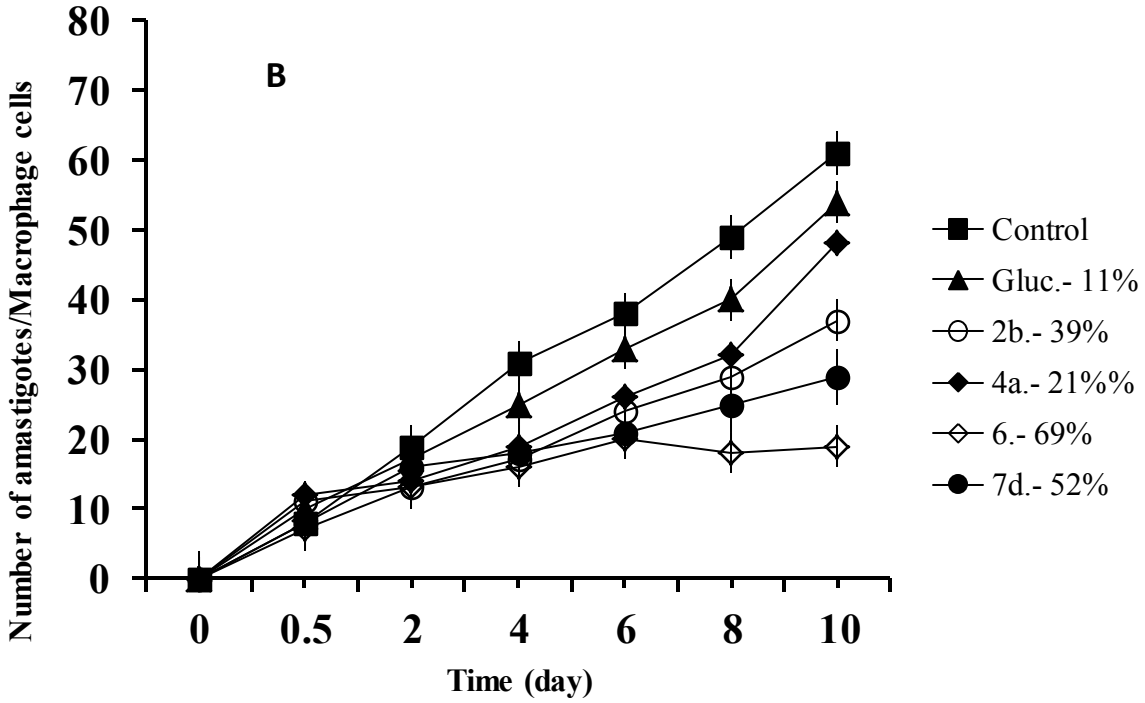


Figure 2. Reduction of the infection of *L. braziliensis* in macrophage cells treated with Glucantime and triazolepyridine salts. (**A**) Rate of infection, (**B**) means number of amastigotes per infected macrophage cells. Measured at IC_{25} .Values are the means of the three separate experiments.

In the case of *L. donovani* obtained results were (**Figure 3**): **6** > **2b** > glucantime respectively (**Figure 3A**) and with the infectivity values of: 76%, 55 and 28% respectively, and the decrease in amastigote numbers was: 66%, 61%, and 38% for **6, 2b,** and glucantime respectively.

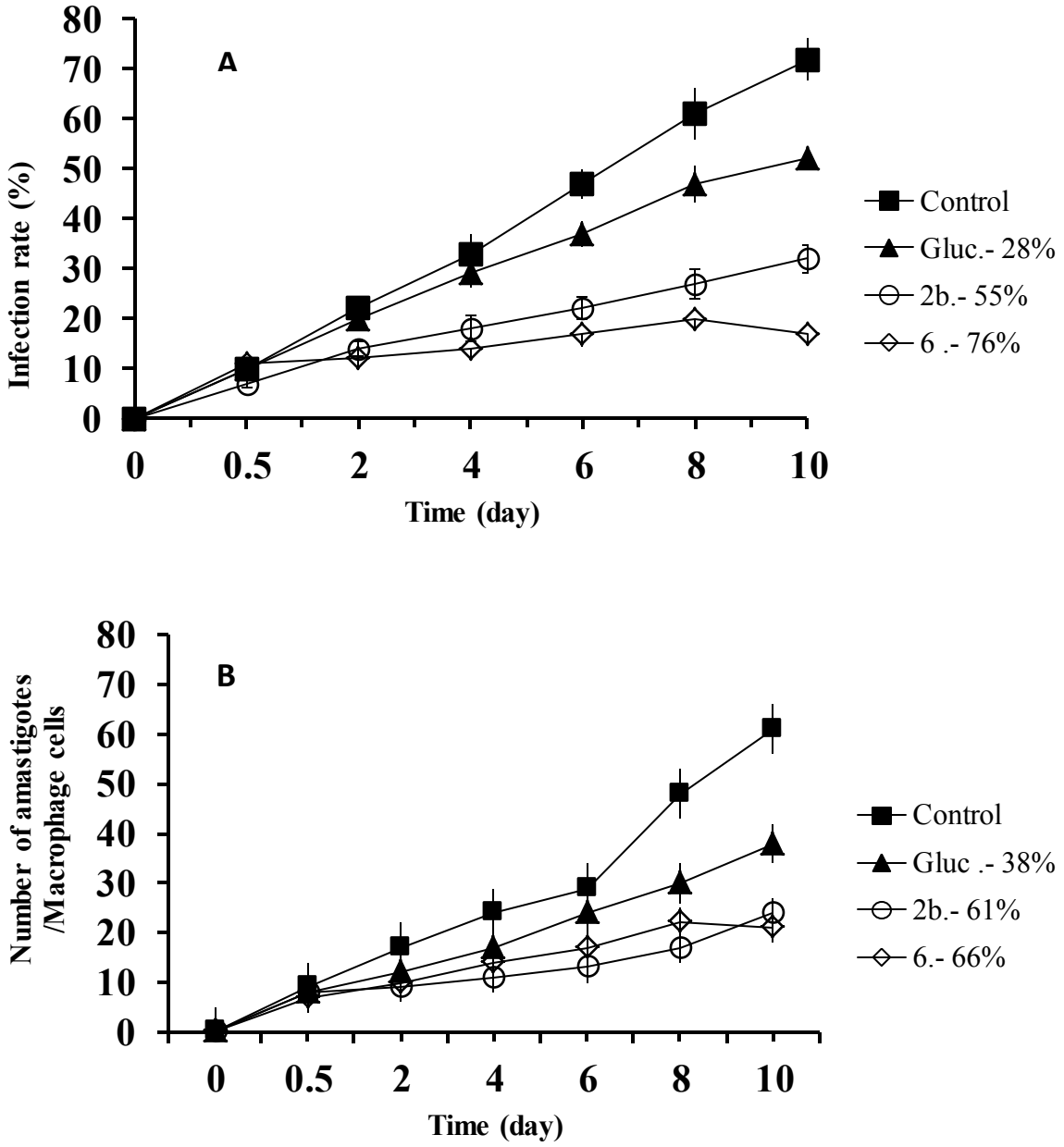


Figure 3. Reduction of the infection of *L. donovani* in macrophage cells treated with Glucantime and triazolepyridine salts. (A) Rate of infection, (B) means number of amastigotes per infected macrophage cells. Measured at IC_{25} .Values are the means of the three separate experiments

Metabolite excretion. Since trypanosomatids are unable to completely degrade glucose to CO_2, they excrete a considerable portion of its hexose skeleton as partially oxidised fragments in the form of fermented metabolites, whose nature and percentage depend on the pathway used for glucose metabolism.[32,33] The catabolism products in Leishmania are usually CO_2, succinate, acetate, pyruvate, **D**-lactate, **L**-alanine, and, to a lesser extent, ethanol.[34] Detection of large amounts of succinate as a major end product is an usual

feature, because it rules on glycosomal redox balance, enabling reoxidation of NADH produced in the glycolytic pathway. Succinic fermentation requires 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, **L**-lactate, **L**-alanine, or ethanol according to the degradation pathway followed by each species.[35]

In order to obtain some information concerning the effect of the tested compounds on glucose metabolism in the parasites, we registered the ^{1}H NMR spectra of promastigotes from *L. infantum*, *L. braziliensis* and *L.donovani* after treatment with the selected compounds triazolopyridine salts, and the final excretion products were identified qualitatively and quantitatively. The results were compared with those found with promastigotes maintained in a cell-free medium (control) for three days after inoculation with the parasite. The characteristic presence of acetate, **D**-lactate, succinate and **L**-alanine was confirmed in the control experiments performed on the three species. As expected, succinate and acetate were the most abundant end products identified. However, after treatment of the parasites with compounds, the excretion of catabolites was substantially altered at the dosages employed. Figures 4, 5 and 6 displays these modifications with respect to the control observed at the height of the spectral peaks corresponding to the most representative final excretion products.

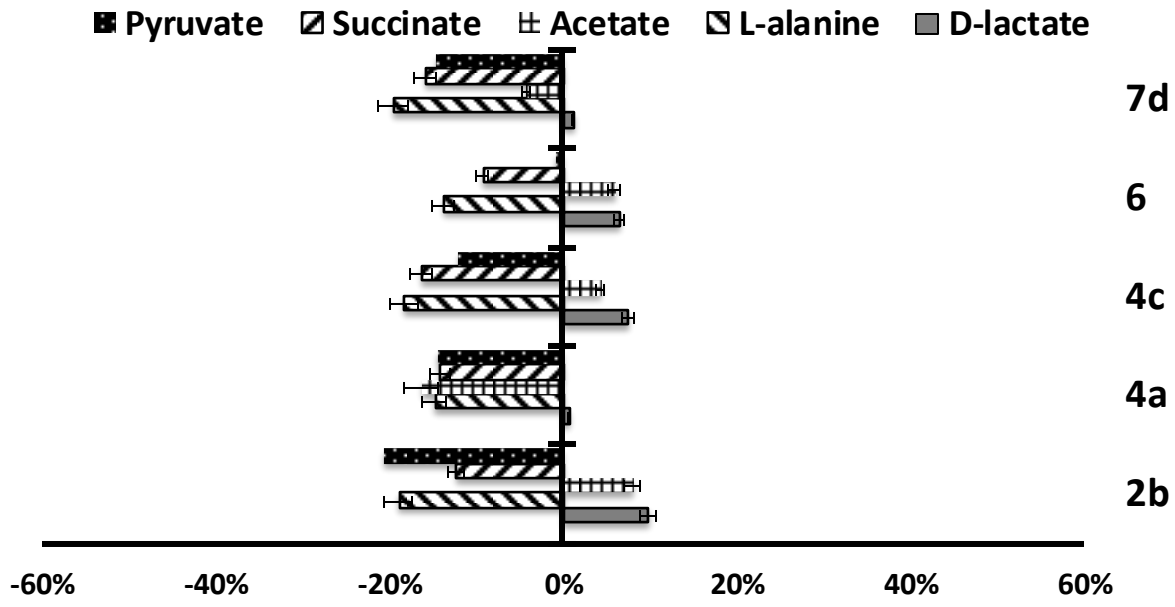


Figure 4. Variation percentages in the area of the peaks corresponding to excreted catabolites by *L. infantum* promastigotes in the presence of compounds **2b, 4a, 4c, 6** and **7d** at their IC_{25} compared to a control sample after 96 h of incubation.

Marked differences in the catabolic pathway appeared, and that seemed to be connected with the leishmanicidal activity commented above. Mainly, each compound has a certain effect in catabolyte production. In the case of *L. infantum* (**Figure 4**), compounds **2b**, **4c** and **6**, trigger an increase in the production of acetate, D-lactate. It is well known that acetate, D-lactate, L-alanine and ethanol originate from the transformation of PEP in pyruvate in the presence of pyruvate kinase or pyruvate phosphate dikinase.[33] Therefore, it seems possible that these compounds were interacting with the pyruvate kinase enzymes and modifying the glucose metabolism of the parasite at the pyruvate stage.[33] Compounds **4a** and **7d** do not trigger the increase of those catabolytes, instead, there is a remarkable reduction in their production.

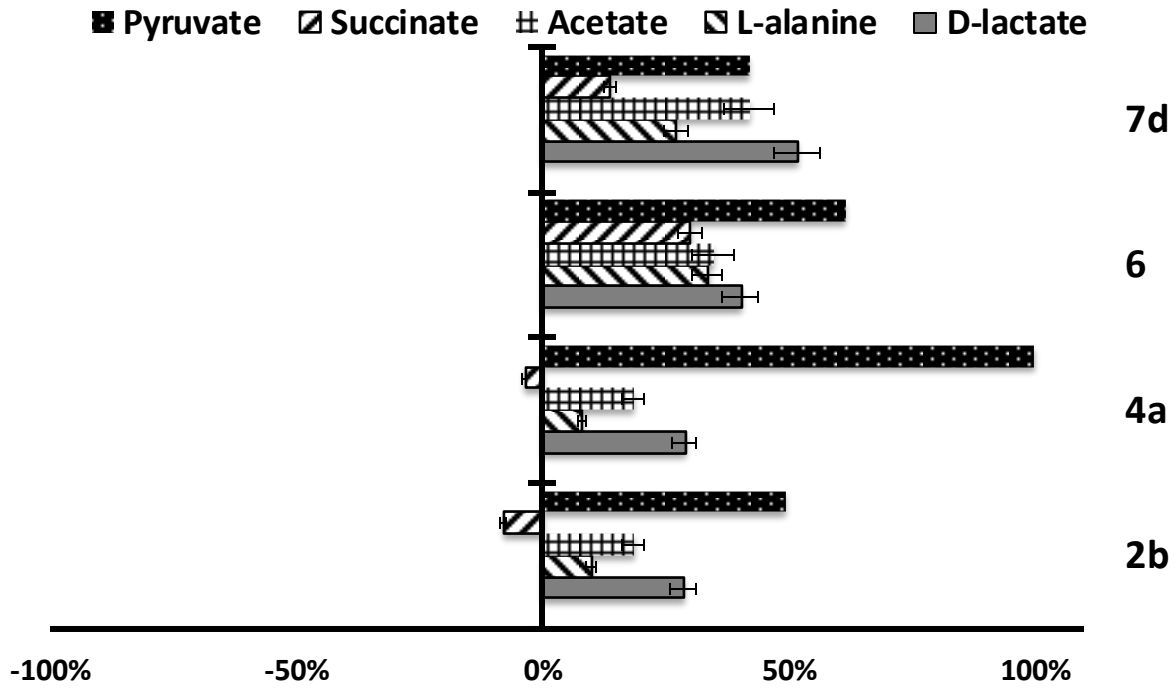


Figure 5. Variation percentages in the area of the peaks corresponding to excreted catabolites by *L. brazilinesis* promastigotes in the presence of compounds **2b, 4a, 6** and **7d** at their IC_{25} compared to a control sample after 96 h of incubation.

On the other hand, in the case of *L. braziliensis* (**Figure 5**), the action of the compounds is different. All excreted metabolytes: pyruvate, acetate, d-alanine and d-lactate, yield an enhanced production as mentioned above for compounds **2b**, **4c** and **6** in *L. infantum*. This effect also occurs in compound **2b** for *L. donovani* (**Figure 6**) what makes us think that those compounds are acting over pyruvate kinase in *L. braziliensis* and *L. donovani*. In the case of *L. donovani* catabolyte production is strongly inhibited by compounds **6**.

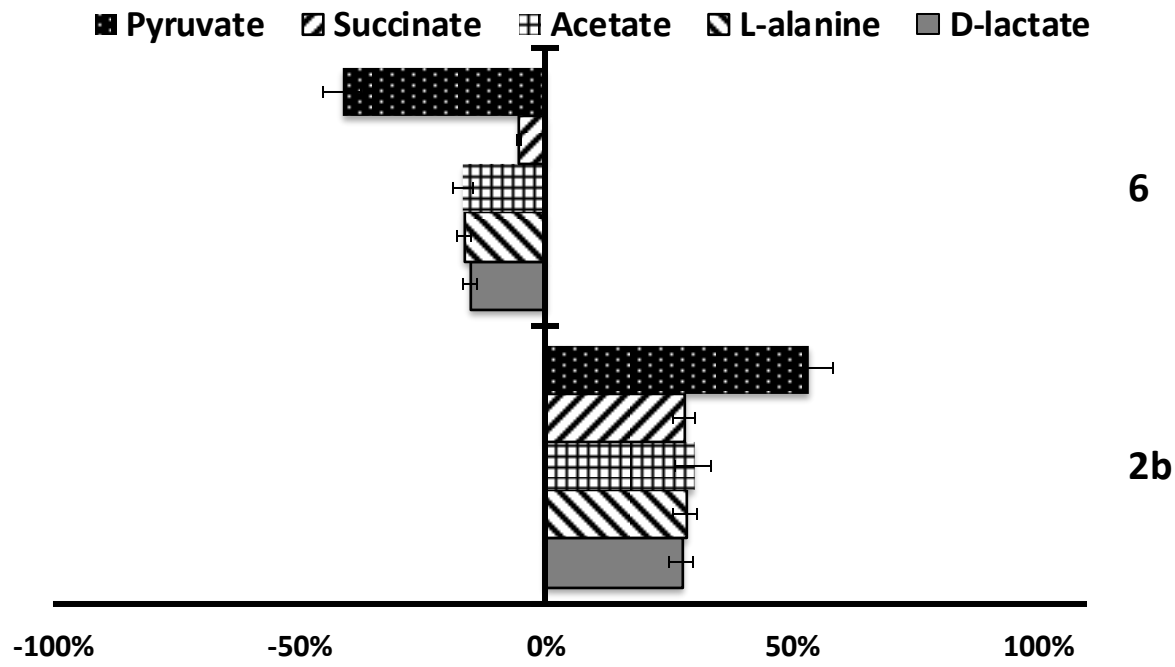


Figure 6. Variation percentages in the area of the peaks corresponding to excreted catabolites by *L.donovani* promastigotes in the presence of compounds **2b** and **6** at their IC_{25} compared to a control sample after 96 h of incubation.

SOD enzymatic inhibition in the parasites and in human erythrocytes. We tested the effect of compounds on Fe-SOD isolated from the three species of Leishmania over a range of concentrations from 1 to 100 μM. We used promastigote forms of the three species, which excreted Fe-SOD when cultured in a medium lacking inactive FBS.[36] The inhibition data obtained are displayed in **Figures 7**, **8**, **9** and **10**, and the corresponding IC_{50} values are included in order to make interpretation the results easier. For comparison **Figure 7** shows the effect of the same compounds on CuZn-SOD obtained from human erythrocytes.

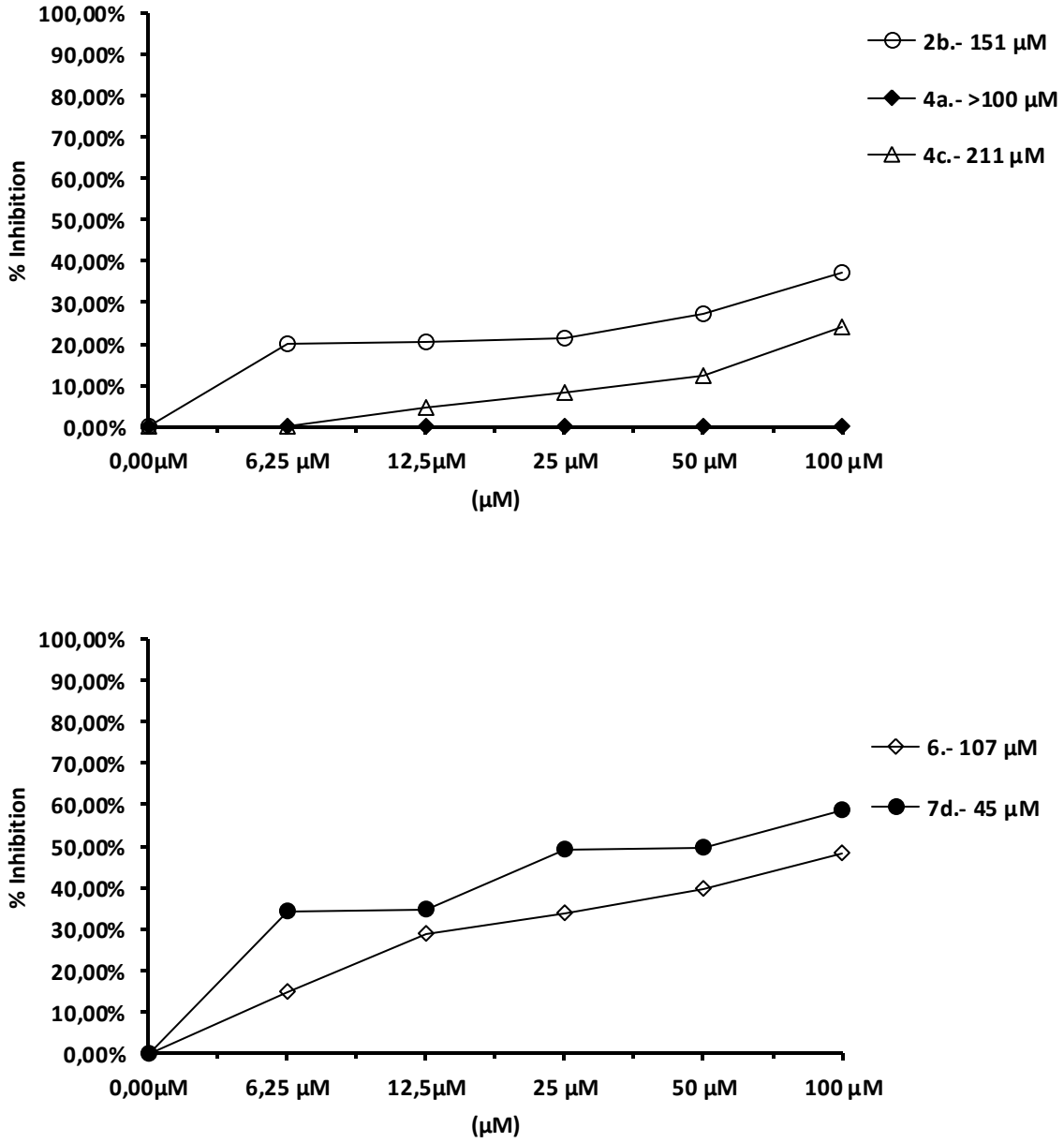


Figure 7. I*n vitro* inhibition (%) of CuZn-SOD from human erythrocytes for compounds **2b, 4a, 4c, 6, 7d**. Differences between the activities of the control homogenate and those ones incubated with compounds were obtained according to the Newman-Keuls test. Values are the average of three separate rate determinations.

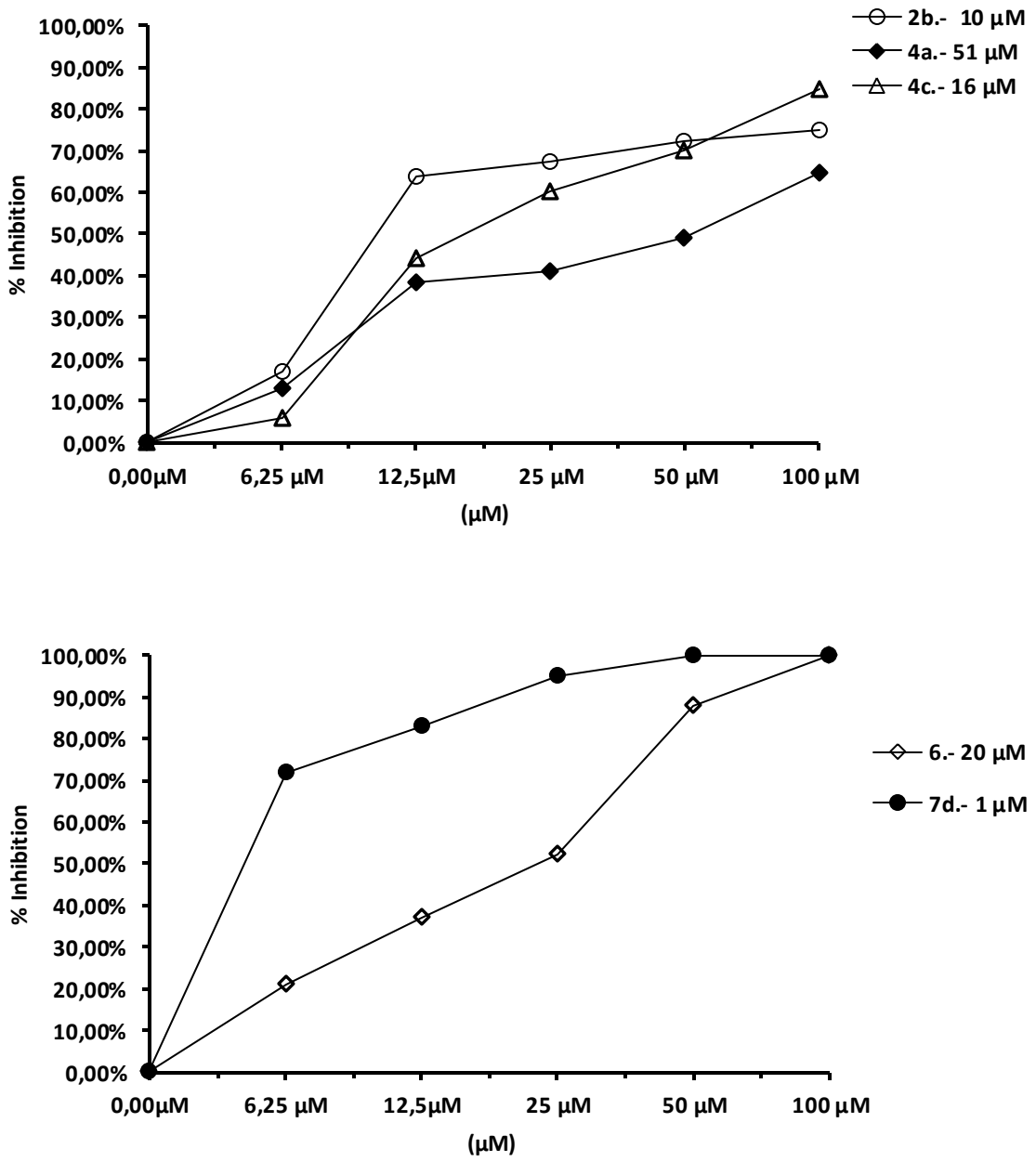


Figure 8. *In vitro* inhibition (%) of Fe-SOD from *L. infantum* promastigotes for the same compounds. Differences between the activities of the control homogenate and those ones incubated with compounds were obtained according to the Newman-Keuls test. Values are the average of three separate rate determinations.

The most remarkable result (**Figure 8**) was the significant inhibition of Fe-SOD activity found by the compounds **2b**, **4a**, **4c**, **6** and **7**d, whereas their inhibition of human CuZn-SOD was clearly lower. If we consider the IC_{50} calculated for *L. infantum*, inhibition of Fe-SOD by compounds **2b, 4a, 4c**, **6** and **7d** was respectively 15.1-, >19.6-, 13.2-, 5.3- and

45-fold higher than inhibition of CuZn-SOD, and respective values of 37.8-, 19.6-, 586.1-, 42800-, 12857.1-fold higher were obtained in the case of *L. braziliensis* (**Figure 9**).

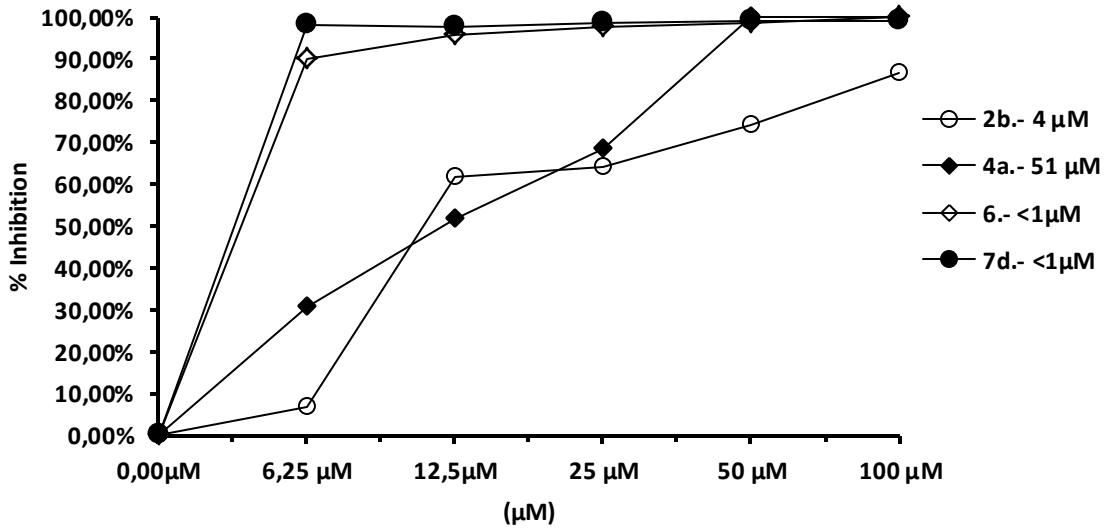


Figure 9. *In vitro* inhibition (%) of Fe-SOD from *L. braziliensis* promastigotes for compounds **2b, 4a, 6, 7**. Differences between the activities of the control homogenate and those ones incubated with compounds were obtained according to the Newman-Keuls test. Values are the average of three separate rate determinations.

In the case of *L. donovani*, (**Figure 10**) results for compounds **2b** and **6** were 3.5- and 6.7-fold higher than those in the case of Cu/Zn-SOD. We can also conclude that compound **6** is a more efficient Fe-SOD inhibitor in *L. braziliensis* than in *L. infantum* and *L donovani* (IC_{50} 0.0025 µM, 20 µM and 16 µM, respectively), the same occurs with compound **7d**, that is a major inhibitor of Fe-SOD of *L. braziliensis* than in *L. infantum*.

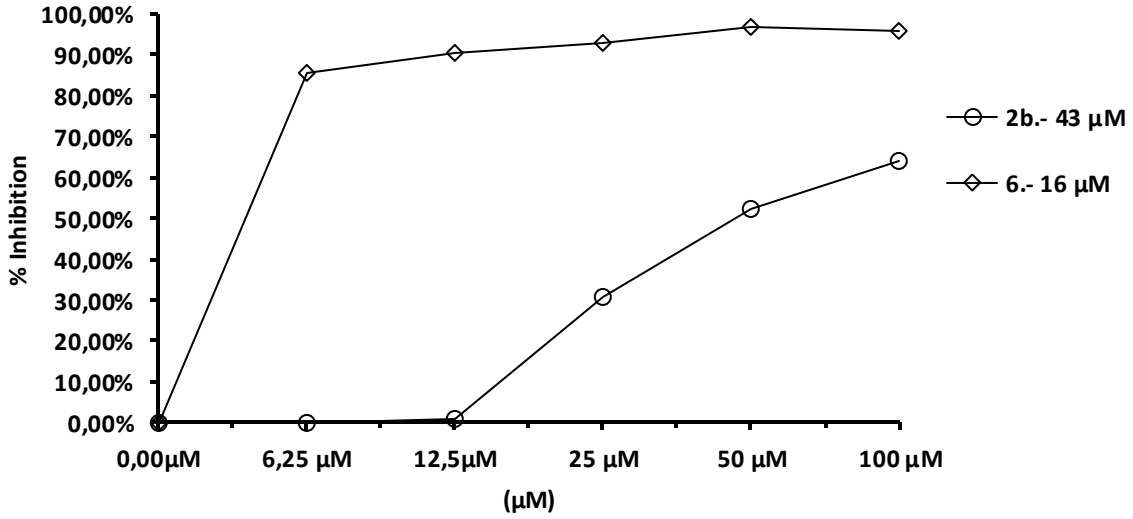


Figure 10. *In vitro* inhibition (%) of Fe-SOD from *L. donovani* promastigotes for compounds **2b, 6**. Differences between the activities of the control homogenate and those ones incubated with compounds were obtained according to the Newman-Keuls test. Values are the average of three separate rate determinations.

CONCLUSION

In summary, we described a novel family of triazolopyridinium salts with interesting antileishmanial activity. The activity was tested on *Leishmania infantum*, *Leishmania braziliensis* and *Leishmania donovani* parasites, using promastigotes and intracellular amastigotes forms. The cytotoxicity of the tested compounds on J774.2 macrophage cells was also measured. Five of the tested compounds (**2b**, **4a, 4c**, **6**, **7d**) showed selectivity indexes higher than those of the reference drug Glucantime for the three *Leishmania* species. These compounds are potent inhibitors of Fe-SOD in the three parasite species considered whereas their impact on human CuZn-SOD was low. The high activity, low toxicity, stability, low cost of the starting materials and straightforward synthesis make these compounds appropriate molecules for the development of affordable antileishmanicidal agents.

EXPERIMENTAL SECTION

Materials and Measurements: Starting materials, if commercially available, were purchased and used as such, provided that suitable checks (melting ranges, refractive

indices and gas chromatography) had confirmed the claimed purity. When known compounds had to be prepared by literature procedures, pertinent references are given. Melting points or ranges (m.p.) given were determined on a Büchi B-545 heated stage and found to be reproducible after recrystallization, unless stated otherwise ("decomp"). ^{1}H and (^{1}H decoupled), ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 500 or 300 and 101 or 75 MHz, respectively. Chemical shifts are reported in δ units, parts per million (ppm), and were measured relative to the signals for residual water/methanol. Coupling constants (J) are given in Hz. COSY experiments were performed for all compounds. IR spectra were recorded using a Thermoscientific Nicolet FT IR iS10 ATR. HRMS TRIPLETOF^{T}5600 (ABSciex): LC/MS.Elemental analyses (C, H, N) were run on a CE Instrument EA 1110 CHNS analyzer**.** The solvents used were of spectroscopic or equivalent grade. Water was twice distilled and passed through a Millipore apparatus. The compounds were isolated as amorphous solids with variable amounts of hydratation.

General procedure

At 25 ºC [1,2,3]triazolo[1,5-*a*]pyridine **1a** or 3-methyl[1,2,3]triazolo[1,5-*a*]pyridine **1b** (2 mmol, 4 eq) and the corresponding alkylating agent (0.5 mmol, 1 eq) were diluted in DMF (1 mL) and heated at 90 º without stirring for 9 h in a sealed tube (see **Table 3**). Then the reaction mixture was allowed to reach 25 ºC and a solid was precipitated. The remaining liquid phase was removed by decantation, the solid was washed 3 times with DMF and then 3 more times with ethyl acetate, ultrasonication was employed each time for the complete removal of remaining reagents. The solid material was then dried under reduced pressure for 24 h. In Table 3 salts yields are given. See supporting information for all figures of NMR spectra.

Table 3: General conditions and yields for the synthesis of compounds **2a-7d.**

trizolopyiridine		alkylating agent		product (mg)	yield
1a	240 mg	1,3-dibromopropane	100 mg	**2a** 140	69%
1a	241 mg	1,4-dibromobutane	106 mg	**2b** 180	79%
1a	242 mg	1,5-dibromopentane	112 mg	**2c** 150	63%
1a	243 mg	1,6-dibromohexane	124 mg	**2d** 120	50%
1a	244 mg	1,4-bis(bromomethyl)benzene	130 mg	**3a** 171	68%
1a	245 mg	1,3-bis(bromomethyl)benzene	130 mg	**3b** 235	93%
1a	246 mg	1,2-bis(bromomethyl)benzene	130 mg	**3c** 175	70%
1b	260 mg	1,3-dibromopropane	100 mg	**4a** 140	60%
1b	260 mg	1,4-dibromobutane	106 mg	**4b** 143	59%
1b	260 mg	1,5-dibromopentane	112 mg	**4c** 134	52%
1b	260 mg	1,6-dibromohexane	124 mg	**4d** 150	58%
1b	260 mg	1,4-bis(bromomethyl)benzene	130 mg	**5a** 240	90%
1b	260 mg	1,3-bis(bromomethyl)benzene	130 mg	**5b** 242	90%
1b	260 mg	1,2-bis(bromomethyl)benzene	130 mg	**5c** 171	64%
1b	260 mg	4,4'-bis(bromomethyl)-1,1'-biphenyl	170 mg	**6** 190	62%
1b	130 mg	benzylbromide	240 mg	**7a** 185	60%
1b	130 mg	(2-bromoethyl)benzene	255 mg	**7b** 190	61%
1b	130 mg	(3-bromopropyl)benzene	267 mg	**7c** 170	51%
1b	130 mg	(4-bromobutyl)benzene	270 mg	**7d** 170	49%

2,2'-(Propane-1,3-diyl)bis([1,2,3]triazolo[1,5-*a*]pyridin-2-ium) bromide 2a. Mp 264-266ºC. ^{1}H NMR (500 MHz, D_2O) δ 9.19 (s, 1H/partially deuterated), 9.08 (d, *J* = 7.1 Hz, 2H), 8.27 (dd, *J* = 9.1, 1.2 Hz,, 2H), 7.96 – 7.91 (m, 2H), 7.80 (t, *J* = 7.1 Hz 2H), 5.11 (t, *J* = 6.8 Hz, 4H), 3.06 (dd, *J* = 6.8 Hz, 2H). ^{13}C NMR (126 MHz, D_2O) δ = 135.74 (2C), 130.86 (2CH), 125.84 (2CH), 123.80 (2CH), 123.01 (2CH), 119.71 (2CH), 51.01 ($2CH_2$), 28.81 ($2CH_3$). IR (ATR, cm^{-1}) 3076,3036, 1646, 1633, 1525, 1508, 1471, 1355, 1324, 1160, 1130, 1050, 1011, 954, 860, 798, 752. HRMS ESI-[TOF] for $C_{15}H_{16}N_6^{2+}$ calcd. 140.0713; found 140.0711. Anal. Calcd. for $C_{15}H_{16}N_6Br_2$·(MeOH) C 40.70, H 4.27, N 17.80; found C 40.38, H 3.72, N 18.04.

2,2'-(Butane-1,4-diyl)bis([1,2,3]triazolo[1,5-*a*]pyridin-2-ium) bromide 2b. Mp 256-258ºC. ^{1}H NMR (500 MHz, D_2O) δ 9.03 (s, 2H), 8.97 (ddd, *J* = 7.3, 1.9, 1.0 Hz, 2H), 8.17 (dt, *J* = 9.0, 0.9 Hz, 2H), 7.82 (ddd, *J* = 9.0, 7.1, 0.8 Hz, 2H), 7.69 (td, *J* = 7.1, 1.2 Hz, 2H),

4.90 (brs, 4H), 2.23 (brs, 4H). ^{13}C NMR (126 MHz, D_2O) δ = 135.70 (2C), 130.64 (2CH), 125.85 (2CH), 123.31 (2CH), 122.72 (2CH), 119.63 (2CH), 53.45 ($2CH_2$), 25.87 ($2CH_2$). IR (ATR, cm^{-1}) 3036, 3010, 2931, 1646, 1525, 1508, 1475, 1454, 1422, 1358, 1257, 1162, 1127, 1051, 991, 914, 859, 829, 757. HRMS ESI-[TOF] for $C_{16}H_{18}N_6^{2+}$ calcd. 147.0791; found 147.0787. Anal. Calcd for $C_{16}H_{18}N_6Br_2 \cdot 0.5(H_2O)$ C 41.49, H 4.13, N 18.14; found C 41.75, H 3.80, N 17.73.

2,2'-(Pentane-1,5-diyl)bis([1,2,3]triazolo[1,5-*a*]pyridin-2-ium) bromide 2c. Mp 240-241ºC ^{1}H NMR (500 MHz, D_2O) δ 9.00 (s, 2H/partilly deuterated), 8.95 (dd, *J* = 7.0, 0.7 Hz, 2H), 8.16 (dd, *J* = 9.0, 1.1 Hz, 2H), 7.83 – 7.78 (m, 2H), 7.69 (dd, *J* = 7.1, 1.2 Hz, 2H), 4.82 (t, *J* = 7.1 Hz, 4H), 2.17 (m, 4H), 1.48 – 1.42 (m, 2H). ^{13}C NMR (126 MHz, D_2O) δ = 135.65 (2C), 130.55 (2CH), 125.80 (2CH), 123.11 (2CH), 122.60 (2CH), 119.58 (2CH), 53.96 ($2CH_2$), 28.31 ($2CH_2$), 22.26 (CH_2). IR (ATR, cm^{-1}) 3021, 2868, 1644, 1523, 1473, 1444, 1369, 1244, 1130, 1042, 918, 833, 800, 758. HRMS ESI-[TOF] for $C_{17}H_{20}N_6^{2+}$ calcd. 154.0869; found 154.0871. Anal. Calcd. for $C_{17}H_{20}N_6Br_2$ C 43.61, H 4.31, N 17.95; found C 43.87, H 3.99, N 17.77.

2,2'-(Hexane-1,6-diyl)bis([1,2,3]triazolo[1,5-*a*]pyridin-2-ium) bromide 2d. Mp 232-233ºC. ^{1}H NMR (500 MHz, D_2O) δ 8.95 (dd, *J* = 7.1, 1.2 Hz 2H), 8.15 (dt, *J* = 9.3, 1.1 Hz, 2H), 7.83 – 7.77 (m, 2H), 7.67 (td, *J* = 7.1, 1.2 Hz, 2H), 4.79 (t, *J* = 7.1 Hz, 4H), 2.09-2.06 (m, 2H), 1.42 – 1.39 (m,4H). ^{13}C NMR (126 MHz, D_2O) δ = 135.53 (2C), 130.48 (2CH), 125.77 (2CH), 122.53 (2CH), 119.51 (2CH), 54.18 ($2CH_2$), 28.73 ($2CH_2$), 24.76 ($2CH_2$). IR (ATR, cm^{-1}) 3617(H_2O), 3375(H_2O), 3067, 2931,2867, 1649, 1583, 1526, 1441, 1378, 1246, 1143, 1052, 919, 836, 760. HRMS ESI-[TOF] for $C_{18}H_{222}N_6^{2+}$ calcd. 161.0947; found 161.0943. Anal. Calcd. for $C_{18}H_{22}N_6Br_2$ 0.5·(H_2O) C 44.01, H 4.72, N 17.11; found C 44.43, H 4.20, N 16.77.

2,2'-(1,4-Phenylenebis(methylene))bis([1,2,3]triazolo[1,5-*a*]pyridin-2-ium) bromide 3a. Mp 256-257ºC. ^{1}H NMR (300 MHz, MeOH) δ 9.35 (s, 2H), 9.21 (d, *J* = 7.1 Hz, 2H), 8.36 (d, *J* = 9.0 Hz, 2H), 8.01 – 7.95 (m, 2H), 7.90 – 7.84 (m, 2H), 7.78 (s, 4H), 6.20 (s, 4H). ^{13}C NMR (75 MHz, MeOH) δ = 137.16 (2C), 135.15 (2C), 131.95 (2CH), 131.50 (4CH), 127.34 (2CH), 124.73 (2CH), 124.17 (2CH), 121.09 (2CH), 58.32 ($2CH_2$). IR (ATR, cm^{-1}) 3617(H_2O), 3375(H_2O), 3067, 3015, 2931, 1648, 1526, 1421, 1323, 1246, 1145, 1049, 918, 826, 757. HRMS ESI-[TOF] for $C_{20}H_{18}N_6^{2+}$ calcd. 171.0791; found

171.0790. Anal. Calcd for $C_{20}H_{20}N_6Br_2$ 0.5·(H_2O) 0.5·(MeOH) C 46.18 H 3.88, N 16.15; found C 46.41 H 2.77, N 15.90

2,2'-(1,3-Phenylenebis(methylene))bis([1,2,3]triazolo[1,5-*a*]pyridin-2-ium) bromide 3b. Mp 219-220ºC. ^{1}H NMR (300 MHz, D_2O) δ 8.82 (dd, *J* = 7.1, 0.8 Hz, 2H), 8.16 (d, *J* = 9.1 Hz, 2H), 7.86(dd, *J* = 9.1, 7.1 Hz, 2H), 7.70 (m, 6H), 6.32 (s, 4H). ^{13}C NMR (75 MHz, D_2O) δ = 133.23 (2C), 133.10 (2C), 132.60 (2CH), 131.76 (2CH), 131.38 (4CH), 125.97 (2CH), 123.55 (2CH), 120.02 (2CH), 55.15 ($2CH_2$). IR (ATR, cm^{-1}) 3373(H_2O), 3045, 2976, 2928, 1646, 1525, 1479, 1434, 1420, 1262, 1182, 1146, 1045, 841, 794, 759, 729. HRMS ESI-[TOF] for $C_{20}H_{18}N_6^{2+}$ calcd. 171.0791; found 171.0797. Anal. Calcd. for $C_{20}H_{18}N_6Br_2$ 0.5·(H_2O) C 46.99, H 3.75, N 16.44; found C 46.60, H 3.84, N 16.00.

2,2'-(1,2-Phenylenebis(methylene))bis([1,2,3]triazolo[1,5-*a*]pyridin-2-ium) bromide 3c. Mp 221-224ºC^{1}H NMR (300 MHz, D_2O) δ 9.02 (dd, *J* = 7.1, 0.9 Hz, 2H), 8.22 (dd, *J* = 9.1, 1.1 Hz, 2H), 7.92 – 7.86 (m, 2H), 7.80 – 7.61 (m, 6H), 6.10 (s, 4H). ^{13}C NMR (75 MHz, D_2O) δ = 136.07 (2C), 133.49 (2C), 131.10 (2CH), 130.98 (2CH), 130.77 (2CH), 130.41 (2CH), 126.21 (2CH), 123.25 (2CH), 120.05 (2CH), 57.49 ($2CH_2$). IR (ATR, cm^{-1}) 3050, 3000, 2943, 1645, 1523, 1447, 1341, 1260, 1207, 1153, 1137, 1047, 914, 818, 773, 762, 725. HRMS ESI-[TOF] for $C_{20}H_{18}N_6^{2+}$ calcd. 171.0791; found 171.0799. Anal. Calcd. $C_{20}H_{18}N_6Br_2$ 0.5·(H_2O) C 46.99, H 3.75, N 16.44; found C 46.90, H 3.69, N 16.66.

2,2'-(Propane-1,3-diyl)bis(3-methyl-[1,2,3]triazolo[1,5-*a*]pyridin-2-ium) bromide 4a. Mp 249-251ºC. ^{1}H NMR (500 MHz, D_2O) δ 8.96 (dd, *J* = 7.1, 1.2 Hz, 2H), 8.20(d, *J* = 9.1, Hz, 2H), 7.88 – 7.81 (m, 2H), 7.75 (dd, *J* = 7.1, 1.2 Hz, 2H), 5.02 (t, *J* = 6.8 Hz, 4H), 2.99 (t, *J* = 6.8 Hz, 3H), 2.92 (s, 3H). ^{13}C NMR (126 MHz, D_2O) δ 134.33 (2C), 133.51 (2C), 129.41 (2CH), 125.41 (2CH), 123.03 (2CH), 119.24 (2CH), 47.95 ($2CH_2$), 26.90 (CH_2), 8.04 ($2CH_3$) IR (ATR, cm^{-1}) 3452(H_2O), 3404(H_2O), 3031, 2942, 1647, 1620, 1432, 1383, 1263, 1151, 928, 865, 740. HRMS ESI-[TOF] for $C_{17}H_{20}N_6^{2+}$ calcd. 154.0869; found 154.0869. Anal. Calcd for $C_{17}H_{20}N_6Br_2$ 2·H_2O C 40.50, H 4.80, N 16.67; found C 40.08, H 4.41, N 16.59.

2,2'-(Butane-1,4-diyl)bis(3-methyl-[1,2,3]triazolo[1,5-*a*]pyridin-2-ium) bromide 4b. Mp 256-257ºC. ^{1}H NMR (300 MHz, D_2O) δ 8.95 (dt, *J* = 7.0, 1.0 Hz, 2H), 8.20 (dt, *J* = 9.1, 1.2 Hz, 2H), 7.86 – 7.79 (m, 2H), 7.72 (td, *J* = 7.1, 1.3 Hz, 2H), 4.88 (m, 4H), 2.87 (s, 6H), 2.35 – 2.19 (m, 2H). ^{13}C NMR (75 MHz, D_2O) δ 134.69 (2C), 133.39 (2C), 129.55 (2CH), 125.84 (2CH), 123.11 (2CH), 119.57 (2CH), 50.61 ($2CH_2$), 25.59 ($2CH_2$), 8.44

($2CH_3$). IR ATR, cm^{-1}) 3452(H_2O), 3404(H_2O), 3027, 2984, 1645, 1539, 1480, 1383, 1267, 1255, 1157, 930, 859, 742. HRMS ESI-[TOF] for $C_{18}H_{22}N_6^{2+}$ calcd. 161.0947; found 161.0944. Anal. Calcd for $C_{18}H_{22}N_6Br_2$ 2· H_2O C 41.72, H 5.06, N 16.22; found C 41.78, H 5.47, N 15.68.

2,2'-(Pentane-1,5-diyl)bis(3-methyl-[1,2,3]triazolo[1,5-*a*]pyridin-2-ium) bromide 4c. Mp 255-257ºC. ^{1}H NMR (300 MHz, D_2O) δ 8.93 (d, *J* = 7.1 Hz, 2H), 8.19 (dt, *J* = 9.1, 1.2 Hz, 2H), 7.85 – 7.78 (m, 2H), 7.71 (td, *J* = 7.1, 1.3 Hz, 2H), 4.92 (m, 4H), 2.85 (s, 6H), 2.19 (m, 4H), 1.57 (m, 4H). ^{13}C NMR (75 MHz, D_2O) δ 134.64 (2C), 133.20 (C), 129.46 (2CH), 125.78 (2CH), 123.00 (2CH), 119.52 (2CH), 51.14 ($2CH_2$), 28.08 ($2CH_2$), 22.84 (CH_2), 8.40 ($2CH_3$). IR (ATR, cm^{-1}) 3452(H_2O), 3404(H_2O), 3027, 2984, 1645, 1598, 1479, 1404, 1255, 1105, 930, 866, 743. HRMS ESI-[TOF] for $C_{19}H_{24}N_6^{2+}$ calcd. 168.1026; found 168.1024. Anal. Calcd. for $C_{19}H_{24}N_6Br_2$ C 54.99, H 4.87, N 16.94; found C 45.81, H 4.80, N 17.10

2,2'-(Hexane-1,6-diyl)bis(3-methyl-[1,2,3]triazolo[1,5-*a*]pyridin-2-ium) bromide 4d. Mp 259-260ºC. ^{1}H NMR (300 MHz, D_2O) δ 8.94(dd, *J* = 7.1, 1.2 Hz, 2H), 8.18 (dt, *J* = 9.0, 1.2 Hz, 2H), 7.84 – 7.76 (m, 2H), 7.70 (td, *J* = 7.1, 1.2 Hz, 2H), 2.84 (s, 6H), 4.85 (m, 4H) 2.10 (t, *J* = 7.0 Hz,4H), 1.54 – 1.44 (m, 4H). ^{13}C NMR (75 MHz, D_2O) δ 134.61 (2C), 133.13 (2C), 129.41 (2CH), 125.77 (2CH), 122.94 (2CH), 119.47 (2CH), 51.39 ($2CH_2$), 28.52 ($2CH_2$), 25.33 ($2CH_2$), 8.34 ($2CH_3$). IR (ATR, cm^{-1}) 3452(H_2O), 3404(H_2O), 3027, 1646, 1558, 1480, 1397, 1255, 1143, 933, 866, 743. HRMS ESI-[TOF] for $C_{20}H_{26}N_6^{2+}$ calcd. 175.1104; found 175.1103. Anal. Calcd. for $C_{20}H_{26}N_6Br_2$ C 47.08, H 5.14, N 16.47; found C 47.19, H 4.91, N 15.85.

2,2'-(1,4-Phenylenebis(methylene))bis(3-methyl-[1,2,3]triazolo[1,5-*a*]pyridin-2-ium) bromide 5[a]. Mp 268-270ºC. ^{1}H NMR (300 MHz, MeOH/D_2O) δ 9.14 (d, *J* = 7.0 Hz, 2H), 8.35 (dt, *J* = 9.0, 1.2 Hz, 2H), 7.96 – 7.89 (m, 2H), 7.84 (td, *J* = 7.0, 1.4 Hz, 2H), 7.64 (s, 4H), 6.13 (s, 4H), 2.92 (s, 6H). ^{13}C NMR (75 MHz, MeOH/D_2O) δ 136.38 (2C), 135.05 (2C), 131.23 (4CH), 131.12 (2CH), 127.51 (2CH), 124.73 (2CH), 121.11 (2CH), 55.91 ($2CH_2$), 9.66 ($2CH_3$). IR (ATR, cm^{-1}) 3458(H_2O), 3006, 1646, 1435, 1344, 1255, 1128, 824. HRMS ESI-[TOF] for $C_{22}H_{22}N_6^{2+}$ calcd. 185.0947; found 185.0942. Anal. Calcd. for $C_{22}H_{22}N_6Br_2$ 0.5·H_2O C 49.00, H 4.30, N 15.58; found C 49.24, H 4.30, N 15.25.

2,2'-(1,3-Phenylenebis(methylene))bis(3-methyl-[1,2,3]triazolo[1,5-*a*]pyridin-2-ium) bromide 5b. Mp 241-242ºC. ^{1}H NMR (300 MHz, D_2O) δ 8.63 (d, *J* = 7.1 Hz, 2H), 8.09 (d,

J = 9.1 Hz, 2H), 7.75 – 7.65 (m, 2H), 7.61 – 7.48 (m, 4H), 7.41 (m, 2H), 6.17 (s, 4H), 2.81 (s, 6H). ^{13}C NMR (75 MHz, D_2O) δ 140.79 (2C), 134.67 (2C), 134.13 (2C), 131.78 (CH), 131.13 (2CH), 130.91 (CH), 130.02 (CH), 125.53 (2CH), 123.60 (2CH), 119.62 (2CH), 52.87 ($2CH_2$), 8.73 ($2CH_3$). IR (ATR, cm^{-1}) 3479(H_2O), 3359(H_2O), 3009, 1646, 1435, 1334, 1157, 1129, 931, 824. HRMS ESI-[TOF] for $C_{22}H_{22}N_6^{2+}$ calcd. 185.0947; found 185.0943. Anal. Calcd. for $C_{22}H_{22}N_6Br_2$ 0.5·H_2O C 49.00, H 4.30, N 15.58; found C 49.01, H 4.36, N 15.38.

2,2'-(1,2-Phenylenebis(methylene))bis(3-methyl-[1,2,3]triazolo[1,5-*a*]pyridin-2-ium) bromide 5c. Mp 250-251ºC. ^{1}H NMR (300 MHz, D_2O) δ 8.95 (d, *J* = 7.0 Hz, 2H), 8.19 (d, *J* = 9.0 Hz, 2H), 7.87 – 7.80 (m, 2H), 7.74 (t, *J* = 7.0 Hz, 2H), 7.62 – 7.47 (m, 4H), 6.03 (s, 4H), 2.81 (s, 6H). ^{13}C NMR (75 MHz, D_2O) δ = 133.59 (2C), 133.30 (2C), 130.87 (2C) 130.64 (2CH), 129.83 (2CH), 129.75 (2CH), 129.14 (2CH), 125.88 (2CH), 123.33 (2CH), 119.60 (2CH), 54.56($2CH_2$), 8.58 ($2CH_3$). IR (ATR, cm^{-1}) 3364(H_2O), 3009, 2970, 2926, 1647, 1435, 1329, 1208, 1090, 951, 818. HRMS ESI-[TOF] for $C_{22}H_{22}N_6^{2+}$ calcd. 185.0947; found 185.0952. Anal. Calcd. for $C_{22}H_{22}N_6Br_2$ C 49.83, H 4.18, N 15.85; found C 50.03, H 3.94, N 15.25.

2,2'-([1,1'-Biphenyl]-4,4'-diylbis(methylene))bis(3-methyl-[1,2,3]triazolo[1,5-*a*]pyridin-2-ium) bromide 6. Mp 156-157 ºC. ^{1}H NMR (300 MHz, D_2O) δ 8.95 (d, *J* = 6.7 Hz, 2H), 8.17 (d, *J* = 9.1 Hz, 2H), 7.81 (t, *J*=6.7Hz, 1H), 7.71 (m, 6H), 7.54-7.52 (m, 4H), 6.03 (s, 4H), 2.81 (s, 3H), 2.80(s, 3H). ^{13}C NMR (75 MHz, D_2O) δ 140.83 (C), 134.86 (C), 133.44 (C), 131.86 (C), 129.68 (CH), 129.57 (CH), 128.04 (CH), 125.86 (CH), 123.25 (CH), 119.56 (CH), 54.64 (CH_2), 8.57 (CH_3). IR (ATR, cm^{-1}) 3417(H_2O), 3322(H_2O), 3103, 3009, 1643, 1504, 1438, 1399, 1268, 1204, 1141, 1062, 924, 849, 813, 758. HRMS ESI-[TOF] for $C_{28}H_{26}N_6^{2+}$ calcd. 223.1104; found 223.1097. Anal. Calcd. for $C_{28}H_{26}N_6Br_2$ 2·H_2O Calcd. C 52.35, H 4.71, N 13.08; found C 52.23, H 4.65, N 13.13.

2-Benzyl-3-methyl-[1,2,3]triazolo[1,5-*a*]pyridin-2-ium bromide 7[a]. Mp 100-101 ºC. **[1]**H NMR (300 MHz, D_2O) δ 8.95 (d, *J* = 7.1 Hz, 1H), 8.18 (d, *J* = 9.0 Hz, 1H), 7.91 – 7.65 (m, 2H), 7.47 (s, 5H), 6.00 (s, 2H), 2.81 (s, 3H). ^{13}C NMR (75 MHz, D_2O) δ 134.96 (C), 133.44 (C), 132.24 (C), 129.79 CH), 129.67 CH), 128.88 (CH), 127.76 (CH), 125.86 (CH), 123.20 (CH), 119.57 (CH), 55.00 (CH_2), 8.57 (CH_3). IR (ATR, cm^{-1}) 3453(H_2O), 3383(H_2O), 3120, 3011, 2973, 2934, 1646, 1621, 1501, 1440, 1324, 1271, 1204, 1157, 866, 752, 730. HRMS ESI-[TOF] for $C_{14}H_{14}N_3^+$ calcd. 224.1182; found 224.1177. Anal

Calcd. for $C_{14}H_{14}N_3Br_2$ 1.5·H_2O Calcd. C 50.77, H 5.17, N 12.69; found 50.52, H 5.40, N 12.73.

3-Methyl-2-phenethyl-[1,2,3]triazolo[1,5-*a*]pyridin-2-ium bromide 7b. Mp. 129-131 ºC. ^{1}H NMR (300 MHz, D_2O) δ 8.92 (d, *J* = 7.0 Hz, 1H), 8.09 (d, *J* = 9.0 Hz, 1H), 7.81 – 7.75 (m, 1H), 7.70 (td, *J* = 7.0, 1.3 Hz, 1H), 7.30 (m, 3H), 7.19 – 7.13 (m, 2H), 5.03 (t, *J* = 6.6 Hz, 2H), 3.41 (t, *J* = 6.6 Hz, 2H), 2.47 (s, 3H). ^{13}C NMR (75 MHz, D_2O) δ 136.77 (C), 134.27 (C), 133.64 (C), 129.46 (CH), 129.34 (CH), 129.24 (CH), 127.83 (CH), 125.82 (CH), 123.12 (CH), 119.57 (CH), 52.83 (CH_2), 35.26 (CH_2), 8.03 (CH_3). IR (ATR, cm^{-1}) 3447(H_2O), 3383(H_2O), 3122, 3009, 2973, 2931, 1646, 1615, 1499, 1443, 1327, 1268, 1204, 1157, 866, 752, 730. HRMS ESI-[TOF] for $C_{15}H_{16}N_3^+$calcd. 238.1339; found 238.1332. Anal Calcd. for $C_{15}H_{16}N_3Br_2$ .H_2O Calcd. C 53.58, H 5.40, N 12.50; found 53.17, H 4.56, N 12.55.

3-Methyl-2-(3-phenylpropyl)-[1,2,3]triazolo[1,5-*a*]pyridin-2-ium bromide 7c. Mp 132-135 ºC. ^{1}H NMR (300 MHz, D_2O) δ 8.87 (d, *J* = 7.0 Hz, 1H), 8.05 (d, *J* = 8.9 Hz, 1H), 7.79 – 7.72 (m, 1H), 7.67 (td, *J* = 7.0, 1.3 Hz, 1H), 7.21-7.20 (m, 4H), 7.08 (m, 1H), 4.87 – 4.69 (m, 1H), 2.83 (t, *J* = 7.0 Hz, 2H), 2.74 (s, 3H), 2.55 – 2.46 (m, 2H). ^{13}C NMR (75 MHz, D_2O) δ 140.32 (C), 134.39 (C), 133.16 (C), 129.33 (2CH), 128.58 (CH), 126.62 (CH), 125.66 (CH), 122.92 (CH), 119.35 (CH), 51.21 (CH_2), 32.15 (CH_2), 29.15 (CH_2), 8.35 (CH_3). IR (ATR, cm^{-1}) 3042, 2995, 2961, 1640, 1499, 1446, 1396, 1332, 1274, 1243, 1157, 1141, 1038, 930, 874,846, 763, 741, 699. HRMS ESI-[TOF] for $C_{16}H_{18}N_3^+$ calcd. 252.1495; found 252.1483. Anal. Calcd. for $C_{16}H_{18}N_3Br$ ·(CH_2Cl_2) Calcd. C 53.49, H 5.28, N 11.01; found C 53.65, H 5.04, N 11.63

3-Methyl-2-(4-phenylbutyl)-[1,2,3]triazolo[1,5-*a*]pyridin-2-ium bromide 7d. Mp 173-175 ºC. ^{1}H NMR (300 MHz, D_2O) δ 8.89 (d, *J* = 7.1 Hz, 1H), 8.14 (dt, *J* = 9.1, 1.2 Hz, 1H), 7.89 – 7.76 (m, 1H), 7.69 (td, *J* = 7.1, 1.3 Hz, 1H), 7.37 – 7.14 (m, 5H), 4.72 (t, *J* = 7.1 Hz, 2H), 2.76 (s, 1H), 2.69 (t, *J* = 7.4 Hz, 2H), 2.07 (dt, *J* = 15.1, 7.2 Hz, 2H), 1.86 – 1.55 (m, 2H). ^{13}C NMR (75 MHz, D_2O) δ 142.40 (C), 134.50 (C), 133.00 (C), 129.39 (CH), 128.91 (CH), 128.88 (CH), 126.45 (CH), 125.73 (CH), 122.94 (CH), 119.49 (CH), 51.46 (CH_2), 34.53 (CH_2), 27.98 (CH_2), 27.52 (CH_2), 8.32 (CH_3). IR (ATR, cm^{-1}) 2995, 2964, 1643, 1604, 1496, 1446, 1399, 1332, 1268, 1232, 1154, 1135, 1027, 930, 860, 791, 766, 752, 721, 694. HRMS ESI-[TOF] for $C_{17}H_{20}N_3^+$ calcd. 266.1652; found 266.1645. Anal. Calcd. for $C_{17}H_{20}N_3Br$ Calcd. C 58.97, H 5.82, N 12.14; found C 58.68, H 5.55, N 12.47.

Biological evaluation.

Parasite strain and culture. Promastigote forms of *L. infantum* (MCAN/ES/2001/UCM-10), *L. braziliensis* (MHOM/BR/1975/M2904) and *L. donovani* (MHOM/PE/84/LC26) were cultured in vitro in medium trypanosomes liquid (MTL) supplemented with 10% inactive fetal calf serum (FCS) kept in an air atmosphere at 28°C, in Roux flasks (Corning, USA) with a surface area of 75 cm^2, according to the methodology described by Gonzalez et al.[37]

In vitro activity assays. Compounds to be tested were first dissolved in dimethylsulfoxide (DMSO, Panreac, Barcelona, Spain) at a concentration of 0.1% and then assayed for toxicity and inhibitory effects on parasite and mammalian cells growth as previously described by Gonzalez et al.[37]

Cell culture and cytotoxicity tests. The macrophage line J774.2 [European collection of cell cultures (ECACC) number 91051511] was derived in 1968 from a tumor in a female BALB/c mouse. The macrophages were grown in minimal essential medium (MEM) plus glutamine (2 mM) and 20% inactive FCS, with a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

The cytotoxicity testing on macrophages was performed by flow cytometric analysis according to a method previously described.[30] The percentage of viable cells was calculated with respect to the control culture. The IC50 was calculated using linear regression analysis from the Kc values of the concentrations employed.

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

Amastigote assay: intracellular form. J774.2 Macrophage cells were grown and seeded at a density of 1×10^4 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultured for 2 days. The adherent macrophages were then infected with promastigotes of L. infantum, L. braziliensis and L. donovani in the stationary growth phase, at a ratio of 10:1 and maintained for 24 h at 37°C in air containing 5% CO2. Non-

phagocytosed parasites were removed by washing, and the infected cultures were incubated with the testing compounds (concentrations ranging from 1 to 100 μM) and then cultured for 72 h in MEM plus glutamine (2 mM) and 20% inactive FCS. Compound activity was determined from the percentage reductions in amastigote number in treated versus untreated cultures in methanol-fixed and Giemsa-stained preparations. Values are the means of three separate determinations.[38]

Infectivity assay. Adherent macrophage cells grown as described above were infected in vitro with promastigote forms of L. infantum, L. braziliensis or L. donovani, at a ratio of 10:1. The compounds to be tested (IC25 concentrations) were added immediately after infection, and incubated for 12 h at 37°C in 5% CO2. Nonphagocytosed parasites and compounds were removed by washing, and then the infected cultures were grown for 10 days in fresh medium. Cultures were washed every 48 h and fresh culture medium was added. Compound activity was determined on the basis of both the percentage of infected cells and the number of amastigotes per infected cell in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analysing more than 500 host cells distributed in randomly chosen microscopic fields. Values are the means of three separate determinations.

Metabolite excretion. Cultures of L. infantum, L. braziliensis and L. donovani promastigotes (initial concentration 5×10^5 cells/mL) received the IC25 dose of each compound (except for control cultures). After incubation for 72 h at 28°C the cells were centrifuged at 400 g for 10 min. The supernatants were collected to determine the excreted metabolites by ^{1}HNMR, and chemical shifts were expressed in ppm, using sodium 2,2-dimethyl-2-silapentane-5-sulphonate as the reference signal. The chemical displacements used to identify the respective metabolites were consistent with those described previously by some of the co-authors.[39]

Fe-SOD enzymatic inhibition. Parasites were collected in the logarithmic growth phase by centrifugation (400 g for 10 min at room temperature). The pellet obtained after centrifugation, was resuspended in 3 mL of STE buffer (0.25 M sucrose, 25 mM Tris–HCl, 1 mM EDTA, pH 7.8) and the cells were lysed by three cycles of sonication for 30 s each at 60 W. The sonicated homogenate was centrifuged at 1500 g for 5 min at 4°C, and the pellet was washed three times in ice-cold STE buffer.

This fraction was centrifuged (2500 g for 10 min at 4°C) and the supernatant was collected. Then, the supernatant was subjected to ice-cold ammonium sulphate precipitation between 35 and 85% salt concentration and the resulting precipitate was dissolved in 2.5 mL of distilled water and desalted by chromatography in Sephadex G-25 column (GE Healthcare Life Sciences®, PD 10 column) previously equilibrated with 2 mL of distilled water, taking it up to a final volume of 3.5 mL35. The protein content was quantified using the Sigma Bradford test, which uses bovine serum albumin (BSA) as a standard.[40] Iron and copper−zinc superoxide dismutases actyvities were determined using a previously described method,[41] that measures the reduction in nitroblue tetrazolium (NBT) by superoxide ions. According to the protocol, 845 μL of stock solution [3 mL of L-methionine (300 mg, 10 mL−l), 2 mL of NBT (1.41 mg, 10 mL−1) and 1.5 mL of Triton X-100 1% (v/v)] were added into each well, along with 30 μL of the parasite homogenate fraction, 10 μL of riboflavine (0.44 mg, 10 mL−l), and an equivalent volume of the different concentrations of the compounds being tested. Seven different concentrations were used for each agent, from 0.1 to 100 μM. In the control experiment the volume was made up to 1000 μL with 50 mM potassium phosphate buffer (pH 7.8, 3 mL), and 30 μL of the parasite homogenate fraction were added to the mixtures containing the compounds. Then, the absorbance (A0) was measured at 560 nm in a UV spectrophotometer. Afterward, each well was illuminated with UV light for 10 min under constant stirring and the absorbance (A1) was measured again. The human CuZn-SOD and substrates used in these assays were obtained from Sigma Chemical Co. The resulting data were analysed using the Newman-Keuls test.

AUTOR INFORMATION

Corresponding Authors

***E-mail: belen.abarca@uv.es, rafael.ballesteros@uv.es.....

Author Contributions

A. M-M. and R. B-G. contributed equally

ACKNOWLEDGMENTS

We are grateful to the Ministerio de Ciencia e Innovación (Spain) (Project CONSOLIDER-INGENIO SUPRAMED CSD 2010-00065), and to Universidad de Valencia (Valencia,

Spain) (Project UV-INV-AE15-332846) for its financial support. R.B-G. acknowledges to Ministerio de Economía y Competitividad (Spain) for a Contrato de Formación Posdoctoral 2013.

REFERENCES

(1) Croft, S. L.; Sundar, S.; Fairlamb, A. H. Drug Resistance in Leishmaniasis. *Clin. Microbiol. Rev*. **2006**, *19*, 111–126.

(2) dos Santos, D. R. L.; Vitral, C. L.; de Paula, V. S.; Marchevsky, R. S.; Lopes, J. F.; Gaspar, A. M. C.; Saddi, T. M.; Júnior, N. C. de M.; Guimarães, F. de R.; Júnior, J. G. C.; Ximenes, L. L. L.; Souto, F. J. D.; Pinto, M. A. Serological and Molecular Evidence of Hepatitis E Virus in Swine in Brazil. *Vet. J.* **2009**, *182*, 474–480.

(3) Cavalli, A.; Bolognesi, M. L. Neglected Tropical Diseases: Multi-Target-Directed Ligands in the Search for Novel Lead Candidates against Trypanosoma and Leishmania. *J. Med. Chem*. **2009**, *52*, 7339–7359.

(4) *Control of the Leishmaniases: Report of a Meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22-26 March **2010***; WHO Expert Committee on the Control of the Leishmaniases, World Health Organization, Eds.; WHO technical report series; World Health Organization: Geneva, **2010**.

(5) Espuelas, S.; Plano, D.; Nguewa, P.; Font, M.; Palop, J. A.; Irache, J. M.; Sanmartín, C. Innovative Lead Compounds and Formulation Strategies as Newer Kinetoplastid Therapies. *Curr. Med. Chem*. **2012**, *19*, 4259–4288.

(6) Singh, T. U.; Choudhury, S.; Parida, S.; Maruti, B. S.; Mishra, S. K. Arachidonic Acid Inhibits Na^+-K^+-ATPase via Cytochrome P-450, Lipoxygenase and Protein Kinase C-Dependent Pathways in Sheep Pulmonary Artery. Vascul. Pharmacol. **2012**, 56 (1–2), 84–90.

(7) Tron, G.C.; Pirali, T.; Billington, R.A.; Canonico, P.L.; Sorba, G.; Genazzani, A.A. Click chemistry reactions in medicinal chemistry: applications of the 1,3-dipolar cycloaddition between azides and alkynes *Med. Res. Rev*. **2008**, *28*, 278-308.

(8) Agalave, S. G.; Maujan, S. R.; Pore, V. S. Click Chemistry: 1,2,3-Triazoles as Pharmacophores. *Chem Asian J* **2011**, *6*, 2696–2718.

(9) Almajan, G. L.; Barbuceanu, S.-F.; Almajan, E.-R.; Draghici, C.; Saramet, G. Synthesis, Characterization and Antibacterial Activity of Some Triazole Mannich Bases Carrying Diphenylsulfone Moieties. *Eur. J. Med. Chem*. **2009**, *44*, 3083–3089.

(10) Phillips, O.A.; Udo, E.E.; Abdel-Hamid, M.E.; Varghese, R. Synthesis and antibacterial activity of novel 5-(4-methyl-1H-1,2,3-triazole) methyl oxazolidinones *Eur. J. Med. Chem*. **2009**, *44*, 3217-3227.

(11) Bauer, K. S.; Cude, K. J.; Dixon, S. C.; Kruger, E. A.; Figg, W. D. Carboxyamido-Triazole Inhibits Angiogenesis by Blocking the Calcium-Mediated Nitric-Oxide Synthase-Vascular Endothelial Growth Factor Pathway. *J. Pharmacol. Exp. Ther*. **2000**, *292*, 31–37.

(12) Kumar, A.; Ahmad, I.; Chhikara, B. S.; Tiwari, R.; Mandal, D.; Parang, K. Synthesis of 3-Phenylpyrazolopyrimidine-1,2,3-Triazole Conjugates and Evaluation of Their Src Kinase Inhibitory and Anticancer Activities. *Bioorg. Med. Chem. Lett*. **2011**, *21*, 1342–1346.

(13) Giffin, M. J.; Heaslet, H.; Brik, A.; Lin, Y.-C.; Cauvi, G.; Wong, C.-H.; McRee, D. E.; Elder, J. H.; Stout, C. D.; Torbett, B. E. A copper(I)-Catalyzed 1,2,3-Triazole Azide-Alkyne Click Compound Is a Potent Inhibitor of a Multidrug-Resistant HIV-1 Protease Variant. *J. Med. Chem*. **2008**, *51*, 6263–6270.

(14) Aher, N. G., Pore, V. S., Mishra, N. N., Kumar, A., Shukla, P. K., Sharma, A., Bhat, M. K. Synthesis and antifungal activity of 1,2,3-triazole containing fluconazole analogues *Bioorg. Med. Chem. Lett*. 2009, *19*, 759-763.

(15) Patil, V., Guerrant, W. Chen, P. C., Gryder, B., Benicewicz, D. B., Khan, S. I., Tekwani, B. L., Oyelere, A. K. Antimalarial and antileishmanial activities of histone deacetylase inhibitors with triazole-linked cap group *Bioorg. Med. Chem*. **2010**, *18*, 415-425

(16) Tahghighi, A.; Razmi, S.; Mahdavi, M.; Foroumadi, P.; Ardestani, S. K.; Emami, S.; Kobarfard, F.; Dastmalchi, S.; Shafiee, A.; Foroumadi, A. Synthesis and Anti-Leishmanial Activity of 5-(5-Nitrofuran-2-Yl)-1,3,4-Thiadiazol-2-Amines Containing N-[(1-Benzyl-1H-1,2,3-Triazol-4-yl)methyl] Moieties. *Eur. J. Med. Chem*. **2012**, *50*, 124–128.

(17) Guimarães, T. T., Pinto, M. d. C. F. R., Lanza, J. S., Melo, M. N., Monte-Neto, R. L. do, Melo, I. M. M. de, Diogo, E. B. T., Ferreira, V. F. Camara, C. A., Valença, W. O., Oliveira, R. N. de, Frézard, F., Silva Junior., E. N. da. Potent naphthoquinones against antimony-sensitive and -resistant Leishmania parasites: Synthesis of novel α- and nor-α-lapachone-based 1,2,3-triazoles by copper-catalyzed azide–alkyne cycloaddition, *Eur. J. Med. Chem*. **2013**, *63*, 523-530.

(18) Suryawanshi, S. N.; Tiwari, A.; Kumar, S.; Shivahare, R.; Mittal, M.; Kant, P.; Gupta, S. Chemotherapy of Leishmaniasis. Part XII: Design, Synthesis and Bioevaluation of

Novel Triazole Integrated Phenyl Heteroterpenoids as Antileishmanial Agents. *Bioorg. Med. Chem. Lett*. **2013**, *23* (10), 2925–2928.

(19) a) da Silva Jr. E. L.; Menna-Barreto, R.F.S.; Pinto, M.d.C.F.V.; Silva, R.S.F.; Teixeira, D.V.; de Souza, M.C.B.V.; De Simone, C.A.; De Castro, S.L.; Ferreira, V.F. and Pinto, A.V. Naphthoquinoidal [1,2,3]-triazole, a new structural moiety active against Trypanosoma cruzi *Eur. J. Med. Chem*. **2008**, *43*, 1774-1780; b) Patil, V.; Wuerrant,W.; Chen, P.C.; Gryder, B.; Benicewicz, D.B.; Khan, S.I., Tekwani, B.L., Oyelere, A. K. Antimalarial and antileishmanial activities of histone deacetylase inhibitors with triazole-linked cap group. *Bioorg. Med. Chem*. **2010**, *18*, 415-425; c) Tahghighi, A.; Razmi, S.; Mahdavi, M.; Foroumadi, P.; Ardestani, S.K.; Emami, S.; Kobarfard, F.; Dastmalchi, S.; Shafiee, A. and Foroumadi, A. Synthesis and anti-leishmanial activity of 5-(5-nitrofuran-2-yl)-1,3,4-thiadiazol-2-amines containing N-[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] moieties. *Eur. J. Med. Chem*. **2012**, *50*, 124-128

(20) a) Aravinda, T.; Bhojya, N.H.S.; Prakash, N.H.R. 1,2,3-Triazole Fused Quinoline-Peptidomimetics: Studies on Synthesis, DNA Binding and Photonuclease Activity. *Int. J. Pept. Res. Ther*. **2009**, *15*, 273-279; b) Cui, S.-F.; Ren, Y.; Zhang, S.-L.; Peng, X.-M.; Damu, G. L.V.; Geng, R.-X.; Zhou, C.-H. Synthesis and biological evaluation of a class of quinolone triazoles as potential antimicrobial agents and their interactions with calf thymus DNA. *Bioorg. Med. Chem. Lett*. **2013**, *23*, 3267-3272.

(21) Li, X.; Lin, Y.; Wang, Q.; Yuan, Y.; Zhang, H.; Qian, X. The Novel Anti-Tumor Agents of 4-Triazol-1,8-Naphthalimides: Synthesis, Cytotoxicity, DNA Intercalation and Photocleavage. *Eur. J. Med. Chem*. **2011**, *46*, 1274–1279.

(22) a) Lemke, K.; Wojciechowski, M.; Laine, W.; Bailly, C.; Colson, P.; Baginski, M.; Larsen, A. K.; Skladanowski, A. Induction of Unique Structural Changes in Guanine-Rich DNA Regions by the Triazoloacridone C-1305, a Topoisomerase II Inhibitor with Antitumor Activities. *Nucleic Acids Res*. **2005**, *33*, 6034–6047. b) Zabost, E.; Nowicka, A. M.; Mazerska, Z.; Stojek, Z. Influence of Temperature and Interactions with Ligands on Dissociation of dsDNA and Ligand-dsDNA Complexes of Various Types of Binding. An Electrochemical Study. *Phys. Chem. Chem Phys* **2012**, *14*, 3408–3413.

(23) Cholody, W. M.; Martelli, S.; Konopa, J. 8-Substituted 5-[(aminoalkyl)amino]-6H-v-triazolo[4,5,1-de]acridin-6-ones as potential antineoplastic agents. Synthesis and biological activity. *J. Med. Chem*. **1990**, *33*, 2852-2856.

(24) a) Abarca, B.; Ballesteros-Garrido, R. 1,2,3-Triazoles Fused to Aromatic Rings. Topics in Heterocyclic Chemistry 40, pp 325–378; Dehaen, W., Bakulev, V. A., Eds.;

Springer International Publishing, 2015. b) Jones, G.; Abarca B. *Adv*. *Heterocycl. Chem*. **2010**, *100*, 195-252, Elsevier Inc.

(25) Adam, R.; Bilbao-Ramos, P.; López-Molina, S.; Abarca, B.; Ballesteros, R.; González-Rosende, M. E.; Dea-Ayuela, M. A.; Alzuet-Piña, G. Triazolopyridyl ketones as a novel class of antileishmanial agents. DNA binding and BSA interaction. *Bioorg. Med. Chem*. **2014**, *22*, 4018-4027.

(26) Adam, R.; Bilbao-Ramos, P.; Abarca, B.; Ballesteros, R.; González-Rosende, M. E.; Dea-Ayuela, M. A.; Estevan, F.; Alzuet-Piña, G. Triazolopyridopyrimidines: an emerging family of effective DNA photocleavers. DNA binding. Antileishmanial activity. *Org. Biomol. Chem*. **2015**, *13*, 4903-4917.

(27) Lapier, M., PhD Thesis, October 2015, University of Chile, Santiago, Chile

(28) a) Abarca, B.; Asensio, A.; Ballesteros, R.; Bosch, J.; Jones, G.; Mojarrad, F.; Metni, M.R.; Richardson C.M. Triazolopyridines. Part 10. Site of Quaternization salts of [1,2,3]Triazolo[1,5-*a*]pyridines. *J. Chem. Res. (M)*, **1990**, 347-354, *J. Chem. Res. (S)*, **1990,** 9; b) G. Jones, C. M. Richardson, P. C. Yates, G. Hajós, G. Timari, Theoretical interpretations of some experimental observations in reactions of triazolopyridines and their quaternary salts, *Tetrahedron* **1993**, *49*, 4307-4314.

(29) a) Bower, J. D.; Ramage, G.R. Heterocyclic systems related to pyrrocoline. Part II. The preparation of polyazaindenes by dehydrogenative cyclisations. *J. Chem. Soc*. **1957**, 4506. b) Boyer, H.; Goebel, N. Notes- The Identification of $C_{12}H_8N_4O$, an Oxidation Product from α-Pyridil Monohydrazone *J. Org. Chem*. **1960**, *25*, 304-305. Note that it is now commercially available (ASM Research Chemicals)

(30) Sánchez-Moreno, M.; Marín, C.; Navarro, P.; Lamarque, L.; García-España, E.; Miranda, C.; Huertas, O.; Olmo, F.; Gómez-Contreras, F.; Pitarch, J.; Arrebola, F. In Vitro and in Vivo Trypanosomicidal Activity of Pyrazole-Containing Macrocyclic and Macrobicyclic Polyamines: Their Action on Acute and Chronic Phases of Chagas Disease. *J. Med. Chem*. **2012**, *55*, 4231–4243.

(31) Nwaka, S.; Ramirez, B.; Brun, R.; Maes, L.; Douglas, F.; Ridley, R. Advancing Drug Innovation for Neglected Diseases-Criteria for Lead Progression. *PLoS Negl Trop Dis* **2009**, *3*, e440.

(32) Ginger, M. L. Trypanosomatid Biology and Euglenozoan Evolution: New Insights and Shifting Paradigms Revealed through Genome Sequencing. *Protist* **2005**, *156*, 377–392.

(33) Bringaud, F.; Rivière, L.; Coustou, V. Energy Metabolism of Trypanosomatids: Adaptation to Available Carbon Sources. *Mol. Biochem. Parasitol.* **2006**, *149*, 1–9.

(34) Cazzulo, J. J. Aerobic Fermentation of Glucose by Trypanosomatids. *FASEB J.* **1992**, *6*, 3153–3161.

(35) Michels, P. A. M.; Bringaud, F.; Herman, M.; Hannaert, V. Metabolic Functions of Glycosomes in Trypanosomatids. *Biochim. Biophys. Acta* **2006**, *1763*, 1463–1477.

(36) Longoni, S. S.; Sánchez-Moreno, M.; López, J. E. R.; Marín, C. Leishmania Infantum Secreted Iron Superoxide Dismutase Purification and Its Application to the Diagnosis of Canine Leishmaniasis. *Comp. Immunol. Microbiol. Infect. Dis.* **2013**, *36*, 499–506.

(37) González, P.; Marín, C.; Rodríguez-González, I.; Hitos, A. B.; Rosales, M. J.; Reina, M.; Díaz, J. G.; González-Coloma, A.; Sánchez-Moreno, M. In Vitro Activity of C20-Diterpenoid Alkaloid Derivatives in Promastigotes and Intracellular Amastigotes of Leishmania Infantum. *Int. J. Antimicrob. Agents* **2005**, *25*, 136–141.

(38) Sánchez-Moreno, M.; Sanz, A. M.; Gómez-Contreras, F.; Navarro, P.; Marín, C.; Ramírez-Macias, I.; Rosales, M. J.; Olmo, F.; Garcia-Aranda, I.; Campayo, L.; Cano, C.; Arrebola, F.; Yunta, M. J. R. In Vivo Trypanosomicidal Activity of Imidazole- or Pyrazole-Based Benzo[g]phthalazine Derivatives against Acute and Chronic Phases of Chagas Disease. *J. Med. Chem.* **2011**, *54*, 970–979.

(39) Sánchez-Moreno, M.; Gómez-Contreras, F.; Navarro, P.; Marín, C.; Olmo, F.; Yunta, M. J. R.; Sanz, A. M.; Rosales, M. J.; Cano, C.; Campayo, L. Phthalazine Derivatives Containing Imidazole Rings Behave as Fe-SOD Inhibitors and Show Remarkable Anti-T. Cruzi Activity in Immunodeficient-Mouse Mode of Infection. *J. Med. Chem.* **2012**, *55*, 9900–9913.

(40) Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72*, 248–254.

(41) Beyer, W. F.; Fridovich, I. Assaying for Superoxide Dismutase Activity: Some Large Consequences of Minor Changes in Conditions. *Anal. Biochem.* **1987**, *161*, 559–566.

In vitro antileishmanial activity and iron superoxide dismutase inhibition of arylamine Mannich base derivatives

ALVARO MARTIN-MONTES[1], MERY SANTIVAÑEZ-VELIZ[2,3], ELSA MORENO-VIGURI[2,3], RUBÉN MARTÍN-ESCOLANO[1], CARMEN JIMÉNEZ-MONTES[1], CATALINA LOPEZ-GONZALEZ[1], CLOTILDE MARÍN[1], CARMEN SANMARTÍN[2,3], RAMÓN GUTIÉRREZ SÁNCHEZ[4], MANUEL SÁNCHEZ-MORENO[1]* *and* SILVIA PÉREZ-SILANES[2,3]*

[1] *Departamento de Parasitología, Instituto de Investigación Biosanitaria (ibs.GRANADA), Hospitales Universitarios De Granada/Universidad de Granada, Granada, Spain*
[2] *Universidad de Navarra, Instituto de Salud Tropical, Campus Universitario, 31008 Pamplona, Spain*
[3] *Departamento de Química Orgánica y Farmacéutica, Facultad de Farmacia y Nutrición, Universidad de Navarra, Campus Universitario, 31008 Pamplona, Spain*
[4] *Department of Statistics, University of Granada, Severo Ochoa s/n, 18071 Granada, Spain*

(*Received 13 February 2017; revised 1 June 2017; accepted 4 June 2017; first published online 9 August 2017*)

SUMMARY

Leishmaniasis is one of the world's most neglected diseases, and it has a worldwide prevalence of 12 million. There are no effective human vaccines for its prevention, and treatment is hampered by outdated drugs. Therefore, research aiming at the development of new therapeutic tools to fight leishmaniasis remains a crucial goal today. With this purpose in mind, we present 20 arylaminoketone derivatives with a very interesting *in vitro* and *in vivo* efficacy against *Trypanosoma cruzi* that have now been studied against promastigote and amastigote forms of *Leishmania infantum*, *Leishmania donovani* and *Leishmania braziliensis* strains. Six out of the 20 Mannich base-type derivatives showed Selectivity Index between 39 and 2337 times higher in the amastigote form than the reference drug glucantime. These six derivatives affected the parasite infectivity rates; the result was lower parasite infectivity rates than glucantime tested at an IC_{25} dose. In addition, these derivatives were substantially more active against the three *Leishmania* species tested than glucantime. The mechanism of action of these compounds has been studied, showing a greater alteration in glucose catabolism and leading to greater levels of iron superoxide dismutase inhibition. These molecules could be potential candidates for leishmaniasis chemotherapy.

Key words: *Leishmania infantum*, *Leishmania donovani*, *Leishmania braziliensis*, iron superoxide dismutase, arylamine derivatives, Mannich base derivatives.

INTRODUCTION

Leishmaniasis caused by the intracellular protozoan *Leishmania* is one of the world's most neglected diseases (WHO, 2016).

Although the immunology, biology and genetics of the parasites causing these diseases have been studied extensively, there are no effective human vaccines for their prevention, and treatment of kinetoplastid infections is hampered by outdated drugs (Uliana *et al*. 2017). The use of these drugs has been limited due to their elevated cost, side-effects, variable degree of efficacy, route of administration, long treatment duration and the emergence of drug-resistant strains. Therefore, research aiming at the development of new therapeutic tools to fight leishmaniasis remains a crucial goal today (Menezes *et al*. 2015).

* Corresponding authors: Facultad de Farmacia y Nutrición, Departamento de Química Orgánica y Farmacéutica, Universidad de Navarra, Campus Universitario, 31008 Pamplona, Spain. E-mail: sperez@unav.es and Facultad de Ciencias, Departamento de Parasitología, Universidad de Granada, 18071 Granada, Spain. E-mail: msanchem@ugr.es

The design of new potential drugs for *Leishmania* treatment claims to understand the essential metabolic biochemical pathways and crucial parasite-specific enzymes. In this context, enzymes that can help to avoid the damage caused by oxidative stress have emerged as interesting targets (Coimbra *et al*. 2016; Singh *et al*. 2016). The most interesting ones are those that present biochemical and structural differences with their human counterparts (Hunter *et al*. 2003; Piacenza *et al*. 2009; Menna-Barreto and de Castro, 2014). It has been shown that superoxide dismutase (Fe-SOD) enzyme plays an important role in the defence of trypanosomatids against oxidative agents. It is exclusive to the parasite, and parasitic protozoan survival is closely related to the ability of this enzyme to evade toxic radical damage originated by their host (Turrens, 2004; Bodyl and Mackiewicz, 2008; Sanz *et al*. 2008; Sánchez-Moreno *et al*. 2011).

From a chemical point of view, thiophene entity is a promising scaffold in medicinal chemistry due to its broad spectrum as an anti-inflammatory, analgesic or antibacterial (Issa *et al*. 2009; Arun *et al*. 2010; Puterová and Krutosilová, 2010). Moreover,

Parasitology (2017), **144**, 1783–1790.
doi:10.1017/S0031182017001123

the potential of thiophene derivatives as leishmanicidal agents only or in combination with other moieties has also been reported (Félix *et al*. 2016), and the leishmanicidal properties for a range of benzodioxole derivatives have also been described (Parise-Filho *et al*. 2012; Fernandes *et al*. 2015). Naphthalene derivatives have already been described for their antileishmanial activity (Mori-Yasumoto *et al*. 2012; Manzano *et al*. 2016). The interest in Mannich base-type derivatives as drugs or drug candidates is well known, and their antitrypanosomal action has been reported (Lee *et al*. 2005; Wenzel *et al*. 2009; Mahal *et al*. 2017). Moreover, Mannich reaction is an important tool for C–C bond formation in organic chemistry, widely used for the preparation of β-aminoketones used as antiparasitic agents. So, taking into account the potential of these scaffolds, we decided to explore the antitrypanosomal capacity of a new family of Mannich base derivatives.

Recently, our research group has described the *in vitro* and *in vivo* anti-*Trypanosoma cruzi* activity of 20 arylaminoketone Mannich base-type compounds obtained by condensation of the corresponding arylamines and different aromatic rings with interest in medicinal chemistry, including thiophene, benzothiophene, benzodioxole and naphthalene (see Supplementary Material) (Moreno-Viguri *et al*. 2016). This family of compounds has shown promising activity in the infective forms of the parasites, and no genotoxicity or mutagenicity was observed in the primary screening. The mechanism of action of these compounds has been studied at metabolic levels by ^{1}H nuclear magnetic resonance (^{1}H NMR), and the study has been completed by testing their activity against Fe-SOD. These molecules could be potential candidates for *Leishmania* therapy (Turrens 2004; Sanchez-Moreno *et al*. 2015) because they show selectivity over Fe-SOD. Therefore, we decided to test these molecules against promastigote and amastigote forms of *Leishmania infantum*, *Leishmania donovani* and *Leishmania braziliensis* strains.

MATERIALS AND METHODS

Chemistry

The synthesis of the arylaminoketone Mannich base compounds (**1**–**20**) was previously described (Moreno-Viguri *et al*. 2016). The desired compounds were prepared by condensation of the corresponding methylketone with the appropriate arylamine via Mannich reaction in acidic medium and using 1,3-dioxolane as the solvent and the formaldehyde source. Purification of the compounds was performed in all cases using flash column chromatography eluting in gradient with CH_2Cl_2/methanol. Spectroscopic data were the same as those described in (Moreno-Viguri *et al*. 2016), and the adequate purity of the compounds was confirmed by the analytical data.

Parasite strain and culture

Promastigote forms of *L. infantum* (MCAN/ES/2001/UCM-10), *L. braziliensis* (MHOM/BR/1975/M2904) and *L. donovani* (LCR-L 133 LRC), Jerusalem (Israel), were cultured *in vitro* in medium trypanosomes liquid (MTL) supplemented with 10% inactive fetal calf serum and kept in an air atmosphere at 28 °C in Roux flasks (Corning, New York, USA) with a surface area of 75 cm^2, following the methodology described by González *et al*. (2005).

In vitro activity assays

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

Cell culture and cytotoxicity tests

The macrophage line J774.2 [European Collection of Authenticated Cell Cultures (ECACC) number 91051511] was used for the cytotoxicity test. The macrophages were cultured and the cytotoxicity testing was performed by the flow cytometry analysis according to a method previously described by Kirkinezos and Moraes (2001)

Promastigote and amastigote assay

The compounds were dissolved in the culture medium to give final concentrations of 100, 50, 25, 10 and 1 μM. The effects of each compound against the promastigote forms at the different concentrations were tested according to the methodology described by González *et al*. (2005). The inhibition effect was expressed as the IC_{50} value, i.e. the concentration required to result in 50% inhibition, calculated by the linear regression analysis.

In the case of amastigote forms, J774.2 macrophage cells were cultured and seeded at a density of 1×10^4 cells per well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultured for 2 days, according to the method described by Sánchez-Moreno *et al*. (2012).

Infectivity assay

Adherent macrophage cells grown as described above were infected *in vitro* with promastigote forms of *L. infantum*, *L. braziliensis* and *L. donovani* at a ratio of 10 : 1. The tested compounds (IC_{25}

concentrations) were added immediately after infection, and incubated for 12 h at 37 °C in 5% CO_2 (Gonzalez *et al*. 2005). Compounds and non-phagocytozed parasites were removed by washing, and then the infected cultures were cultured for 10 days in fresh medium. Cultures were washed every 48 h and fresh culture medium was added. Compound activity was determined on the basis of both the percentage of infected cells and the number of amastigotes per infected cell in treated and untreated cultures in methanol-field and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analysing more than 200 host cells distributed in randomly chosen microscopic fields.

Metabolite excretion

Cultures of *L. infantum*, *L. braziliensis* and *L. donovani* promastigotes (initial concentration 5×10^5 cells mL^{-1}) received the IC_{25} dose of each compound (except for control cultures). The methodology used was described by Fernandez-Becerra *et al*. (1997).

Superoxide dismutase (SOD) inhibition studies

Promastigotes of *Leishmania* spp. were grown in tissue-culture flasks and an axenic medium, as described above, until reaching a population of approximately 1×10^7 parasites mL^{-1}. Cells were harvested at the logarithmic growth phase by centrifugation (1500 ***g*** for 10 min at room temperature). The pellet of cells was washed twice in the MTL medium without serum, and the cells were counted, distributed into aliquots of 5×10^9 parasites mL^{-1} in MTL medium without serum, and allowed to grow for 24 h.

After 24 h, the promastigote culture was centrifuged (1500 ***g*** for 10 min) and the supernatant was filtered (Minisart®, Φ 20 μm). The filtered supernatant was subjected to ice-cold ammonium sulphate precipitation at 35% salt concentration. Following centrifugation, the resultant supernatant was then treated with 85% ice-cold ammonium sulphate and the second precipitate was 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 (Fraction P85e).

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

RESULTS

In vitro *antileishmanial evaluation*

In a first step, we assayed the *in vitro* antileishmanial activity of compounds **1**–**20** on both extra- and intracellular forms of the parasites. Table 1 shows the IC_{50} values obtained after 72 h of exposure when compounds **1**–**20** were tested on extra- and intracellular forms of *L. infantum*, *L. braziliensis* and *L. donovani*. Toxicity values against J774.2 macrophage after 72 h of culture were also calculated and Selectivity Index (SI) values for the amastigote form have also been included in Table 1. Results obtained for the reference drug glucantime were included in all cases for comparison. An overall analysis of the biological data evidenced that nine of the screened compounds (**3, 4, 7, 11, 12, 14, 17, 18** and **19**) showed high activity against at least one of three *Leishmania* species in both promastigote and amastigote forms. For example, the SI of compound **3** exceeded that of the reference drug in *L. infantum* by 150-fold, by 2337-fold in *L. braziliensis* and by 1215-fold in *L. donovani*. Different authors have claimed that compounds having SI values >20 can be considered ideal candidates for further development as leishmanicidal drugs (Nwaka and Hudson, 2006). This requirement is satisfied by compounds **3, 4, 6, 7, 10** and **17** (**17** only in *L. donovani*).

Infectivity assay

In order to gain a better insight into the activities of the lead compounds **3, 4, 6, 7, 10** and **17**, their effect on the infectivity and intracellular replication of

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

	Activity IC_{50} (μM)[a]							SI[c]		
	Leishmania infantum		*Leishmania braziliensis*		*Leishmania donovani*					
Comp.	Promastigote	Amastigote	Promastigote	Amastigote	Promastigote	Amastigote	Macrophage toxicity IC_{50} (μM)[b]	*L. infantum amastigote*	*L. braziliensis amastigote*	*L. donovani amastigote*
Glucantime	18·0 ± 3·1	24·2 ± 2·6	25·6 ± 1·7	30·4 ± 6·1	26·6 ± 5·4	33·3 ± 1·2	15·2 ± 1·0	0·6	0·5	0·5
1	146·5 ± 9·8	41·6 ± 2·1	25·2 ± 0·9	27·5 ± 0·9	33·8 ± 0·1	30·8 ± 1·4	236·9 ± 11·3	6 (9)	9 (17)	8 (15)
2	67·7 ± 6·7	51·6 ± 3·4	49·7 ± 2·6	22·6 ± 1·7	38 ± 4·1	24·7 ± 1·8	157·2 ± 11·4	3 (5)	7 (14)	6 (13)
3	14·4 ± 0·8	16·9 ± 0·9	2·2 ± 0·0	1·3 ± 0·2	1·0 ± 0·1	2·5 ± 0·8	1518·8 ± 75·2	89·9 (150)	1168 (2337)	607 (1215)
4	19·1 ± 1·4	21·6 ± 2·2	3·1 ± 0·0	1·5 ± 0·2	1·3 ± 0·2	4·8 ± 0·7	693·9 ± 41·9	36 (50)	463 (925)	145 (289)
5	17·9 ± 1·6	23·8 ± 1·7	141·3 ± 1·9	51·5 ± 4·8	63·4 ± 2·4	40·6 ± 3·5	176·4 ± 31·7	7 (12)	3 (7)	4 (9)
6	28·6 ± 1·8	23·5 ± 1·6	30·8 ± 2·6	25·5 ± 1·6	31·7 ± 3·1	26·9 ± 1·8	553·7 ± 23·9	23 (39)	18 (36)	21 (41)
7	3·1 ± 0·6	2·7 ± 0·5	60·1 ± 4·2	27·5 ± 1·6	30·6 ± 3·2	20·7 ± 1·5	721·9 ± 50·0	267 (446)	26 (52)	35 (70)
8	34·7 ± 4·7	27·9 ± 2·0	14·6 ± 3·5	12·6 ± 0·8	90·7 ± 6·9	33·7 ± 1·5	129·8 ± 6·4	5 (8)	9 (18)	4 (8)
9	44·6 ± 2·6	35·8 ± 2·3	76·4 ± 5·8	41·5 ± 1·8	70·8 ± 4·6	29·7 ± 2·3	70·8 ± 6·1	2 (3)	2 (3)	2 (5)
10	21·7 ± 1·2	30·7 ± 10·7	37·5 ± 1·7	29·5 ± 1·8	107·7 ± 41·0	61·6 ± 4·2	1753·8 ± 76·4	57 (95)	59 (119)	34 (68)
11	8·9 ± 1·7	10·4 ± 0·8	7·9 ± 0·7	5·8 ± 1·1	8·9 ± 3·1	3·5 ± 0·5	4·8 ± 0·1	0 (1)	1 (2)	1 (3)
12	15·6 ± 0·7	10·6 ± 0·6	19·8 ± 1·1	14·5 ± 0·8	20·7 ± 2·1	15·5 ± 0·5	65·8 ± 1·8	6 (10)	4 (9)	4 (8)
13	21·8 ± 1·0	16·8 ± 0·8	24·8 ± 1·4	13·8 ± 0·8	27·9 ± 1·5	26·9 ± 2·0	137·9 ± 6·6	8 (14)	10 (20)	5 (10)
14	11·9 ± 0·8	8·7 ± 0·4	13·7 ± 0·8	7·9 ± 0·6	16·9 ± 1·2	10·4 ± 0·7	25·7 ± 2·4	3 (5)	3 (6)	2 (5)
15	23·2 ± 5·1	16·5 ± 0·5	24·3 ± 1·6	12·9 ± 0·6	27·8 ± 2·2	18·9 ± 1·1	30·1 ± 1·4	2 (3)	2 (5)	2 (3)
16	20·8 ± 1·4	17·8 ± 1·2	29·8 ± 1·6	24·5 ± 1·8	33·8 ± 0·9	26·8 ± 1·5	74·3 ± 5·3	4 (7)	3 (5)	3 (5)
17	13·7 ± 0·9	11·5 ± 1·4	56·7 ± 1·7	30·0 ± 2·5	0·1 ± 0·0	2·4 ± 0·7	112·4 ± 13·8	10 (16)	4 (8)	47 (94)
18	26·1 ± 1·7	18·5 ± 2·3	92·0 ± 5·3	36·3 ± 2·5	5·5 ± 0·1	8·3 ± 1·4	43·6 ± 5·5	2 (4)	1 (1)	7 (14)
19	9·6 ± 1·1	4·8 ± 0·3	24·2 ± 2·1	11·7 ± 1·4	52·9 ± 3·2	15·7 ± 3·5	0·3 ± 0·0	0 (0)	0 (0)	0 (0)
20	21·8 ± 0·4	28·6 ± 1·7	29·8 ± 1·7	31·6 ± 2·0	36·8 ± 1·4	31·9 ± 1·7	275·8 ± 17·2	9 (16)	9 (17)	9 (17)

Results are averages of four separate determinations, ±S.D.

[a] IC_{50}: is the concentration required to give 50% inhibition, calculated by linear regression analysis.

[b] Against J774.2 macrophages after 72 h of culture.

[c] Selectivity Index (SI) = IC_{50} macrophages toxicity/IC_{50} activity on extracellular or intracellular forms of the parasite. In parenthesis: compound SI/reference drug SI.

RESULTS

amastigotes was subsequently determined. Macrophage cells were grown and infected with promastigotes in the stationary phase. The parasites invaded the cells and underwent morphological conversion to amastigotes within 1 day after infection. On day 10, the rate of host cell infection reached its maximum (control experiment). We used the IC_{25} of each product as the test dosage. Figure 1 shows the effect of the studied derivatives on the infection and growth rates of the three *Leishmania* species. A measure of the average number of amastigotes per infected macrophage led to similar conclusions: in the case of *L. infantum* (Fig. 1A), all compounds were more effective than glucantime. Amastigote numbers obtained on *L. braziliensis* (Fig. 1B) also showed that all compounds were clearly more effective than glucantime under the tested conditions. It was also observed that the infection rate decreased with respect to the control and, furthermore, the six compounds (**3, 4, 6, 7, 10** and **17**), were also remarkably more effective in decreasing parasite infectivity than glucantime at an IC_{25} dose.

Metabolite excretion

Trypanosomatids are unable to completely degrade glucose to CO_2, so that they excrete part of the hexose skeleton into the medium as partially oxidized fragments. The nature and percentage of the oxidized fragments depend on the pathway used for glucose metabolism (Turrens, 2004). The catabolism products in *Leishmania* species are principally succinate, acetate, D-lactate and L-alanine (Kirkinezos and Moraes, 2001).

In order to acquire information regarding the effects of **3, 4, 6, 7, 10** and **17** on the glucose metabolism of the parasite, we obtained the ^{1}H NMR spectrum of three species of *Leishmania* (*L. infantum*, *L. braziliensis* and *L. donovani*) promastigotes treated with the test compounds (compound **17** only in *L. donovani*); the final excretion products were qualitatively and quantitatively identified. Figure 2 shows the results obtained and the comparison with those found for untreated control promastigotes.

All the compounds induce an increase in succinate production in the three species of *Leishmania* ranging from 16·1 to 251·3% (compound **17** only in *L. donovani*) as can be observed in Fig. 2. This effect is observed in *L. donovani* to a lesser extent (Fig. 2C) except for compound **10** that presents a higher accumulation of succinate in *L. donovani* than in *L. infantum* and *L. braziliensis*.

SOD enzymatic inhibition in the Leishmania *parasites and in human erythrocytes*

Considering the obtained results, we decided to test the effects of these compounds on Fe-SOD isolated from *L. infantum*, *L. braziliensis* and *L. donovani* over a range of concentrations, from 0·1 to 100 μM. We used promastigote forms of both species, which excrete Fe-SOD when cultured in a medium lacking inactive FBS (Kirkinezos and Moraes, 2001). The inhibition data obtained are shown in Fig. 3(A–C), and the corresponding IC_{50} values are included for easier evaluation of the displayed graphs; for comparison, Fig. 3A shows the effects of the same compounds on CuZn-SOD obtained from human erythrocytes.

Regarding the SOD enzymatic inhibition in the *Leishmania* parasites and in human erythrocytes (Fig. 3), the most remarkable result was the inhibitory effect on Fe-SOD found for the highly antileishmanial compounds **3, 4** and **7** in the three species tested, whereas their inhibition of human CuZn-SOD was clearly lower. If we consider the IC_{50} calculated for *L. infantum*, inhibition of Fe-SOD by compounds **3, 4** and **7** was 25-, 11- and 29-fold higher, respectively, than inhibition of CuZn-SOD. Compound **3** showed a Fe-SOD inhibition 25, 66 and 10 times higher than CuZn-SOD inhibition in *L. infantum, L. braziliensis and L. donovani*, and compound **7** showed the respective values of 29, 14 and 36. Therefore, compounds **3** and **7** could be considered the most selective inhibitors of Fe-SOD.

DISCUSSION

As explained above (Moreno-Viguri *et al*. 2016), previous studies have indicated that arylaminoketone Mannich base-type compounds may be considered prospective chemotherapeutic drugs in the treatment of Chagas disease caused by *T. cruzi* parasites (Turrens, 2004). We now comment on the results obtained regarding the antiparasitic activity of compounds **1**–**20** (Table 1) against three significant species of *Leishmania*: *L. infantum*, *L. braziliensis* and *L. donovani*. It was shown that the inhibition activities against intracellular forms of the parasites of studied compounds (**17** with efficacy only against *L. donovani*) were higher than those found for the reference drug glucantime, whereas the effect on extracellular forms was more random. Regarding the toxicity in mammalian cells, the tested compounds were found to be much less toxic for macrophages than the reference drug. Therefore, compounds **3, 4, 6, 7, 10** and **17** were considered the lead ones due to their excellent antileishmanial activity and were selected for subsequent studies.

Interestingly, the best SI results for the more representative intracellular forms were obtained in *L. infantum* and *L. donovani*, two species forming part of the *L. donovani* complex, pointing towards a greater specificity towards parasites causing the particularly harmful visceral leishmaniasis in both its European and American versions.

With regard to the structure–activity relationship, in general, derivatives with the benzo[*b*]thiophene

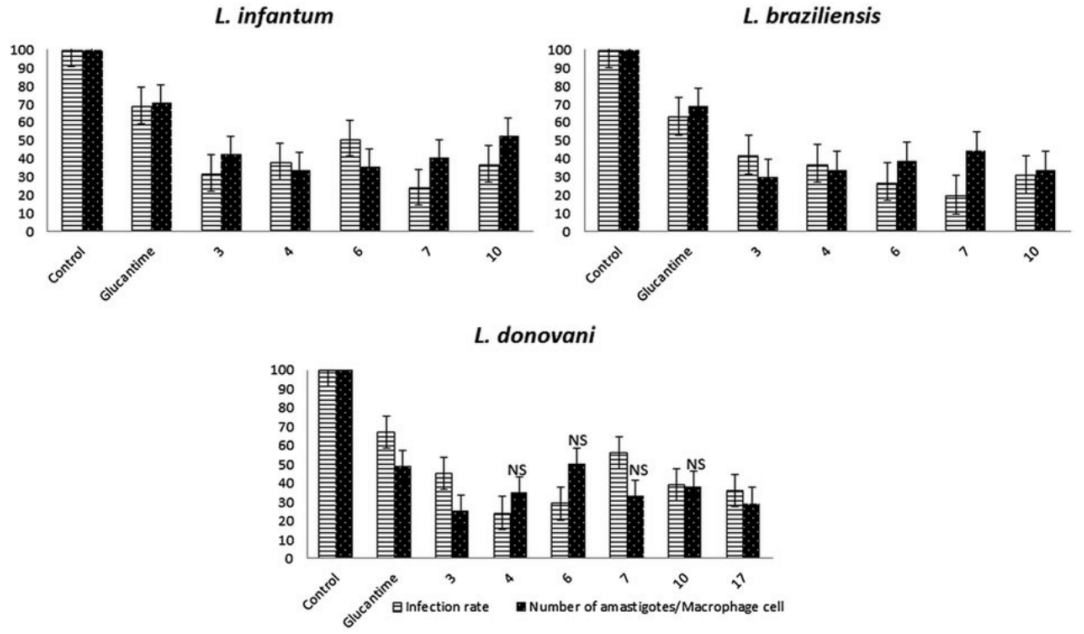


Fig. 1. Effect of arylaminoketone derivatives **3, 4, 6, 7** and **10** on the infection and growth rates and mean numbers of amastigotes per infected J774.2 macrophage cell (at IC_{25} concentration) of *Leishmania infantum* (A), *Leishmania braziliensis* (B) and *Leishmania donovani* (C). Values are the means of three separate experiments. All compounds are statistically significant against glucantime at a P value < 0·05, except compounds labelled as NS.

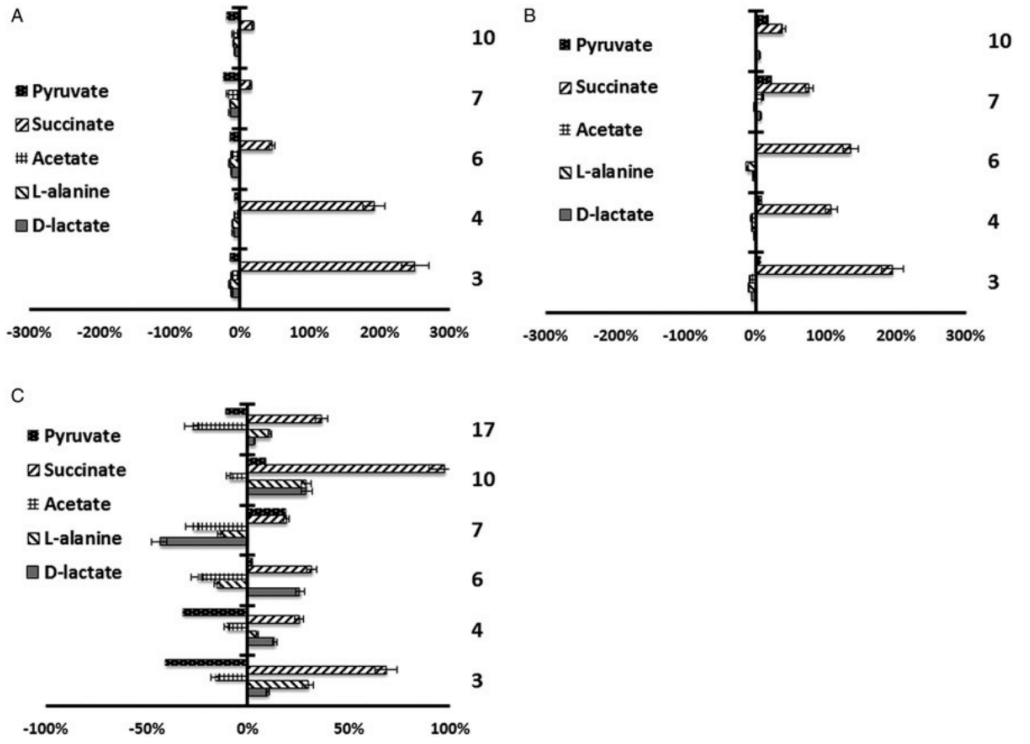


Fig. 2. Variation percentages in the area of the peaks corresponding to excreted catabolites by *Leishmania infantum* (A), *Leishmania brasilienzis* (B) and *Leishmania donovani* (C) promastigotes in the presence of compounds **3, 4, 6, 7, 10** and **17** at their IC_{25} compared with a control sample after 96 h of incubation.

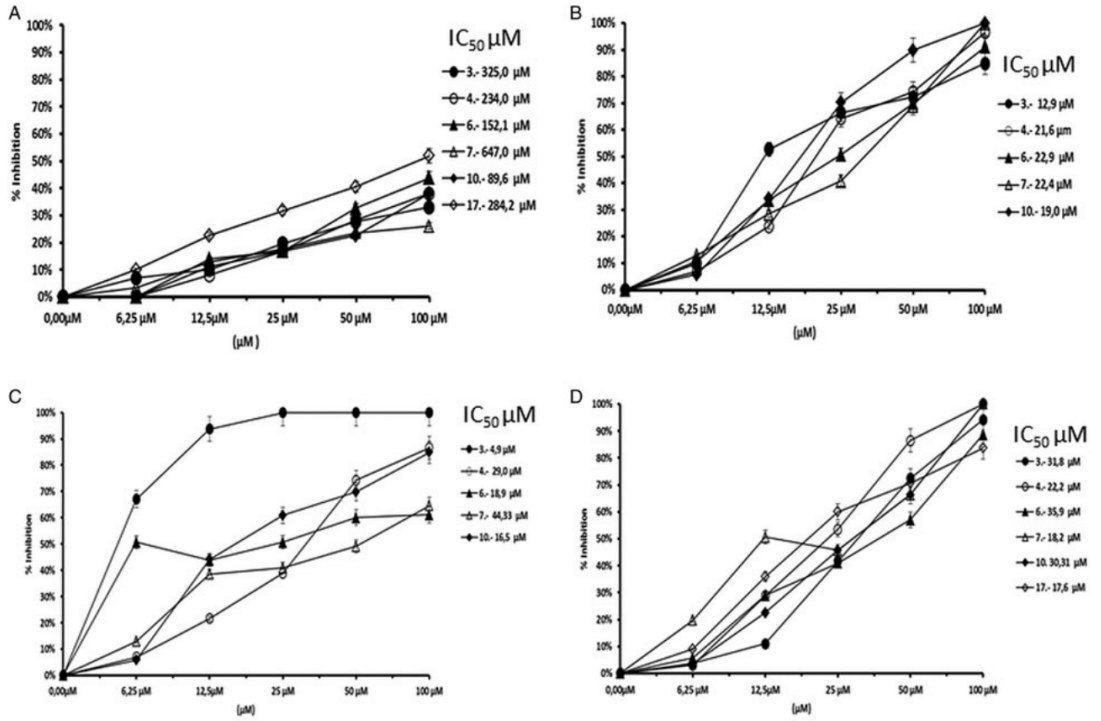


Fig. 3. (A) *In vitro* inhibition of CuZn-SOD in human erythrocytes by compounds **3, 4, 6, 7, 10** and **17**. (B–D) *In vitro* inhibition (%) of Fe-SOD of *Leishmania infantum* (B), *Leishmania braziliensis* (C) and *Leishmania donovani* (D) promastigotes by compounds **3, 4, 6, 7, 10** and **17**. Values are the average of three separate determinations. Differences between the activities of the control homogenate and those incubated with the tested compounds were obtained according to the Newman–Keuls test. IC_{50} was calculated by linear regression analysis.

scaffold are less cytotoxic than the rest of the derivatives.

In the infectivity assay (Fig. 1), all the compounds were more effective in relation to IC_{25} than glucantime. The infection rates decreased with respect to the control and the reference drug glucantime. The measure of the average number of amastigotes per infected macrophage led to similar conclusions. All these data seem to be in line with results previously described for *T. cruzi* (Moreno-Viguri *et al*. 2016).

Regarding the studies to elucidate the possible mechanism of action, the studied compounds produce greater glucose metabolism alteration because they increase succinate excretion (Fig. 2). Detection of large amounts of succinate as a major end product is a usual feature, because it relies on glycosomal redox balance, enabling re-oxidation of the NADH produced in the glycolytic pathways. It is interesting to mention that the increase in succinate with these compounds indicates catabolic changes that could be related to mitochondria malfunction, due to the redox stress produced by inhibition of the mitochondrion-resident Fe-SOD enzyme (Marín *et al*. 2011).

In addition, these compounds led to greater levels of Fe-SOD inhibition. All these data appear to confirm some type of relation between the antileishmanial activity and the Fe-SOD inhibition, coinciding with the results described in previous work (Ginger, 2005). Fe-SOD inhibition could also, at another level, be related to the catabolic changes discussed above because a mitochondrial malfunction, originated from the redox stress produced by inhibition of the mitochondrion-resident Fe-SOD enzyme (Marín *et al*. 2011) should result in severe alteration of pyruvate metabolism, and consequently, a decrease in the production of succinate. Because the Fe-SOD present in mitochondria is an essential part of the antioxidant protective response of the parasite, its inhibition would be related to a decrease in the rate of survival for the parasite.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at https://doi.org/10.1017/S0031182017001123.

FINANCIAL SUPPORT

This work has been carried out with the financial support of Fundación Caja Navarra (Project no. 70314), Fundación María Francisca de Roviralta, FIMA (Fundación para la Investigación Médica Aplicada; Project

ISTUN-API-2011/01) and from the former Spanish Ministry of Science and Innovation (MICINN) and now from the Ministry of Economy and Competitiveness (MINECO) (project Consolider Ingenio CSD2010-00065). M.J. is indebted to the University of Navarra for a grant. R.M-E. is grateful for a FPU Grant (no. FPU14/01537) from the Ministry of Education of Spain.

REFERENCES

Arun, M. I., Balakrishna, K. and Sridhar, K. (2010). Synthesis, characterization and biological activities of some new benzo[b]thiophene derivatives. *European Journal of Medicinal Chemistry* **45**, 825–830.

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

Bodyl, A. and Mackiewicz, P. (2008). Were class C iron-containing superoxide dismutases of trypanosomatid parasites initially imported into a complex plastid? A hypothesis based on analyses of their N-terminal targeting signals. *Parasitology* **135**, 1101–1110.

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

Coimbra, E. S., Antinarelli, L. M., Silva, N. P., Souza, I. O., Meinel, R. S., Rocha, M. N., Soares, R. P. and da Silva, A. D. (2016). Quinoline derivatives: synthesis, leishmanicidal activity and involvement of mitochondrial oxidative stress as mechanism of action. *Chemico-Biological Interactions* **25**, 50–57.

Félix, M. B., de Souza, E. R., de Lima, M., Frade, D. K., de Serafim, V. L., Rodrigues, K. A., Néris, P. L., Ribeiro, F. F., Scotti, L., Scotti, M. T., de Aquino, T. M., Mendonça, F. J., Jr. and de Oliveira, M. R. (2016). Preliminary antifungal and cytotoxic evaluation of synthetic cycloalkyl[b]thiophene derivatives with PLS-DA analysis. *Bioorganic and Medicinal Chemistry* **24**, 3972–3977.

Fernandes, I. A., de Almeida, L., Ferreira, P. E., Marques, M. J., Rocha, R. P., Coelho, L. F., Carvalho, D. T. and Viegas, C. (2015). Synthesis and biological evaluation of novel piperidine-benzodioxole derivatives designed as potential leishmanicidal drug candidates. *Bioorganic and Medicinal Chemistry Letters* **25**, 3346–3349.

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

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

González, P., Marín, C., Rodríguez-González, I., Hitos, A. B., Rosales, M. J., Reina, M., Díaz, J. G., González-Coloma, A. and Sánchez-Moreno, M. (2005). *In vitro* activity of C20-diterpenoid alkaloid derivatives in promastigotes and intracellular amastigotes of *Leishmania infantum*. *International Journal of Antimicrobial Agents* **25**, 136–141.

Hunter, W. N., Alphey, M. S., Bond, C. S. and Schuttelkopf, A. W. (2003). Targeting metabolic pathways in microbial pathogens: oxidative stress and anti-folate drug resistance in trypanosomatids. *Biochemical Society transactions* **31**, 607–610.

Issa, M. I. F., Mohamed, A. A. R., El-Batran, S., Abd El-Salam, O. M. E. and El-Shenawy, S. M. (2009). Synthesis and pharmacological evaluation of 2-substituted benzo[*b*]thiophenes as anti-inflammatory and analgesic agents. *European Journal of Medicinal Chemistry* **44**, 1718–1725.

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

Lee, B., Bauer, H., Melchers, J., Ruppert, T., Rattray, L., Yardley, V., Davioud-Charvet, E. and Krauth-Siegel, R. L. (2005). Irreversible inactivation of trypanothione reductase by unsaturated Mannich bases: a divinyl ketone as key intermediate. *Journal of Medicinal Chemistry* **48**, 7400–7410.

Mahal, K., Ahmad, A., Schmitt, F., Lockhauserbäumer, J., Starz, K., Pradhan, R., Padhye, S., Sarkar, F. H., Koko, W. S., Schobert, R., Ersfeld, K. and Biersack, B. (2017). Improved anticancer and antiparasitic activity of new lawsone Mannich bases. *European Journal of Medicinal Chemistry* **126**, 421–431.

Manzano, J. I., Cochet, F., Boucherle, B., Gomez-Perez, V., Boumendjel, A., Gamarro, F., Peuchmaur, M. (2016). Arylthiosemicarbazones as antileishmanial agents. *European Journal of Medicinal Chemistry* **123**, 161–170.

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

Menezes, J. P., Guedes, C. E., Petersen, A. L., Fraga, D. B. and Veras, P. S. (2015). Advances in development of new treatment for leishmaniasis. *Biomed Research International* **2015**, 815023.

Menna-Barreto, R. F. and de Castro, S. L. (2014). The double-edged sword in pathogenic trypanosomatids: the pivotal role of mitochondria in oxidative stress and bioenergetics. *Biomedical Research International* **2014**, 614014.

Moreno-Viguri, E., Jiménez-Montes, C., Martín-Escolano, R., Santivañez-Veliz, M., Martin-Montes, A., Azqueta, A., Jimenez-Lopez, M., Zamora Ledesma, S., Cirauqui, N., López de Ceráin, A., Marín, C., Sánchez-Moreno, M. and Pérez-Silanes, S. (2016). *In vitro* and *in vivo* anti-*Trypanosoma cruzi* activity of new arylamine Mannich base-type derivatives. *Journal of Medicinal Chemistry* **59**, 10929–10945.

Mori-Yasumoto, K., Izumoto, R., Fuchino, H., Ooi, T., Agatsuma, Y., Kusumi, T., Satake, M., Sekita, S. (2012). Leishmanicidal activities and cytotoxicities of bisnaphthoquinone analogues and naphthol derivatives from Burman Diospyros burmanica. *Bioorganic & Medicinal Chemistry* **20**, 5215–5219.

Nwaka, S. and Hudson, A. (2006). Innovative lead discovery strategies for tropical diseases. *Nature Reviews Drug Discovery* **5**, 941–955.

Parise-Filho, R., Pasqualoto, K. F., Magri, F. M., Ferreira, A. K., da Silva, B. A., Damião, M. C., Tavares, M. T., Azevedo, R. A., Auada, A. V., Polli, M. C. and Brandt, C. A. (2012). Dillapiole as antileishmanial agent: discovery, cytotoxic activity and preliminary SAR studies of dillapiole analogues. *Archiv der Pharmazie* **345**, 934–944.

Piacenza, L., Zago, M. P., Peluffo, G., Alvarez, M. N., Basombrio, M. A. and Radi, R. (2009). Enzymes of the antioxidant network as novel determiners of *Trypanosoma cruzi* virulence. *International Journal for Parasitology* **39**, 1455–1464.

Puterová, Z., Krutosiková, A. (2010). Substituted 2-aminothiophenes: synthesis, properties and applications. In *Heterocyclic Compounds: Synthesis, Properties and Applications* (ed. Nylund, K. and Johansson, P.), pp. 1–46. Nova Science Publishers, USA.

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

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

Sanchez-Moreno, M., Gomez-Contreras, F., Navarro, P., Marin, C., Ramirez-Macias, I., Rosales, M. J., Campayo, L., Cano, C., Sanz, A. M. and Yunta, M. J. (2015). Imidazole-containing phthalazine derivatives inhibit Fe-SOD performance in *Leishmania* species and are active *in vitro* against visceral and mucosal leishmaniasis. *Parasitology* **142**, 1115–1129.

Sanz, A. M., Gomez-Contreras, F., Navarro, P., Sanchez-Moreno, M., Boutaleb-Charki, S., Campuzano, J., Pardo, M., Osuna, A., Cano, C., Yunta, M. J. and Campayo, L. (2008). Efficient inhibition of iron superoxide dismutase and of *Trypanosoma cruzi* growth by benzo[g]phthalazine derivatives functionalized with one or two imidazole rings. *Journal of Medicinal Chemistry* **51**, 1962–1966.

Singh, K., Garg, G. and Ali, V. (2016). Current therapeutics, their problems and thiol metabolism as potential drug targets in leishmaniasis. *Current Drug Metabolism* **17**, 897–919.

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

Uliana, S. R., Trinconi, C. T. and Coelho, A. C. (2017). Chemotherapy of leishmaniasis: present challenges. *Parasitology*. **20**, 1–17.

Wenzel, I. N., Wong, P. E., Maes, L., Müller, T. J., Krauth-Siegel, R. L., Barrett, M. P., Davioud-Charvet, E. (2009). Unsaturated Mannich bases active against multidrug-resistant *Trypanosoma brucei brucei* strains. *ChemMedChem* **4**, 339–351.

World Health Organization (2016) *Leishmaniasis in high-burden countries: an epidemiological update based on data reported in 2014. Weekly Epidemiological Record* No. 22, 91, 285–296.

AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

CHEMISTRY; BIOSYNTHESIS

Library of Seleno-Compounds as Novel Agents against *Leishmania* Species

Álvaro Martín-Montes,[a] Daniel Plano,[b,c] Rubén Martín-Escolano,[a] Verónica Alcolea,[b,c] Marta Díaz,[b,c] Silvia Pérez-Silanes,[b,d] Socorro Espuelas,[d] Esther Moreno,[d] Clotilde Marín,[a] Ramón Gutiérrez-Sánchez,[e] Carmen Sanmartín,[b,c] Manuel Sánchez-Moreno[a]

Universidad de Granada, Departamento de Parasitología, Facultad de Ciencias, Severo Ochoa s/n, Granada, Spain, and Instituto de Investigación Biosanitaria (ibs. Granada), Hospitales Universitarios de Granada, Granada, Spain[a]; University of Navarra, Faculty of Pharmacy and Nutrition, Departament of Organic and Pharmaceutical Chemistry, Campus Universitario, Pamplona, Spain[b]; IdiSNA, Instituto de Investigación Sanitaria de Navarra, Pamplona, Spain[c]; Institute of Tropical Health, University of Navarra, Pamplona, Spain[d]; Department of Statistics, University of Granada, Severo Ochoa s/n, Granada, Spain[e]

ABSTRACT The *in vitro* leishmanicidal activities of a series of 48 recently synthesized selenium derivatives against *Leishmania infantum* and *Leishmania braziliensis* parasites were tested using promastigotes and intracellular amastigote forms. The cytotoxicity of the tested compounds for J774.2 macrophage cells was also measured in order to establish their selectivity. Six of the tested compounds (compounds 8, 10, 11, 15, 45, and 48) showed selectivity indexes higher than those of the reference drug, meglumine antimonate (Glucantime), for both *Leishmania* species; in the case of *L. braziliensis*, compound 20 was also remarkably selective. Moreover, data on infection rates and amastigote numbers per macrophage showed that compounds 8, 10, 11, 15, 45, and 48 were the most active against both *Leishmania* species studied. The observed changes in the excretion product profile of parasites treated with these six compounds were also consistent with substantial cytoplasmic alterations. On the other hand, the most active compounds were potent inhibitors of Fe superoxide dismutase (Fe-SOD) in the two parasite species considered, whereas their impact on human CuZn-SOD was low. The high activity, low toxicity, stability, low cost of the starting materials, and straightforward synthesis make these compounds appropriate molecules for the development of affordable antileishmanicidal agents.

KEYWORDS *Leishmania*, selenium, superoxide dismutase, glucose metabolism

Received 30 November 2016 **Returned for modification** 8 January 2017 **Accepted** 12 March 2017

Accepted manuscript posted online 20 March 2017

Citation Martín-Montes Á, Plano D, Martín-Escolano R, Alcolea V, Díaz M, Pérez-Silanes S, Espuelas S, Moreno E, Marín C, Gutiérrez-Sánchez R, Sanmartín C, Sánchez-Moreno M. 2017. Library of seleno-compounds as novel agents against *Leishmania* species. Antimicrob Agents Chemother 61:e02546-16. https://doi.org/10.1128/AAC.02546-16.

Address correspondence to Carmen Sanmartín, sanmartin@unav.es, or Manuel Sánchez-Moreno, msanchem@ugr.es.

Leishmaniasis, caused by the intracellular protozoan *Leishmania*, is transmitted by the bite of phlebotomine sand flies and is endemic in 98 countries, with over 350 million people being at risk (1). Environmental, demographic, and human behavioral factors and coinfections contribute to the changing epidemiology of the disease and to its recent worldwide spread. Clinical manifestations are divided into visceral leishmaniasis (VL; or kala-azar) (2) and the forms cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (ML). CL remains the most common form of leishmaniasis both in general and in international travelers (3–5).

The drugs available for the treatment of VL and severe CL include pentavalent antimonials (SbV), deoxycholate, amphotericin B (AMB), and miltefosine (MIL), all of which have high levels of toxicity and/or require long-duration treatment schedules (5, 6). Moreover, with the exception of MIL, all these drugs must be administered by the parenteral route (7).

In the last decade, resistance to SbV has increased mainly due to low rates of compliance with the treatment schedule. In areas where the parasites are resistant to

SbV, AMB is commonly used as it is very active but requires a month of hospitalization for monitoring of renal function. MIL is highly active for the treatment of VL and well tolerated, but its potential teratogenicity cannot be ruled out. Paromomycin (PM) is actually in clinical trials for the treatment of leishmaniasis: it is effective, safe, and very cheap, but 21 intramuscular (i.m.) injections are required. Other oral drugs include azoles and allopurinol, but they did not reach the standards of efficacy or safety. On the basis of the available information, nowadays, a single dose of liposomal AMB is the best available therapy for VL in India, as it is more effective and safer than any other treatment. However, liposomal AMB seems to be less effective in Africa and for the treatment of non-self-curing CL and ML (8, 9). Moreover, there are still the high price and heat instability difficulties in its wide and safe distribution to primary health care centers. Therefore, better treatments for leishmaniasis diseases are needed, and they must be safer, cheaper, and easier to administer than the current drugs.

Recently, enzymes that are involved in evading the damage caused by oxidative stress and that present substantial biochemical and structural differences from their human counterparts have been reported to be interesting targets for novel antileishmanial drugs (10). In this sense, the iron superoxide dismutase (Fe-SOD), an exclusive antioxidant defense of trypanosomatids, is of special importance (11).

On the other hand, it is well-known that several of the most effective antiprotozoal agents were originally developed as anticancer drugs. Thus, among the compounds active against leishmaniasis, some antitumor drugs, such as cisplatin (12), miltefosine (13), and tamoxifen (14), can be found.

In addition, the research activity of our group is focused on the synthesis of new selenium (Se) compounds as antitumor agents, and we have intensively studied their mechanisms of action, with some of them being found to be antioxidants (15–20). Moreover, growing evidence suggests a connection between Se and parasites, particularly trypanosomatids (21–24).

In the light of the information mentioned above, different Se chemical entities with proven antiproliferative activity against cancer (16, 25, 26) were chosen for testing as leishmanicidal agents and were found to exhibit potent and selective effects against some *Leishmania* species (27–30). Due to their antioxidant activity, the Fe-SOD enzyme present in *Leishmania* spp. might be a plausible and good target for these compounds.

Encouraged by the promising results, we postulated that Se moieties can be central scaffolds for designing new compounds for the treatment of infections caused by leishmania. In this work, we report on the *in vitro* leishmanicidal activities (against *Leishmania infantum* and *L. braziliensis*) of 48 selenocyanates and diselenides, as well as their toxicity against macrophages, using meglumine antimonate (Glucantime) as a reference drug. ^{1}H nuclear magnetic resonance (NMR) analysis of the nature and percentage of the excreted metabolites was performed to obtain information about the inhibitory effect of our compounds on the glycolytic pathway, since this represents the prime energy source of the parasite. Finally, the results of an evaluation of their effectiveness as putative inhibitors of Fe-SOD in relation to human CuZn-SOD are also presented.

RESULTS

Chemistry. The 48 compounds described in this work were synthesized in the Department of Organic and Pharmaceutical Chemistry using previously published protocols (29–33). Their physical characteristics and spectroscopic data are in agreement with the published data and are available in the supplemental material.

Biological evaluation. (i) *In vitro* antileishmanial evaluation. In the first step, we assayed the *in vitro* biological activities of Se compounds 1 to 48 (Fig. 1) against both the extra- and intracellular forms of two significant species of *Leishmania*: *L. infantum* (Table 1) and *L. braziliensis* (Table 2).

Extracellular forms are more commonly used due to the ease of working with them, but the activities of compounds against this form are less indicative of leishmanicidal activity. The use of intracellular forms is more cumbersome but provides more accurate

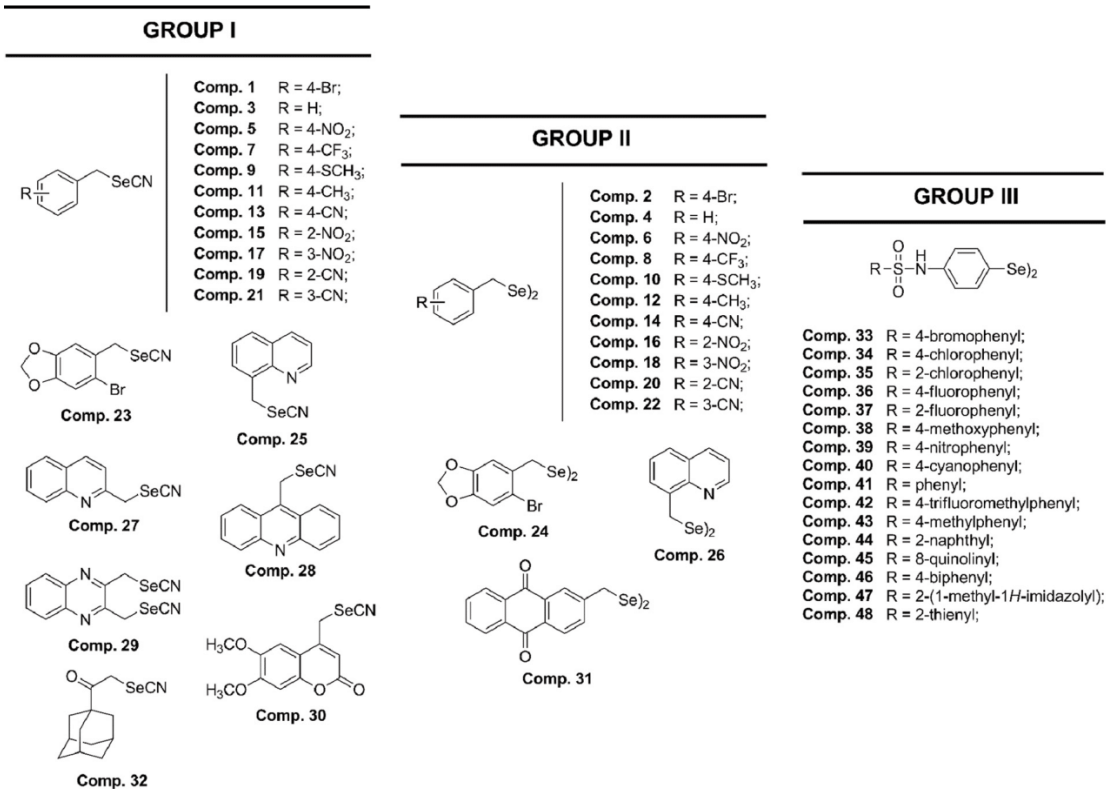


FIG 1 Chemical structures of the Se compounds presented in this work. Comp., compound.

results, as promastigotes are converted to amastigotes in vertebrate host cells (34, 35). Intracellular assays were performed by infecting macrophage cells with promastigotes, which transformed into amastigotes within 1 day after infection.

Table 1 shows the 50% inhibitory concentration (IC_{50}) values obtained after 72 h of exposure when the activities of compounds 1 to 48 against extra- and intracellular forms of *L. infantum* were tested. IC_{50} values for toxicity against J774.2 macrophages after 72 h were also calculated to establish the selectivity indexes (SIs). The results obtained for the reference drug, meglumine antimonate, were included in all cases for comparison. Biological data evidenced that half of the screened compounds (compounds 8, 9, 10, 11, 13, 15, 17, 18, 20, 21, 24, 26, 29, 33, 35, 37, 38, 42, 43, 44, 45, 46, 47, and 48) showed high levels of bioactivity against *L. infantum*, presenting greater potency than the reference drug, meglumine antimonate, against both forms (IC_{50}s, 18.0 μM for promastigotes and 24.2 μM for amastigotes).

In order to obtain a more accurate picture of the features commented on above, we show in Table 1 the SI values calculated from the IC_{50} data, since they are very illustrative of the *in vitro* potential of the tested compounds with respect to that of the reference drug. The number of times that the SI of each compound exceeded the SI of meglumine antimonate is also shown in parentheses. The differences between meglumine antimonate and the tested compounds are clearly revealed. Twelve compounds (compounds 8, 10, 11, 15, 20, 21, 26, 31, 35, 44, 45, and 48) presented notable selectivity index values (SIs $>$ 7) for both forms. These derivatives exhibited substantially better SI values than the reference drug for *L. infantum*, and in the most remarkable case, the SI of compound 8 for the intracellular form of *L. infantum* exceeded that of meglumine antimonate by 237-fold, a relevant data point, which was by far the best SI value.

TABLE 1 *In vitro* activities, toxicities, and SIs of the Se derivatives for extra- and intracellular forms of *Leishmania infantum*

Compound	IC_{50}[a] (μM) for:			SI[d]	
	In vitro activity[b]				
	Promastigote forms	Amastigote forms	Macrophage toxicity[c]	Promastigote forms	Amastigote forms
Meglumine antimonate	18.0 ± 3.1	24.2 ± 2.6	15.2 ± 1.0	0.8	0.6
1	44.1 ± 5.2	31.7 ± 2.0	92.6 ± 5.1	2 (3)	3 (5)
2	38.7 ± 2.9	23.8 ± 2.0	5.9 ± 0.6	0 (0)	0 (0)
3	36.5 ± 2.7	17.9 ± 1.4	61.5 ± 3.5	2 (2)	3 (6)
4	41.8 ± 4.1	16.9 ± 0.9	24.7 ± 2.2	0 (0)	1 (2)
5	23.9 ± 1.7	18.6 ± 1.7	19.6 ± 1.7	0 (0)	1 (2)
6	19.4 ± 1.6	10.4 ± 0.6	29.7 ± 2.6	1 (2)	3 (5)
7	35.9 ± 3.5	28.9 ± 2.2	44.8 ± 3.0	1 (1)	2 (3)
8	0.7 ± 0.2	3.7 ± 0.6	132.8 ± 7.5	190 (237)	36 (60)
9	13.8 ± 0.9	8.3 ± 0.4	51.3 ± 4.1	4 (5)	6 (10)
10	2.1 ± 0.5	4.6 ± 1.0	139.9 ± 8.3	67 (83)	30 (51)
11	1.9 ± 0.3	3.8 ± 0.7	62.9 ± 4.4	33 (41)	17 (28)
12	23.8 ± 1.1	16.4 ± 1.3	8.2 ± 0.7	0 (0)	0 (0)
13	3.5 ± 0.6	6.1 ± 0.3	10.1 ± 0.9	3 (4)	1.6 (3)
14	28.5 ± 1.9	14.8 ± 0.9	9.5 ± 1.1	0 (0)	1 (1)
15	0.8 ± 0.1	2.7 ± 0.3	49.4 ± 3.5	62 (77)	18 (30)
16	23.7 ± 2.6	11.7 ± 0.8	19.2 ± 0.7	0 (0)	2 (3)
17	16.7 ± 0.8	10.3 ± 0.7	19.3 ± 1.2	1 (1)	2 (3)
18	3.6 ± 0.7	5.5 ± 0.6	14.7 ± 1.2	4 (5)	2.6 (4)
19	41.9 ± 2.5	18.7 ± 1.3	70.0 ± 4.7	2 (2)	4 (6)
20	2.7 ± 0.8	3.7 ± 0.4	29.5 ± 2.4	11 (14)	8 (13)
21	1.2 ± 0.2	0.9 ± 0.0	8.3 ± 0.4	7 (9)	9 (14)
22	37.8 ± 2.5	17.5 ± 1.2	27.4 ± 0.9	0 (0)	2 (3)
23	47.0 ± 5.3	19.1 ± 1.6	60.3 ± 4.7	1 (1)	3 (5)
24	6.5 ± 0.7	3.6 ± 0.5	11.8 ± 0.8	2 (2)	3.3 (5)
25	18.4 ± 0.5	14.8 ± 0.7	72.4 ± 5.5	4 (5)	5 (8)
26	6.6 ± 0.4	4.1 ± 0.7	47.9 ± 3.1	7 (9)	11.7 (19)
27	28.6 ± 2.5	20.5 ± 1.7	112.3 ± 7.3	4 (5)	11 (18)
28	83.6 ± 6.8	37.8 ± 2.4	74.2 ± 6.6	0 (0)	2 (3)
29	7.1 ± 0.8	4.2 ± 1.0	22.7 ± 1.6	3 (4)	5.4 (9)
30	26.7 ± 2.0	18.7 ± 1.1	64.0 ± 3.0	2 (3)	3 (6)
31	16.8 ± 1.2	16.8 ± 0.9	185.9 ± 81.6	11 (14)	11 (18)
32	16.5 ± 2.0	13.3 ± 0.8	44.6 ± 2.4	3 (3)	3 (6)
33	7.9 ± 0.6	4.9 ± 0.8	44.1 ± 3.7	6 (7)	9.0 (15)
34	19.7 ± 1.2	15.2 ± 1.1	34.8 ± 2.4	2 (2)	2 (4)
35	3.9 ± 0.7	4.3 ± 0.6	41.8 ± 2.7	11 (13)	10 (16)
36	23.3 ± 1.7	21.6 ± 1.3	59.3 ± 3.7	2 (3)	3 (5)
37	4.8 ± 1.2	2.6 ± 0.2	17.3 ± 0.7	4 (4)	6.6 (11)
38	13.6 ± 0.8	8.9 ± 0.4	78.4 ± 5.7	6 (7)	9 (15)
39	36.5 ± 3.4	30.0 ± 2.2	50.7 ± 3.8	1 (2)	2 (3)
40	25.8 ± 2.7	17.7 ± 1.4	36.2 ± 2.4	1 (2)	2 (3)
41	19.0 ± 1.1	21.8 ± 1.2	9.6 ± 1.3	0 (0)	0 (1)
42	2.5 ± 0.6	3.2 ± 0.3	18.7 ± 0.6	7 (9)	5.8 (10)
43	8.9 ± 0.7	5.8 ± 1.1	28.5 ± 3.8	3 (4)	4.9 (8)
44	4.4 ± 0.3	3.9 ± 0.6	33.4 ± 3.0	8 (9)	8.6 (14)
45	3.7 ± 0.5	2.7 ± 0.3	61.1 ± 4.1	16 (21)	23 (38)
46	8.6 ± 0.8	6.3 ± 1.3	29.7 ± 1.8	3 (4)	4.7 (8)
47	1.1 ± 0.5	2.7 ± 0.5	12.6 ± 0.8	11 (14)	5 (8)
48	1.6 ± 0.1	1.4 ± 0.2	52.8 ± 3.61	33 (41)	38 (63)

[a]Results are the averages from four separate determinations.
[b]IC_{50}, the concentration required to give 50% inhibition, calculated by linear regression analysis from the values of the equilibrium constants (K_c) at the concentrations employed (1, 10, 25, and 200 μM).
[c]Toxicity against J774.2 macrophages after 72 h of culture.
[d]SI, selectivity index, which is calculated as the IC_{50} for macrophage toxicity/IC_{50} for activity against the extracellular or intracellular form of the parasite. The number of times that the SI of the compound exceeded the SI of the reference drug is provided in parentheses.

TABLE 2 *In vitro* activities, toxicities, and SIs for the Se derivatives on extra- and intracellular forms of *Leishmania braziliensis*

Compound	IC_{50}[a] (μM) for:			SI[d]	
	In vitro activity[b]				
	Promastigote forms	Amastigote forms	Macrophage toxicity[c]	Promastigote forms	Amastigote forms
Meglumine antimonate	25.6 ± 1.7	30.4 ± 2.6	15.2 ± 1.0	0.6	0.6
1	43.9 ± 3.7	31.8 ± 2.5	92.6 ± 5.1	2 (3)	3 (5)
2	21.7 ± 2.3	16.8 ± 1.1	5.9 ± 0.6	0 (0)	0 (0)
3	20.8 ± 1.2	17.9 ± 1.3	61.5 ± 3.5	3 (5)	3 (6)
4	37.53.5	26.4 ± 1.6	24.7 ± 2.2	0 (0)	1 (2)
5	15.8 ± 0.6	9.6 ± 0.4	19.6 ± 1.7	1 (2)	2 (3)
6	16.8 ± 1.1	16.8 ± 0.9	29.7 ± 2.6	2 (3)	2 (3)
7	30.7 ± 1.6	22.7 ± 1.6	44.8 ± 3.0	1 (2)	2 (3)
8	1.3 ± 0.3	3.8 ± 0.6	132.8 ± 7.5	102 (170)	35 (58)
9	10.8 ± 0.7	9.4 ± 1.4	51.3 ± 4.1	5 (8)	5.4 (9)
10	4.4 ± 0.1	5.7 ± 0.8	139.9 ± 8.3	32 (53)	24 (41)
11	1.6 ± 0.4	2.8 ± 0.5	62.9 ± 4.4	39 (65)	22 (37)
12	22.7 ± 1.8	15.8 ± 0.8	8.2 ± 0.7	0 (0)	0 (1)
13	4.5 ± 0.4	5.8 ± 1.5	10.1 ± 0.9	2 (3)	1.7 (3)
14	20.7 ± 1.3	19.8 ± 1.3	9.5 ± 1.1	0 (0)	0 (0)
15	0.6 ± 0.1	1.4 ± 0.3	49.4 ± 3.5	82 (137)	35 (59)
16	16.9 ± 1.2	11.7 ± 0.6	19.2 ± 0.7	1 (2)	2 (3)
17	10.5 ± 1.0	8.5 ± 2.0	19.3 ± 1.2	2 (3)	2.3 (4)
18	2.1 ± 0.1	1.9 ± 0.1	14.7 ± 1.2	7 (12)	7.7 (13)
19	8.9 ± 0.6	10.4 ± 1.3	70.0 ± 4.7	8 (13)	7 (11)
20	1.3 ± 0.3	1.1 ± 0.2	29.5 ± 2.4	23 (38)	27 (45)
21	2.0 ± 0.3	1.3 ± 0.2	8.3 ± 0.4	4 (7)	6.4 (11)
22	9.6 ± 1.2	7.5 ± 1.2	27.4 ± 0.9	3 (5)	3.6 (6)
23	20.8 ± 2.5	17.7 ± 0.8	60.3 ± 4.7	3 (5)	3 (6)
24	5.7 ± 0.4	4.3 ± 0.9	11.8 ± 0.8	2 (3)	2.7 (5)
25	13.9 ± 2.0	10.6 ± 1.7	72.4 ± 5.5	5 (9)	7 (11)
26	4.3 ± 0.3	4.8 ± 0.3	47.9 ± 3.1	11 (19)	10 (17)
27	18.4 ± 1.2	12.8 ± 0.4	112.3 ± 7.3	6 (10)	9 (15)
28	37.4 ± 3.4	23.6 ± 1.8	74.2 ± 6.6	2 (3)	3 (5)
29	6.6 ± 0.5	4.2 ± 0.8	22.7 ± 1.6	3 (6)	5.4 (9)
30	20.5 ± 0.9	13.8 ± 0.8	64.0 ± 3.0	3 (5)	5 (8)
31	20.9 ± 2.6	19.7 ± 1.2	185.9 ± 81.6	9 (15)	9 (16)
32	13.7 ± 0.7	6.8 ± 0.2	44.6 ± 2.4	3 (5)	6 (11)
33	6.9 ± 0.6	4.9 ± 0.7	44.1 ± 3.7	6 (11)	9.0 (15)
34	14.5 ± 1.8	10.6 ± 1.7	34.8 ± 2.4	2 (4)	3 (5)
35	2.5 ± 0.7	2.4 ± 0.6	41.8 ± 2.7	17 (28)	17 (29)
36	17.8 ± 0.7	12.2 ± 0.4	59.3 ± 3.7	3 (5)	5 (8)
37	3.7 ± 0.5	2.4 ± 0.4	17.3 ± 0.7	5 (8)	7.2 (12)
38	15.8 ± 0.9	9.9 ± 0.5	78.4 ± 5.7	5 (8)	8 (13)
39	31.5 ± 3.5	17.6 ± 1.4	50.7 ± 3.8	2 (3)	3 (5)
40	24.7 ± 1.7	20.1 ± 2.3	36.2 ± 2.4	2 (2)	2 (3)
41	11.9 ± 1.3	10.8 ± 0.5	9.6 ± 1.3	0 (0)	0 (0)
42	4.7 ± 0.2	3.3 ± 0.6	18.7 ± 0.6	4 (7)	5.7 (9)
43	4.7 ± 0.6	3.7 ± 0.5	28.5 ± 3.8	6 (10)	7.7 (13)
44	2.8 ± 0.8	4.3 ± 0.8	33.4 ± 3.0	8 (13)	10 (17)
45	1.7 ± 0.1	2.0 ± 0.5	61.1 ± 4.1	36 (60)	30 (51)
46	3.3 ± 0.4	3.4 ± 0.7	29.7 ± 1.8	9 (15)	9 (15)
47	0.8 ± 0.2	1.0 ± 0.1	12.6 ± 0.8	16 (26)	13 (21)
48	0.9 ± 0.1	1.8 ± 0.5	52.8 ± 3.6	59 (98)	29 (49)

[a]Results are the averages from four separate determinations.
[b]IC_{50}, the concentration required to give 50% inhibition, calculated by linear regression analysis from the values of the equilibrium constants (K_c) at the concentrations employed (1, 10, 25, and 200 μM).
[c]Toxicity against J774.2 macrophages after 72 h of culture.
[d]SI, selectivity index, which is calculated as the IC_{50} for macrophage toxicity/IC_{50} for activity against the extracellular or intracellular form of the parasite. The number of times that the compound SI exceeded the reference drug SI is provided in parentheses.

Six of the compounds (compounds 8, 10, 11, 15, 45, and 48) stood out as the most active and selective molecules, showing IC_{50}s lower than 5 μM along with SIs higher than 15, and were selected for further studies.

Considering their activity against *L. braziliensis* (Table 2), the results recurred in the two species and in both extra- and intracellular forms, since compounds 8, 10, 11, 15, 45, and 48 showed substantially better results, in terms of activity and selectivity, than the rest of the tested compounds in all cases. For example, the SI of compound 8 exceeded that of the reference drug by 237- and 60-fold for the extra- and intracellular forms of *L. infantum*, respectively, and by 170- and 58-fold for the extra- and intracellular forms of *L. braziliensis*, respectively (Table 2). Compound 10 had an SI similar to that of derivative 8, with the respective values obtained being 83- and 51-fold for *L. infantum* (Table 1) and 53- and 41-fold for *L. braziliensis* (Table 2); compounds 11 and 15 were equally effective against the two species of *Leishmania* and against both the extra- and intracellular forms. Compound 11 had 41- and 28-fold increases in the SIs for the extra- and intracellular forms of*L. infantum*, respectively (Table 1), along with 65- and 37-fold in SIs for the extra- and intracellular forms *L. braziliensis*, respectively (Table 2). On the other hand, compounds 45 and 48 were more selective for the extracellular form of *L. braziliensis* (Table 2). The rest of the tested compounds presented similar values in both species, except compound 20, which was effective against *L. braziliensis* but not against *L. infantum*.

(ii) Leishmanicidal activity in infected macrophages. In order to gain better insight into the activities of the most active compounds, their effects on the infectivity and intracellular replication of amastigotes were subsequently determined. Macrophage cells were grown and infected with promastigotes in the stationary phase. The parasites invaded cells and underwent morphological conversion to amastigotes within 1 day after infection. On day 10, the rate of host cell infection reached its maximum (control experiment). We used the IC_{25} of each product as the test dosage. Different authors have claimed that compounds with SI values less than 20-fold those of the reference drug should be discarded as candidates for more advanced leishmanicidal tests due to their poor selectivity against mammalian cells (36). Therefore, we selected the compounds that fulfilled this requirement (compounds 8, 10, 11, 15, 45, and 48 for *L. infantum* and those compound plus compound 20 for *L. braziliensis*).

As shown in Fig. 2A and B, when the selected compounds were added to macrophages infected with *L. infantum* promastigotes, the infection rate decreased significantly with respect to that for the control; furthermore, the six compounds (compounds 8, 10, 11, 15, 45, and 48) were also remarkably more effective in decreasing infectivity than meglumine antimonate. A measure of the average number of amastigotes per infected macrophage (Fig. 2C and D) led to the same conclusion: all six compounds were more effective than meglumine antimonate.

Infection rates (Fig. 3A and B) and the reduction in amastigote numbers (Fig. 3C and D) obtained with *L. braziliensis* also showed that, in both cases, these six compounds along with compound 20 were clearly more effective than meglumine antimonate, and also in both cases, the order of effectiveness of the compounds was 48 $>$ 45 $>$ 8 $>$ 11 $>$ 10 $>$ 15 $>$ 20 $>$ meglumine antimonate, since the reductions in the infectivity rates calculated were 71%, 62%, 60%, 53%, 50%, 47%, 40%, and 28%, respectively. The decreases in amastigote numbers were 70%, 66%, 60%, 53%, 48%, 48%, 43%, and 30% for compounds 48, 45, 8, 11, 10, 15, and 20 and meglumine antimonate, respectively. When these results are taken into account, it can be concluded that all compounds analyzed were shown to be substantially more active than meglumine antimonate against the two *Leishmania* species tested.

(iii) Studies on the mechanism of action. ***(a) Metabolite excretion effect*.** Since trypanosomatids are unable to completely degrade glucose to CO_2 under aerobic conditions, they excrete much of the hexose skeleton into the medium as partially oxidized fragments in the form of fermented metabolites. The nature and percentages

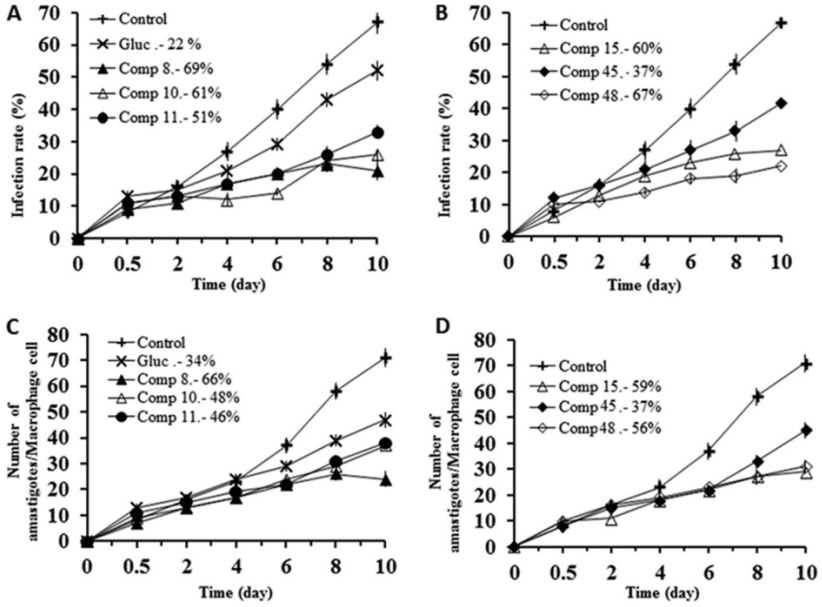


FIG 2 Effects of Se derivatives 8, 10, 11, 15, 45, and 48 on the rates of infection by and growth rates of *L. infantum*. (A, B) Rates of infection; (C, D) mean numbers of amastigotes of *L. infantum* per infected J774.2 macrophage cell (when the compounds were tested at the IC_{25}). Values are the means from three separate experiments. Gluc., Glucantime (meglumine antimonate).

of those excretion products depend on the pathway used for glucose metabolism by each species (37).

The final products of glucose catabolism in *Leishmania* are usually CO_2, succinate, acetate, D-lactate, L-alanine, and, in some cases, ethanol (37). Among them, succinate is the most relevant, because its main role is to maintain the glycosomal redox balance, allowing the reoxidation of NADH produced in the glycolytic pathway. Succinic fermentation requires only half of the phosphoenolpyruvate produced to maintain the NAD^+/NADH balance, and the remaining pyruvate is converted inside the mitochondrion and the cytosol into acetate, D-lactate, L-alanine, or ethanol, according to the degradation pathway followed by a specific species (38).

We report below that the most active compounds caused great damage to the mitochondria of the parasites of both *Leishmania* species, so that their highly disruptive action presumably affected their glucose metabolism and, consequently, modified the percentages of the final excretion products formed. Therefore, we registered the ^{1}H NMR spectra of promastigotes from the *L. infantum* and *L. braziliensis* species after treatment with the selected compounds, and the final excretion products were identified qualitatively and quantitatively. The spectra obtained were compared with those from promastigotes maintained in cell-free medium (control) for 4 days after inoculation with the parasites. The variations in the areas of the signals corresponding to the most significant catabolites are displayed. The expected presence of acetate, D-lactate, L-alanine, and succinate was confirmed in the control experiments performed on both species, and the major metabolite was succinate in all cases, in agreement with data reported in the literature (38). However, relevant differences were found in parasites treated with the derivatives, whereas the presence of meglumine antimonate did not lead to significant alterations in the energetic metabolism (data not shown).

Compounds 10, 11, and 20 induced increases in the levels of succinate production in *L. braziliensis* (Fig. 4 and Table 3), as can be seen by measuring the area under the succinate peak determined by ^{1}H NMR. In the case of *L. infantum*, all the compounds increased the level of succinate production, although product 8 did so to a lesser extent than the other compounds (Fig. 5 and Table 4). In fact, this succinate augmentation is

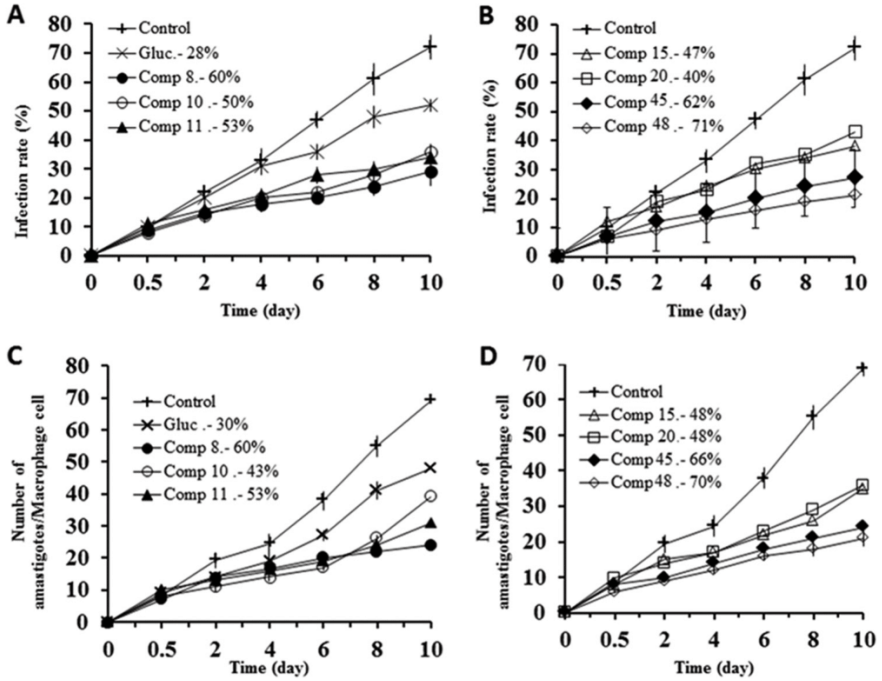


FIG 3 Effects of Se derivatives 8, 10, 11, 15, 20, 45, and 48 on the rates of infection by and growth rates of *Leishmania braziliensis*. (A, B) Rates of infection; (C, D) mean numbers of amastigotes of *L. braziliensis* per infected J774 A.2 macrophage cell (when the compounds were tested at the IC_{25}). Values are the means from three separate experiments.

in agreement with transmission electron microscopy (TEM) evidence, indicating that these derivatives severely damage the mitochondrial structures of the parasites (data not shown).

***(b) SOD enzymatic inhibition in* Leishmania *parasites and in human erythrocytes*.** These results prompted us to evaluate the inhibitory effect of the most promising compounds on the activity of Fe-SOD from *L. infantum* and *L. braziliensis* at concentrations ranging from 0.5 to 100 μM.

To do so, we used the promastigote forms of both species, which excrete Fe-SOD when cultured in a medium lacking inactive fetal bovine serum (39). The inhibition data obtained are shown in Fig. 6A to D, and the corresponding IC_{50}s are included for easier

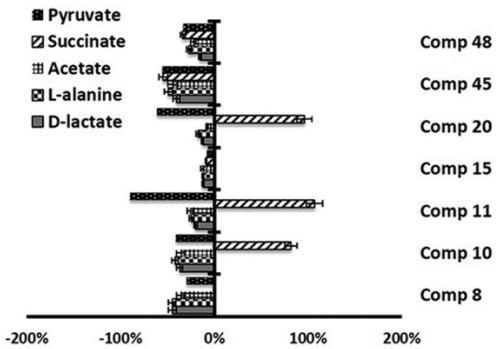


FIG 4 Variation in the area of the peaks (in percent) corresponding to metabolites excreted by *L. braziliensis* forms in the presence of compounds 8, 10, 11, 15, 20, 45, and 48 at their IC_{25}s compared to the area for a control sample, determined by ^{1}H NMR.

TABLE 3 Variation in areas of peaks corresponding to catabolites excreted by *L. braziliensis* promastigotes in presence of compounds with respect to those in control tests

Compound	% variation in area of peaks				
	D-Lactate	L-Alanine	Acetate	Pyruvate	Succinate
Control	0.0	0.0	0.0	0.0	0.0
8	−44.7	−44.9	−36.2	−29.4	−0.2
10	−36.9	−41.7	−36.1	−40.8	81.7
11	−20.0	−24.2	−25.7	−89.6	107.1
15	−12.1	−11.9	−12.7	−7.9	−8.6
20	−12.7	−17.6	−7.7	−61.5	96.2
45	−40.4	−49.0	−44.5	−55.1	−54.4
48	−15.5	−27.4	−22.4	−33.3	−33.6

evaluation of the graphs displayed. For comparison, the graphs in Fig. 7 show the effects of the same compounds on CuZn-SOD obtained from human erythrocytes. The most remarkable result was the significant inhibitory effect of the highly leishmanicidal compounds 8, 10, and 11 found on Fe-SOD from both species and compound 20 in the case of *L. braziliensis*, whereas their inhibition of human CuZn-SOD was clearly lower.

If we consider the IC_{50} calculated for *L. infantum*, the levels of inhibition of Fe-SOD by compounds 8, 10, and 11 were, respectively, 5-, 19- and 16-fold higher than the levels of inhibition of CuZn-SOD, and in the case of *L. braziliensis*, the levels of inhibition by compounds 8, 10, and 11 were 4-, 34-, and 0-fold higher, respectively, and the level of inhibition by compound 20 was 9-fold higher.

DISCUSSION

The present results document the leishmanicidal effects of 48 Se derivatives. After taking into consideration the IC_{50} data shown in Tables 1 and 2, it can be seen that compounds 8, 10, 11, 15, 45, and 48 stand out as the molecules most active and selective against the two *Leishmania* spp. studied (*L. infantum* and *L. braziliensis*). Furthermore, they were much more active than meglumine antimonate against both the extra- and intracellular forms of the parasite. Regarding the evaluation of cytotoxicity against macrophage cells, it was also shown that these six compounds along with compound 20 were clearly more selective than meglumine antimonate against *L. infantum* and *L. braziliensis*. Attending to other studies to elucidate the possible mechanisms of action, it should be noted that these compounds produced great alterations in glucose metabolism in the studied species, according to the ^{1}H NMR data for catabolites. It is feasible that the small size of these compounds allows them to easily penetrate the cristae (tubular invaginations of the inner mitochondrial membrane), leading to subsequent changes in the metabolic pathway, although further

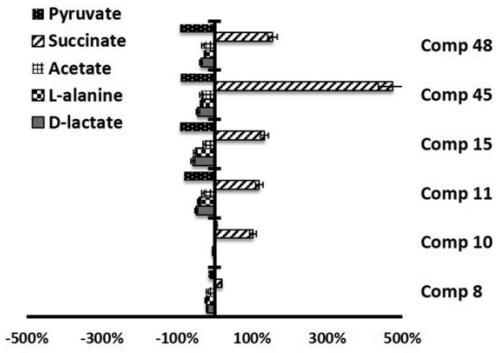


FIG 5 Variation in the area of the peaks (in percent) corresponding to metabolites excreted by *L. infantum* promastigote forms in the presence of compounds 8, 10, 11, 15, 45, and 48 at their IC_{25}s compared to the area for a control sample, determined by ^{1}H NMR.

TABLE 4 Variation in areas of peaks corresponding to catabolites excreted by *L. infantum* promastigotes in presence of compounds with respect to those in control tests

Compound	% variation in area of peaks				
	D-Lactate	L-Alanine	Acetate	Pyruvate	Succinate
Control	0.0	0.0	0.0	0.0	0.0
8	−20.4	−22.5	−18.8	−17.0	18.6
10	1.8	−4.8	1.1	8.3	102.1
11	−48.8	−41.8	−30.6	−80.7	118.9
15	−58.1	−50.7	−27.8	−91.3	132.1
45	−44.6	−34.2	−35.4	−90.3	474.1
48	−36.0	−25.7	−30.3	−91.1	153.6

studies are required to prove this point. In addition, these compounds led to important levels of Fe-SOD inhibition. All these data seem to confirm some kind of relation between parasiticidal activity and inhibition of Fe-SOD, in accordance with results described in a previous work (39). At another level, Fe-SOD inhibition could also be related to the catabolic changes discussed above, since a mitochondrial malfunction might originate from the redox stress produced by inhibition of the mitochondrion-resident Fe-SOD enzyme (40). Since Fe-SOD is a specific chemotherapy target also present in mitochondria, this fact would agree with the hypothesis presented above that these compounds might have a greater ability to pass through the mitochondrial membrane.

From all the results detailed above, we conclude that the Se derivatives (compounds 8, 10, 11, 15, 45, and 48) show interesting *in vitro* leishmanicidal activity which seems

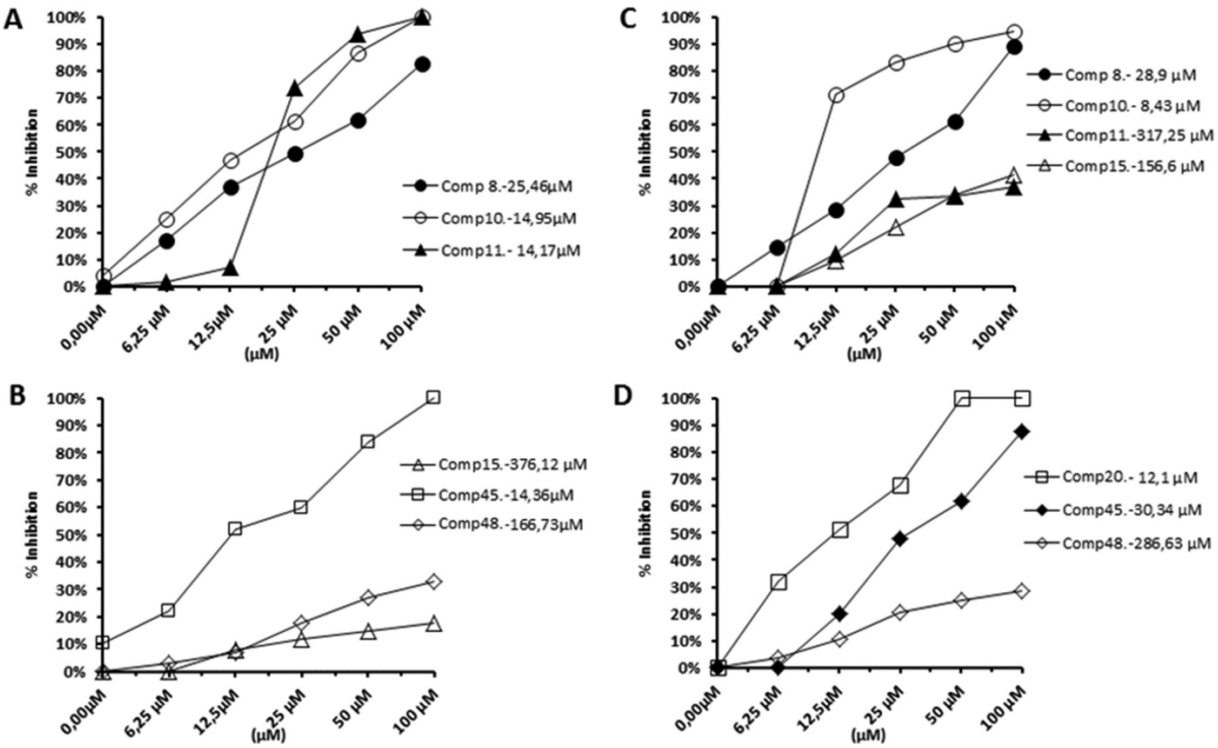


FIG 6 *In vitro* inhibition (in percent) of Fe-SOD from *L. infantum* (A and B) and *L. braziliensis* (C and D) promastigote forms by the Se derivatives. The differences between the activities of the control homogenate and homogenates incubated with the indicated compounds were obtained according to the Newman-Keuls test. The concentrations in the keys are IC_{50}s, which are the concentrations required to give 50% inhibition and which were calculated by linear regression analysis from the values of the equilibrium constants (K_c) at the concentrations employed (0.5 to 100 μM). The values are averages from three different determinations.

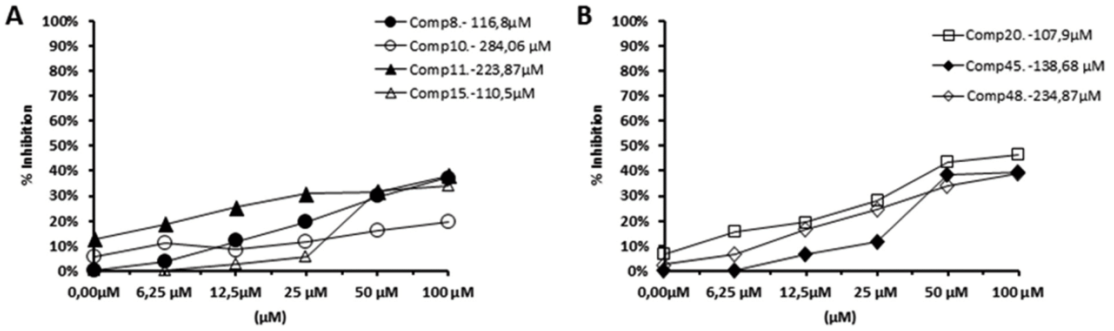


FIG 7 *In vitro* inhibition (in percent) of CuZn-SOD from human erythrocytes by the Se derivatives. The differences between the activities of the control homogenate and homogenates incubated with the indicated compounds were obtained according to the Newman-Keuls test. The concentrations in the keys are IC_{50}s, which are the concentrations required to give 50% inhibition and which were calculated by linear regression analysis from the values of the equilibrium constants (K_c) at the concentrations employed (0.5 to 100 μM). The values are averages from three different determinations.

to be related to their ability to inhibit the parasite Fe-SOD. Interestingly, the ^{1}H NMR data for the catabolites are indicative of substantial alterations in glucose metabolism in the studied species. In fact, this succinate augmentation is in agreement with transmission electron microscopy (TEM) evidence indicating that these derivatives severely damage the mitochondrial structures of the parasites (data not shown). A graphical summary of the conclusions drawn from this work is presented in Fig. 8. On that basis, we think that the activities of these compounds fulfill the requirements needed to justify a more detailed investigation of the nature of the mechanisms involved in their patterns of activity and, furthermore, that they could be considered candidates for the study of their antiparasitic activities at a higher level. Finally, it is worth mentioning that due to their different mechanisms of action, combined therapies should be considered to obtain improved efficacy.

MATERIALS AND METHODS

Chemistry. The Se compounds reported herein can be categorized into three groups (Fig. 1): selenocyanate derivatives, or group I (compounds 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 28, 29, 30, and 32); the diselenide analogs of the selenocyanate derivatives mentioned above, or group II (compounds 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, and 31); and the diphenyldiselenide-derived compounds bearing different sulfonamides substituted on the *para* position, or group III (compounds 33 to 48).

The group I compounds were obtained by the nucleophilic substitution of the halogen atom of the corresponding alkyl or benzyl halides by a selenocyanate group using potassium selenocyanate (KSeCN) under reflux conditions according to previously reported methods (29, 30). Thus, the corresponding organic halide was refluxed for 2 to 4 h in acetone with potassium selenocyanate. The resulting precipitate was filtered off, and the filtrate was evaporated under vacuum. Then, the residue was treated

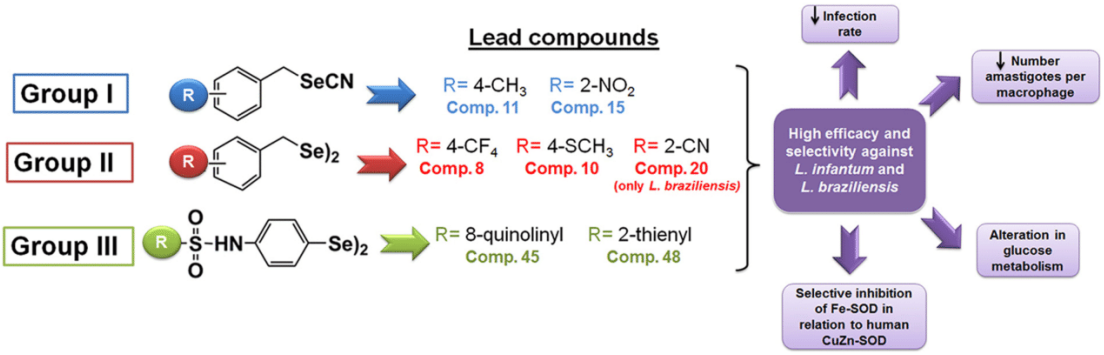


FIG 8 Schematic illustration of conclusions.

with water (2 times with 50 ml each time), filtered, and dried. The solid obtained was purified either by recrystallization or by washing with different solvents.

Group II derivatives were synthesized by reduction of the corresponding selenocyanate from group I using sodium borohydride following the previously described procedure (29, 30). In order to do so, sodium borohydride was added with caution to a stirring solution of the corresponding selenocyanate from group I in absolute ethanol. After 2 h of reaction, the solvent was removed under reduced pressure and the residue was treated with water (50 ml). The mixture was extracted with dichloromethane (3 times with 50 ml each time); the organic fractions were collected, dried over sodium sulfate, and filtered off; and the solvent was removed under vacuum by rotary evaporation. The resulting solid was recrystallized using ethanol.

Finally, targeted compounds of group III were prepared from 4,4′-diselanediylaniline coupled to different substituted sulfonyl chlorides (31). Briefly, the corresponding sulfonyl chloride was added to a mixture of 4,4′-diselanediylaniline and dry ether, and the reaction mixture was stirred at room temperature for 48 to 72 h under a nitrogen atmosphere. The precipitate formed in the reaction mixture was filtered, dried, and purified accordingly by recrystallization or washing with different solvents.

Biological evaluation. (i) Determination of the potential leishmanicidal activity and toxicity of new compounds. ***(a) Axenic cultures.*** The parasites were cultivated in axenic cultures using standard conditions for the promastigote and amastigote forms of *L. infantum* (MCAN/ES/2001/UCM-10) and *L. braziliensis* (MHOM/BR/1975/M2904) strains (41, 42).

(b) Cytotoxicity tests. The viability of J774.2 macrophages and Vero cells was determined by use of a Coulter cell counter using previously published standardized methods (39).

***(c)* In vitro *activity assays*.** For extracellular forms (promastigote assay), promastigotes were collected in the exponential growth phase and distributed in culture trays (with 24 wells) at a final concentration of 5×10^4 parasites/well according to previously reported methods (39).

For intracellular forms (amastigote assay), macrophages of the J774.2 macrophage line (European Collection of Cell Cultures [ECACC] number 91051511) were cultured in 24-well microplates with round coverslips. The assays were performed as previously described (39).

(d) Infectivity assay. The mammalian cells were infected *in vitro* with metacyclic forms of *Leishmania* spp. at a ratio of 10:1 according to information presented in the literature (39). At least three different assays were performed to calculate the SI of each compound, which was determined as the ratio between the IC_{50} obtained in macrophages and the corresponding IC_{50} in promastigotes.

(ii) Studies on the mechanism of action. ***(a) Metabolite excretion.*** According to previous reports (39), cultures of extracellular forms received the selected compounds at a dose of the IC_{25} and were then cultured for 72 h at 28°C. Then, the supernatant was collected to detect the excreted metabolites by ^{1}H NMR.

(b) Enzymatic inhibition of Fe-SOD. For SOD inhibition studies, the parasites cultured were centrifuged and processed as described previously (34, 35). Iron, manganese, and copper-zinc SOD activities were determined using the method described by Beyer and Fridovich (43).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02546-16.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

ACKNOWLEDGMENTS

We express our gratitude to the Spanish MINECO, FEDER (Consolider-Ingenio projects CSD2010-00065-2010 and CTQ2013-48917-C3-1-P), and FIMA (Fundación para la Investigación Médica Aplicada) project ISTUN-API-2011/02 for financial support. R.M.-E. is grateful for an FPU grant (FPU14/01537) from the Ministry of Education of Spain.

REFERENCES

1. Seifert K. 2011. Structures, targets and recent approaches in anti-leishmanial drug discovery and development. Open Med Chem J 5:31–39. https://doi.org/10.2174/1874104501105010031.
2. World Health Organization. 2014. WHO fact sheet no. 375. World Health Organization, Geneva, Switzerland. Accessed 9 September 2016.
3. Kenneth JR, Ray CG, Ahmad N, Drew WL, Plorde JJ. 2010. Sherris medical microbiology: an introduction to infectious diseases, 5th ed. McGraw-Hill, New York, NY.
4. Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, Alvar J, Boelaert M. 2007. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat Rev Microbiol 5:873–882.
5. Lukes J, Mauricio IL, Schonian G, Dujardin JC, Soteriadou K, Dedet JP, Kuhls K, Tintaya KW, Jirku M, Chocholova E, Haralambous C, Pratlong F, Obornik M, Horak A, Ayala FJ, Miles MA. 2007. Evolutionary and geographical history of the Leishmania donovani complex with a revision of current taxonomy. Proc Natl Acad Sci U S A 104:9375–9380. https://doi.org/10.1073/pnas.0703678104.
6. Croft SL, Sundar S, Fairlamb AH. 2006. Drug resistance in leishmaniasis. Clin Microbiol Rev 19:111–126. https://doi.org/10.1128/CMR.19.1.111-126.2006.
7. Ouellette M, Drummelsmith J, Papadopoulou B. 2004. Leishmaniasis: drugs in the clinic, resistance and new developments. Drug Resist Updat 7:257–266. https://doi.org/10.1016/j.drup.2004.07.002.
8. Freitas-Junior LH, Chatelain E, Kim HA, Siqueira-Neto JL. 2012. Visceral leishmaniasis treatment: what do we have, what do we need and how to deliver it? Int J Parasitol Drugs Drug Resist 2:11–19. https://doi.org/10.1016/j.ijpddr.2012.01.003.
9. Loiseau PM, Cojean S, Schrevel J. 2011. Sitamaquine as a putative antileishmanial drug candidate: from the mechanism of action to the risk of drug resistance. Parasite 18:115–119. https://doi.org/10.1051/parasite/2011182115.
10. Menna-Barreto RF, de Castro SL. 2014. The double-edged sword in pathogenic trypanosomatids: the pivotal role of mitochondria in oxidative stress and bioenergetics. Biomed Res Int 2014:614014. https://doi.org/10.1155/2014/614014.

11. Singh K, Garg G, Ali V. 2016. Current therapeutics, their problems and thiol metabolism as potential drug targets in leishmaniasis. Curr Drug Metab 17:897–919. https://doi.org/10.2174/1389200217666160819161444.
12. Tavares J, Ouaissi M, Ouaissi A, Cordeiro-da-Silva A. 2007. Characterization of the anti-Leishmania effect induced by cisplatin, an anticancer drug. Acta Trop 103:133–141. https://doi.org/10.1016/j.actatropica.2007.05.017.
13. Castelo Branco PV, Soares RE, de Jesus LC, Moreira VR, Alves HJ, de Castro Belfort MR, Silva VL, Ferreira Pereira SR. 2016. The antileishmanial drug miltefosine (Impavido(R)) causes oxidation of DNA bases, apoptosis, and necrosis in mammalian cells. Mutat Res Genet Toxicol Environ Mutagen 806:34–39. https://doi.org/10.1016/j.mrgentox.2016.06.007.
14. Doroodgar M, Delavari M, Doroodgar M, Abbasi A, Taherian AA, Doroodgar A. 2016. Tamoxifen induces apoptosis of Leishmania major promastigotes in vitro. Korean J Parasitol 54:9–14. https://doi.org/10.3347/kjp.2016.54.1.9.
15. Dominguez-Alvarez E, Plano D, Font M, Calvo A, Prior C, Jacob C, Palop JA, Sanmartin C. 2014. Synthesis and antiproliferative activity of novel selenoester derivatives. Eur J Med Chem 73:153–166. https://doi.org/10.1016/j.ejmech.2013.11.034.
16. Plano D, Baquedano Y, Ibanez E, Jimenez I, Palop JA, Spallholz JE, Sanmartin C. 2010. Antioxidant-prooxidant properties of a new organoselenium compound library. Molecules 15:7292–7312. https://doi.org/10.3390/molecules15107292.
17. Romano B, Plano D, Encio I, Palop JA, Sanmartin C. 2015. In vitro radical scavenging and cytotoxic activities of novel hybrid selenocarbamates. Bioorg Med Chem 23:1716–1727. https://doi.org/10.1016/j.bmc.2015.02.048.
18. Shaaban S, Arafat MA, Hamama WS. 2014. Vistas in the domain of organoselenocyanates. Arkivoc 2014:470–505. https://doi.org/10.3998/ark.5550190.p008.763.
19. Mecklenburg S, Shaaban S, Ba LA, Burkholz T, Schneider T, Diesel B, Kiemer AK, Roseler A, Becker K, Reichrath J, Stark A, Tilgen W, Abbas M, Wessjohann LA, Sasse F, Jacob C. 2009. Exploring synthetic avenues for the effective synthesis of selenium- and tellurium-containing multifunctional redox agents. Org Biomol Chem 7:4753–4762. https://doi.org/10.1039/b907831b.
20. Shaaban S, Negm A, Ashmawy AM, Ahmed DM, Wessjohann LA. 2016. Combinatorial synthesis, in silico, molecular and biochemical studies of tetrazole-derived organic selenides with increased selectivity against hepatocellular carcinoma. Eur J Med Chem 122:55–71. https://doi.org/10.1016/j.ejmech.2016.06.005.
21. Cassago A, Rodrigues EM, Prieto EL, Gaston KW, Alfonzo JD, Iribar MP, Berry MJ, Cruz AK, Thiemann OH. 2006. Identification of Leishmania selenoproteins and SECIS element. Mol Biochem Parasitol 149:128–134. https://doi.org/10.1016/j.molbiopara.2006.05.002.
22. Lobanov AV, Gromer S, Salinas G, Gladyshev VN. 2006. Selenium metabolism in Trypanosoma: characterization of selenoproteomes and identification of a Kinetoplastida-specific selenoprotein. Nucleic Acids Res 34:4012–4024. https://doi.org/10.1093/nar/gkl541.
23. Noguchi N. 2016. Ebselen, a useful tool for understanding cellular redox biology and a promising drug candidate for use in human diseases. Arch Biochem Biophys 595:109–112. https://doi.org/10.1016/j.abb.2015.10.024.
24. Souza CC, Barreto TDO, da Silva SM, Pinto AW, Figueiredo MM, Rocha OG, Cangussu SD, Tafuri WL. 2014. A potential link among antioxidant enzymes, histopathology and trace elements in canine visceral leishmaniasis. Int J Exp Pathol 95:260–270. https://doi.org/10.1111/iep.12080.
25. Alcolea V, Plano D, Encio I, Palop JA, Sharma AK, Sanmartin C. 2016. Chalcogen containing heterocyclic scaffolds: new hybrids with antitumoral activity. Eur J Med Chem 123:407–418. https://doi.org/10.1016/j.ejmech.2016.07.042.
26. Plano D, Ibanez E, Calvo A, Palop JA, Sanmartin C. 2011. Novel library of selenocompounds as kinase modulators. Molecules 16:6349–6364. https://doi.org/10.3390/molecules16086349.
27. Moreno D, Plano D, Baquedano Y, Jimenez-Ruiz A, Palop JA, Sanmartin C. 2011. Antileishmanial activity of imidothiocarbamates and imidoselenocarbamates. Parasitol Res 108:233–239. https://doi.org/10.1007/s00436-010-2073-x.
28. Fernandez-Rubio C, Campbell D, Vacas A, Ibanez E, Moreno E, Espuelas S, Calvo A, Palop JA, Plano D, Sanmartin C, Nguewa PA. 2015. Leishmanicidal activities of novel methylseleno-imidocarbamates. Antimicrob Agents Chemother 59:5705–5713. https://doi.org/10.1128/AAC.00997-15.
29. Plano D, Baquedano Y, Moreno-Mateos D, Font M, Jimenez-Ruiz A, Palop JA, Sanmartin C. 2011. Selenocyanates and diselenides: a new class of potent antileishmanial agents. Eur J Med Chem 46:3315–3323. https://doi.org/10.1016/j.ejmech.2011.04.054.
30. Baquedano Y, Alcolea V, Toro MA, Gutierrez KJ, Nguewa P, Font M, Moreno E, Espuelas S, Jimenez-Ruiz A, Palop JA, Plano D, Sanmartin C. 2016. Novel heteroaryl selenocyanates and diselenides as potent antileishmanial agents. Antimicrob Agents Chemother 60:3802–3812. https://doi.org/10.1128/AAC.02529-15.
31. Baquedano Y, Moreno E, Espuelas S, Nguewa P, Font M, Gutierrez KJ, Jimenez-Ruiz A, Palop JA, Sanmartin C. 2014. Novel hybrid selenosulfonamides as potent antileishmanial agents. Eur J Med Chem 74:116–123. https://doi.org/10.1016/j.ejmech.2013.12.030.
32. Shaaban S, Negm A, Sobh MA, Wessjohann LA. 2016. Expeditious entry to functionalized pseudo-peptidic organoselenide redox modulators via sequential Ugi/SN methodology. Anticancer Agents Med Chem 16:621–632. https://doi.org/10.2174/1871520615666150916092035.
33. Shaaban S, Negm A, Sobh MA, Wessjohann LA. 2015. Organoselenocyanates and symmetrical diselenides redox modulators: design, synthesis and biological evaluation. Eur J Med Chem 97:190–201. https://doi.org/10.1016/j.ejmech.2015.05.002.
34. Cortazar TM, Coombs GH, Walker J. 2007. Leishmania panamensis: comparative inhibition of nuclear DNA topoisomerase II enzymes from promastigotes and human macrophages reveals anti-parasite selectivity of fluoroquinolones, flavonoids and pentamidine. Exp Parasitol 116:475–482. https://doi.org/10.1016/j.exppara.2007.02.018.
35. Marin C, Boutaleb-Charki S, Diaz JG, Huertas O, Rosales MJ, Perez-Cordon G, Guitierrez-Sanchez R, Sanchez-Moreno M. 2009. Antileishmaniasis activity of flavonoids from Consolida oliveriana. J Nat Prod 72:1069–1074. https://doi.org/10.1021/np8008122.
36. Nwaka S, Ramirez B, Brun R, Maes L, Douglas F, Ridley R. 2009. Advancing drug innovation for neglected diseases—criteria for lead progression. PLoS Negl Trop Dis 3:e440. https://doi.org/10.1371/journal.pntd.0000440.
37. Michels PA, Bringaud F, Herman M, Hannaert V. 2006. Metabolic functions of glycosomes in trypanosomatids. Biochim Biophys Acta 1763:1463–1477. https://doi.org/10.1016/j.bbamcr.2006.08.019.
38. Cazzulo JJ. 1992. Aerobic fermentation of glucose by trypanosomatids. FASEB J 6:3153–3161.
39. Gonzalez P, Marin C, Rodriguez-Gonzalez I, Hitos AB, Rosales MJ, Reina M, Diaz JG, Gonzalez-Coloma A, Sanchez-Moreno M. 2005. In vitro activity of C20-diterpenoid alkaloid derivatives in promastigotes and intracellular amastigotes of Leishmania infantum. Int J Antimicrob Agents 25:136–141. https://doi.org/10.1016/j.ijantimicag.2004.08.010.
40. Bringaud F, Riviere L, Coustou V. 2006. Energy metabolism of trypanosomatids: adaptation to available carbon sources. Mol Biochem Parasitol 149:1–9. https://doi.org/10.1016/j.molbiopara.2006.03.017.
41. Mpamhanga CP, Spinks D, Tulloch LB, Shanks EJ, Robinson DA, Collie IT, Fairlamb AH, Wyatt PG, Frearson JA, Hunter WN, Gilbert IH, Brenk R. 2009. One scaffold, three binding modes: novel and selective pteridine reductase 1 inhibitors derived from fragment hits discovered by virtual screening. J Med Chem 52:4454–4465. https://doi.org/10.1021/jm900414x.
42. Piacenza L, Zago MP, Peluffo G, Alvarez MN, Basombrio MA, Radi R. 2009. Enzymes of the antioxidant network as novel determiners of Trypanosoma cruzi virulence. Int J Parasitol 39:1455–1464. https://doi.org/10.1016/j.ijpara.2009.05.010.
43. Beyer WF, Jr, Fridovich I. 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal Biochem 161:559–566. https://doi.org/10.1016/0003-2697(87)90489-1.

Bioorganic Chemistry 92 (2019) 103274

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

In vitro assessment of 3-alkoxy-5-nitroindazole-derived ethylamines and related compounds as potential antileishmanial drugs

Álvaro Martín-Montes[1*], Benjamín Aguilera-Venegas[2], Rosa Mª Morales-Martín[1], Rubén Martín-Escolano[1], Salvador Zamora-Ledesma[3], Clotilde Marín[1], Vicente J. Arán[4], Manuel Sánchez-Moreno[1*]

1) Departamento de Parasitología, Instituto de Investigación Biosanitaria (ibs.Granada), Hospitales Universitarios de Granada, Universidad de Granada, c/ Severo Ochoa s/n, 18071, Granada, España

2) Department of Inorganic and Analytical Chemistry, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Box 233, Santiago, 8380492, Chile.

3) Cuerpo Académico de Ecología y Diversidad Faunística, Universidad Autónoma de Querétaro, Avenida de las Ciencias s/n, Juriquilla, Querétaro, 76230, Mexico.

4) Instituto de Química Médica (IQM), Consejo Superior de Investigaciones Científicas (CSIC), c/ Juan de la Cierva 3, 28006, Madrid, Spain.

Authors would like to dedicate this work to their colleague Dr. Pilar Navarro (IQM, CSIC, Madrid), who recently retired.

Keywords: antiprotozoal agents, drug discovery, indazoles, *Leishmania*, nitroheterocycles.

Summary

Leishmaniasis is a widespread neglected tropical disease complex that is responsible of one million new cases per year. Current treatments are outdated and pose many problems that new drugs need to overcome. With the goal of developing new, safe, and affordable drugs, we have studied the *in vitro* activity of 12 different 5-nitroindazole derivatives that showed previous activity against different strains of *Trypanosoma cruzi* in a previous work. *T. cruzi* belongs to the same family as *Leishmania* spp., and

treatments for the disease it produces also needs renewal. Among the derivatives tested, compounds **1**, **2**, **9**, **10**, **11**, and **12** showed low J774.2 macrophage toxicity, while their effect against both intracellular and extracellular forms of the studied parasites was higher than the ones found for the reference drug Meglumine Antimoniate (Glucantime®). In addition, their Fe-SOD inhibitory effect, the infection rates, metabolite alteration, and mitochondrial membrane potential of the parasites treated with the selected drugs were studied in order to gain insights into the action mechanism, and the results of these tests were more promising than those found with glucantime, as the leishmanicidal effect of these new drug candidates was higher. The promising results are encouraging to test these derivatives in more complex studies, such as in vivo studies and other experiments that could find out the exact mechanism of action.

Introduction

Leishmaniasis is a widespread neglected tropical disease complex that is endemic in 98 countries and causes about 1 million of new cases and 20,000-30,000 deaths per year. It has three main clinical forms, which range from: a form that causes an ulcer or ulcers or disseminated lesions which leave lifelong scarring when healed (cutaneous leishmaniasis or CL); another form that causes facial deformations and destruction of connective tissue in the mouth, nose, and throat (mucocutaneous leishmaniasis or ML); and the most dangerous form, visceral leishmaniasis or VL, which causes enlargement of the spleen and liver, anemia, and irregular fever. This form is fatal if untreated in a 95% of the cases (1).

CL is found in the Americas, the Mediterranean basin, Africa, and central Asia, while ML is present only in South America (Brazil, Bolivia, and Peru). VL is located especially in India and East Africa, but recently cases have been reported in Brazil. In Madrid (Spain) 560 cases have been reported since 2009, 70% of these cases occurring in immunocompetent patients (2). These cases occur due to the recent overpopulation of non-depredated lagomorphs (*Oryctolagus cuniculus* and *Lepus granatensis*) in natural areas of Madrid and the ecological conditions that allow the vector species, the sandfly *Phlebotomus perniciosus*, to exist (3). The coexistence of a new wild reservoir and the aforementioned vector constitute a new life cycle for the parasite in Spain (4).

Paraguay was thought to be free of leishmaniasis, but 19 cases of VL were reported in 2018. This is quite remarkable because the clinical form that was thought to be found there was the mucocutaneous form. This finding, together with 87 cases diagnosed in the Madrid community, rank it as the location with most cases in Spain, forcing the

authorities to allow the hunting of wild animals as a control measure (5) to curb the expansion of the disease.

The parasite currently found in the Iberian Peninsula is *L. infantum*, which causes the most life-threatening clinical form, VL and also CL. An estimated 40% of the cases in Spain are VL (6).

Current treatments are based mainly on pentavalent antimonials such as sodium stibogluconate and meglumine antimoniate (7), which are outdated drugs that present many negative effects such as hepatotoxicity and nephrotoxicity. The lack of vaccines and effective treatments cause an urgent need to search for new drugs that can overcome the problems that current drugs have, such as aforementioned toxicity, long administration periods and overprice. For this reason, understanding the biochemistry of the parasite (such as enzymatic reactions or metabolic pathways and other inner processes) is critical in order to design new, effective drugs, because current drugs (such as pentavalent antimonials) date from the 1960s and the parasites have developed resistance.

One of the *Leishmania* targets is a kinetoplastid exclusive protein known as iron superoxide dismutase (Fe-SOD), which acts as a protector against the host oxidative burst defense. This enzyme differs from its vertebrate counterpart in the iron atom that is associated with it; vertebrates possess Cu/Zn-SOD, thus making Fe-SOD a perfect therapeutic target due to the parasite dependence on its protection in order to survive and divide (8, 9). Another possible target is the parasite glycolysis, which takes place in the mitochondria, as sometimes drugs can interfere with the metabolic pathway by altering enzymatic function or the mitochondrial membrane potential, ending in a lack of energy that could compromise parasite survival.

In recent years, we have studied the synthesis and antiprotozoal properties of many 5-nitroindazole derivatives. In connection with the current work, the antileishmanial activity of some 5-(indazol-1-yl)pentyl- and 3-oxapentylamines has been reported (10, 11). Additionally, we have recently described the antichagasic activity of some primary, secondary, and tertiary 2-(5-nitroindazol-1-yl)ethylamines (12).

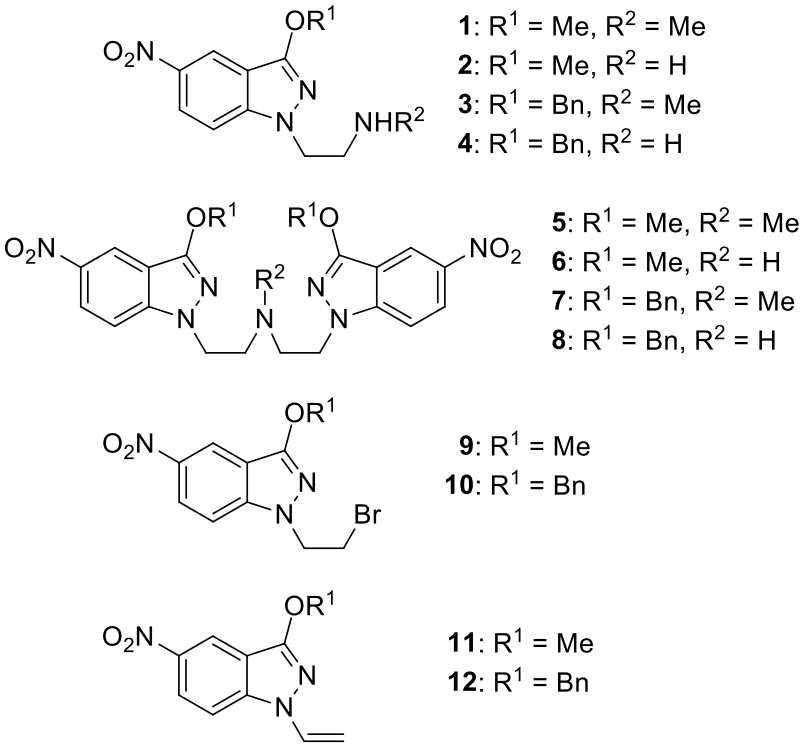


Figure 1: 5-Nitroindazole derivatives studied in the current work.

In the present work, we have studied the effects of the above-mentioned 2-(indazolyl)ethylamines (**1**–**8**), as well as some related reaction intermediates (**9**, **10**) and byproducts (**11**, **12**) (Figure 1), against both forms of three *Leishmania* species, each causing one of the aforementioned clinical forms (*L. infantum* for cutaneous leishmaniasis, *L. braziliensis* for mucocutaneous leishmaniasis, and *L. donovani* for visceral leishmaniasis), their metabolite alteration, their SOD activity, and their mitochondrial membrane potential.

Materials and methods

Chemistry

The amines **1**–**8** (Figure 1) were prepared from 3-methoxy- or 3-benzyloxy-5-nitroindazole according to previously reported procedures. Alkylation of the aforementioned 3-alkoxy derivatives with 1,2-dibromoethane afforded the corresponding 3-alkoxy-1-(2-bromoethyl) indazoles **9**, **10**, and 3-alkoxy-1-vinylindazoles **11**, **12** were obtained as byproducts in this process. Finally, the reaction of 2-bromoethyl derivatives **9** and **10** with ammonia or the required amines afforded the final compounds **1**–**8** (12).

RESULTS

Parasite strain and culture

Extracellular forms (promastigotes) of the three *Leishmania* species were harvested in Roux flasks (Corning, USA) with 75 cm^2 surface with Medium Trypanosomes Liquid (MTL) supplemented with 10% inactivated fetal calf serum and kept at 27 ºC in an air atmosphere. The strains used were MHOM/BR/1975/M2904 for *L. braziliensis*, MCAN/ES/2001/UCM-10 for *L. infantum* and LCR-L 133 LRC, Jerusalem, Israel for *L. donovani*, following a previously described methodology (13).

In vitro *activity assays*

The compounds tested were dissolved in dimethyl sulfoxide (DMSO, Panreac, Barcelona, Spain) and added to the cultures of mammalian cells or extracellular and intracellular forms of the parasite in order to give the required final concentrations as described (13,14); the final concentration of DMSO in cultures was 0.01% (v/v).

For the amastigote inhibitory effect, J774.2 macrophages were harvested in 24-well sterile microplates (Nunc®) with a round coverslip in the bottom of each well at a concentration of 10^4 cells per well. After the cells were fixed to the coverslip, they were infected with promastigotes (10 promastigotes per J774.2 macrophage) of each species studied. At the same time, the drugs were added in order to reach final concentrations of 12.5, 25, 50, and 100 µM. Promastigotes underwent conversion to amastigotes one day after infection. Those microplates were maintained for 2 days, and then coverslips were collected, fixed to a microscope slide, and stained with Giemsa for subsequent examination under an optical microscope.

Results are the median of three separated experiments.

Effects on infected J774.2 macrophages and infection rates

The compounds that showed a selectivity index (SI) 20 times or more higher than that of the reference drug and an IC_{50} value close to (or lower than) 10 µM were chosen for further studies because they were more selective towards parasite cells (15). The first of those studies concerned the effect of the drug on the parasite's ability to infect and divide inside a host cell. We cultured macrophage cells of the J774.2 cell line and then infected them with promastigotes during the stationary phase. One day later, promastigotes underwent morphological conversion to amastigotes, and 10 days after infection they reached their peak. We used IC_{25} as the test dosage following the method described in (16).

Superoxide dismutase (SOD) inhibition studies

RESULTS

As SOD is a key enzyme for fighting oxidative stress, many life forms rely on it to avoid damage caused by reactive oxygen species (ROS) generated by cell oxidative burst defense mechanism. Kinetoplastids evolved to have a version of this enzyme linked to an iron atom, thus making it different from the vertebrate counterpart that appears linked to a copper or zinc atom (Cu/Zn-SOD). Because of the parasite dependence on this molecule and its difference from the human host homologue, this enzyme constitutes a suitable therapeutic target. For these reasons, we studied the effect of the chosen drugs against the activity of both parasite and human erythrocytes SOD, which could partially explain their action mechanism.

We performed this experiment extracting Fe-SOD from the parasites as described (16); commercial Cu/Zn-SOD extracted from human erythrocytes was purchased from Sigma-Aldrich®. We measured the protein quantity by the Sigma Bradford test (17) and analyzed the SOD activity following the method described by Beyer and Fridovich (18).

Metabolite excretion

In order to determine whether the drugs have some effect on the main metabolic pathway (glycolysis) of the parasite, we measured the quantity of the most important metabolites by ^{1}H-NMR using the method reported by Fernandez-Becerra et al. (19).

Effects on the mitochondrial membrane potential

Another approach for understanding the possible action mechanism is to determine whether the leishmanicidal effect of the compounds can be caused by an alteration of the mitochondrial membrane potential. To confirm this, we treated cultures with IC_{25} of each drug, in a way similar to the approach followed for the metabolite excretion test, and were incubated for 72 h at 27 ºC. Afterwards, the cultures were centrifuged (800xg for 10 min) and the pellets were collected, stained with Rho 123 (Sigma-Aldrich®), and analyzed by flow cytometry. Changes in fluorescence intensity were calculated using the equation VI = (TM-CM)/CM, meaning: VI (variation index, TM (median fluorescence for treated parasites) and CM (median fluorescence for untreated parasites) (20).

Results and discussion

In vitro *activity assays*

First, the compounds cytotoxicity in macrophages belonging to the J774.2 cell line was determined. We found that most compounds were at least half as toxic as the reference drug glucantime, except compound **4**, which proved to be severely cytotoxic. With those results, we proceeded to test compounds with the promastigote forms, for

which the results were not as decisive as for those of the amastigote form, but served as a preliminary filter for the screening process. We gathered the IC_{50} data and used it to calculate the selectivity index, dividing the IC_{50} value for J774.2 macrophages by the value for promastigotes. The compounds that registered a good SI value according to Nwaka criteria (15) were tested again with the amastigote forms, and the same calculations were performed. Compounds **1**, **2**, **9**, **10**, **11**, and **12** showed much better activity than the reference drug; for instance, compound **2** was 134 times more active than glucantime against *L. infantum* and 77 times against *L. braziliensis* amastigotes. On the other hand, compound **10** was the most active against *L. donovani* amastigotes, being 200 times more active than glucantime.

For the above-mentioned reasons, we made further studies with compounds **1**, **2**, **9**, **10**, **11**, and **12**.

Compounds	Activity IC_{50} (µM)		J774.2 Macrophage toxicity IC_{50} (µM)	Selectivity index	
	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**
1	**13.2±5.4**	**16.8±0.8**	**289.7±32.7**	**22 (27)**	**17 (29)**
2	**3.5±0.01**	**6.1±0.1**	**489.2±66.8**	**140 (174)**	**80 (134)**
3	**4.3±0.0**	**nd**	**32.9±3.6**	**8 (10)**	**nd**
4	**17.7±4.2**	**nd**	**0.01±0.0**	**0 (0)**	**nd**
5	**9.9±1.5**	**nd**	**79.3±7.1**	**8 (10)**	**nd**
6	**9.9±2.6**	**nd**	**29.8±2.1**	**3 (4)**	**nd**
7	**36.2±3.4**	**nd**	**103.6±8.6**	**3 (4)**	**nd**
8	**2.5±0.6**	**nd**	**30.6±2.4**	**12 (15)**	**nd**
9	**11.5±1.1**	**21.8±1.0**	**234.5±18.7**	**20 (25)**	**11 (18)**
10	**13.1±2.2**	**14.4±0.6**	**587.8±89.6**	**45 (56)**	**41 (68)**
11	**9.3±0.1**	**43.7±1.5**	**667.8±74.5**	**72 (90)**	**15 (25)**
12	**1.7±0.3**	**18.0±1.6**	**306.8±18.9**	**180 (225)**	**17 (28)**

Table 1. *In vitro* activity, toxicity, and selectivity index for the 5-nitroindazole derivatives on extra- and intracellular forms of *L. infantum*. Numbers in brackets show the number of times drugs are more effective than the reference drug.

Compounds	Activity IC_{50} (µM)		J774.2 Macrophage toxicity IC_{50} (µM)	Selectivity index	
	Promastigote forms	Amastigote forms		Promastigote forms	Amastigote forms
Glucantime	**25.6±1.7**	**30.4±2.6**	**15.2± 1.0**	**0.6**	**0.6**
1	**10.6±2.0**	**27.9±1.2**	**289.7±32.7**	**27 (40)**	**10 (17)**
2	**8.1±0.8**	**10.6±0.6**	**489.2±66.8**	**60 (101)**	**46 (77)**
3	**7.4±1.6**	**nd**	**32.9±3.6**	**4 (7)**	**nd**
4	**1.2±0.2**	**nd**	**0.01±0.0**	**0 (0)**	**nd**
5	**51.3±3.6**	**nd**	**79.3±7.1**	**1 (3)**	**nd**
6	**27.6±3.5**	**nd**	**29.8±2.1**	**1 (2)**	**nd**
7	**22.6±1.9**	**nd**	**103.6±8.6**	**5 (8)**	**nd**
8	**6.8±0.7**	**nd**	**30.6±2.4**	**4 (8)**	**nd**
9	**5.5±0.6**	**11.7±1.3**	**234.5±18.7**	**43 (71)**	**20 (33)**
10	**11.4±1.1**	**25.5±2.0**	**587.8±89.6**	**52 (86)**	**23 (38)**
11	**14.1±3.7**	**19.8±1.4**	**667.8±74.5**	**47 (79)**	**34 (56)**
12	**23,2±2,8**	**30.7±2.3**	**306.7±18.9**	**13 (22)**	**10 (17)**

Table 2. *In vitro* activity, toxicity, and selectivity index for the 5-nitroindazole derivatives on extra- and intracellular forms of *L. braziliensis.* Numbers in brackets show the number of times drugs are more effective than the reference drug.

Compounds	Activity IC_{50} (μM)		J774.2 Macrophage toxicity IC_{50} (μM)	Selectivity index	
	Promastigote forms	Amastigote forms		Promastigote forms	Amastigote forms
Glucantime	**26.6±5.4**	**33.3±1.2**	**15.2± 1.0**	**0.7**	**0.6**
1	**6.6±2.5**	**18.3±1.1**	**289.7±32.7**	**44 (63)**	**16 (26)**
2	**7.1±0.9**	**14.6±0.8**	**489.2±66.8**	**69 (98)**	**33 (56)**
3	**5.7±0.7**	**nd**	**32.9±3.6**	**6 (8)**	**nd**
4	**11.4±2.5**	**nd**	**0.01±0.0**	**0 (0)**	**nd**
5	**26.9±3.3**	**nd**	**79.3±7.1**	**3 (4)**	**nd**
6	**3.6±0.2**	**nd**	**29.8±2.1**	**8 (12)**	**nd**
7	**21.6±1.7**	**nd**	**103.6±8.6**	**5 (7)**	**nd**
8	**27.2±1.8**	**nd**	**30.6±2.4**	**1 (2)**	**nd**
9	**14.1±2.3**	**55.2±2.5**	**234.5±18.7**	**17 (24)**	**4 (7)**
10	**1.6±0.0**	**4.9±0.6**	**587.8±89.6**	**367 (525)**	**120 (200)**
11	**37.7±3.0**	**36.5±2.6**	**667.8±74.5**	**18 (25)**	**18 (30)**
12	**21.6±1.6**	**27.9±1.7**	**306.7±18.9**	**14 (20)**	**11 (18)**

Table 3. *In vitro* activity, toxicity, and selectivity index for the 5-nitroindazole derivatives on extra- and intracellular forms of *L. donovani.* Numbers in brackets show the number of times drugs are more effective than the reference drug.

For the currently studied compounds we have not been able to find a clear relationship between structure and *in vitro* activity. It is clear that bis(indazolylethyl)amines (**5**–**8**) are not good antileishmanial agents owing the their low SI. On the other hand, the structures of the most efficient compounds are heterogeneous, including a primary amine (**2**), a secondary amine (**1**), two 1-(2-bromoethyl)indazoles (**9**, **10**) and two 1-vinyl derivatives (**11**, **12**).

For simple 2-(indazolylethyl)amines, 3-benzyloxy derivatives (**3**, **4**) were much more toxic for macrophages than the corresponding 3-methoxy derivatives (**1**, **2**), leading to the very low values of SI found for the former. However, for 2-bromoethyl (**9**, **10**) and vinyl derivatives (**11**, **12**), the effect of the substituent at position 3 appears to be low.

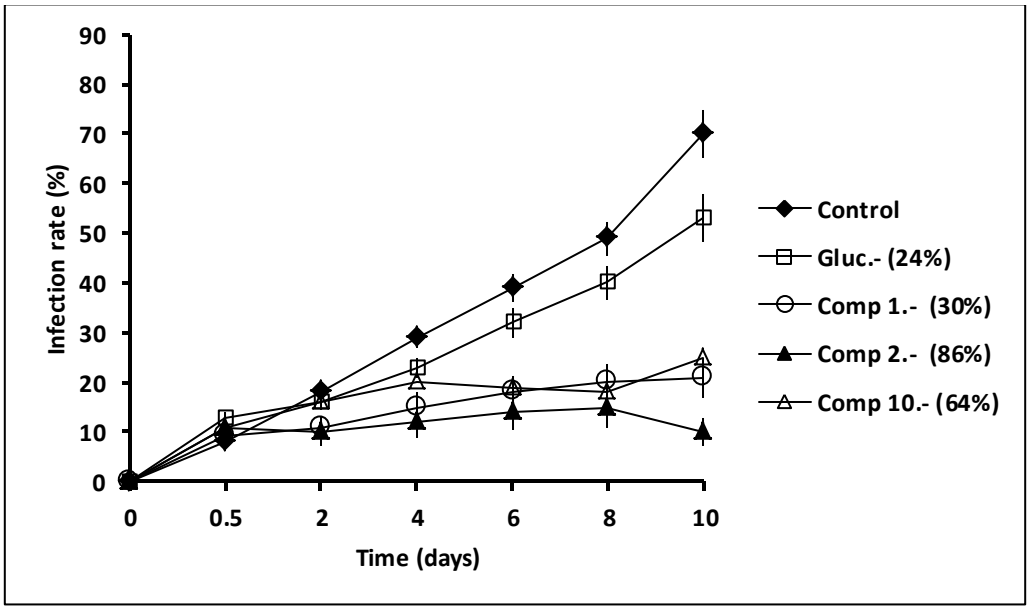
The activity of 5-(indazol-1-y)-3-oxapentylamines and related 3-alkoxy-1-alkylindazoles against *L. infantum* and *L. braziliensis* has been previously studied (11). In general,

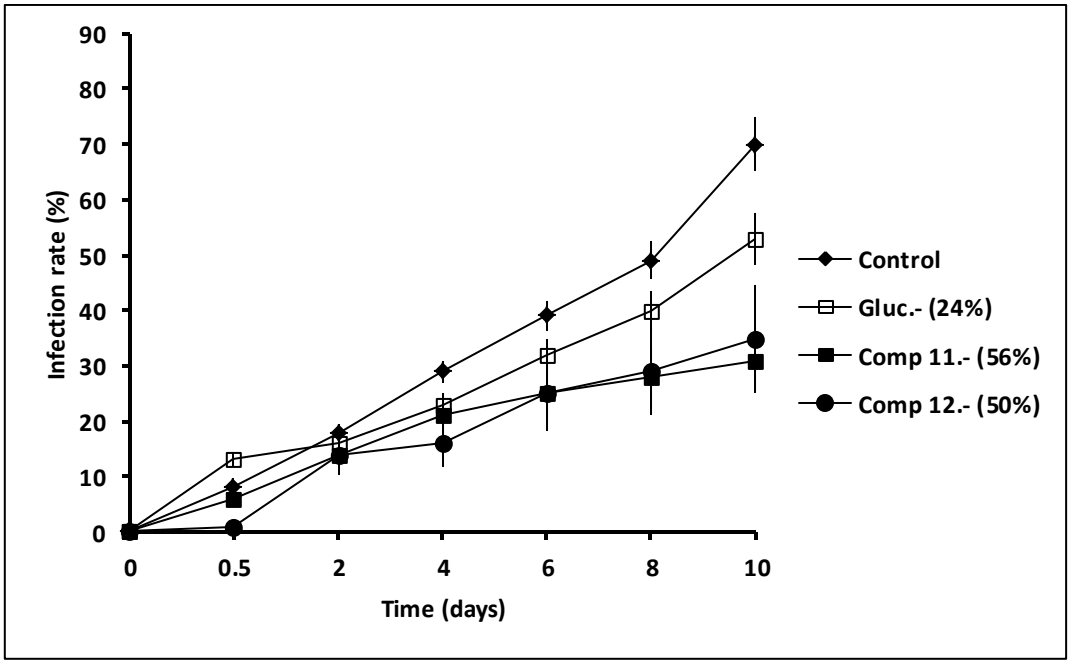
activities of these compounds against promastigotes and amastigotes (IC_{50} = 9–56 μM) are similar to those found for the current products; nevertheless, these initially reported indazoles are quite toxic for macrophages and, consequently, their SI are much lower (SI < 22). On the other hand, activities against *Leishmania* spp. of some 3-alkoxy-1-[5-(dialkylamino)pentyl and 3-oxapentyl]indazoles have been published (10); however, IC_{50} and SI values were not determined in this article, so the provided activity data cannot be compared directly with those obtained in the present work.

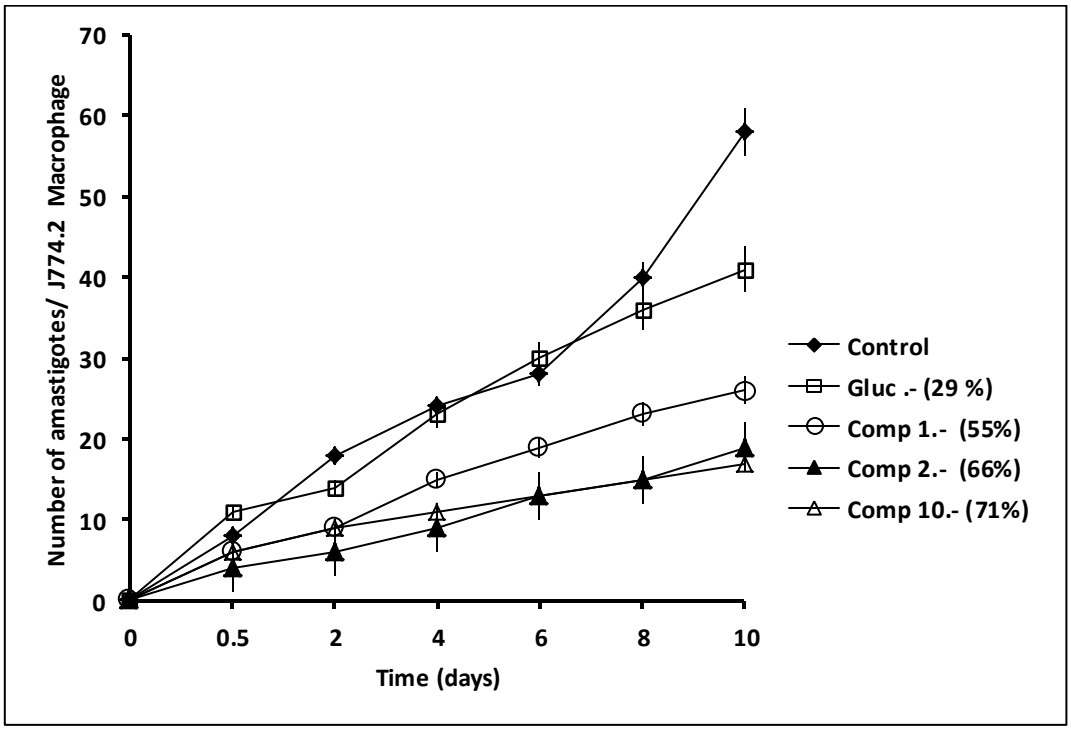
In vitro *infectivity*

We tested the drug ability to reduce parasite infection and division. We display four charts for each parasite, two showing the infection rate through the days the experiment lasted, and the others showing the number of amastigotes per infected cell through 10 days. Overall, a notable reduction in those parameters was found for each product in every parasite. This reduction was higher than the one caused by the reference drug.

Compound **2** was the most effective at reducing the infection rate in all the species studied. In *L. infantum,* the infection rate was lowered by 86%, by 73% in *L. braziliensis*, and by 49% in the case of *L. donovani*. With respect with the number of amastigotes per infected cell, the results were more varied. That is, compound **10** reached a reduction of 71% in *L. infantum*, compound **9** caused a 72% reduction in *L. braziliensis*, and compound **11** reduced the number of amastigotes 64% in *L. donovani*.




Infection rate (%)
90
80
70
60
50
40
30
20
10
0
0
0.5
2
4
6
8
10
Time (days)
Control
Gluc.- (24%)
Comp 11.- (56%)
Comp 12.- (50%)


Number of amastigotes/ J774.2 Macrophage
70
60
50
40
30
20
10
0
0
0.5
2
4
6
8
10
Time (days)
Control
Gluc .- (29 %)
Comp 1.- (55%)
Comp 2.- (66%)
Comp 10.- (71%)

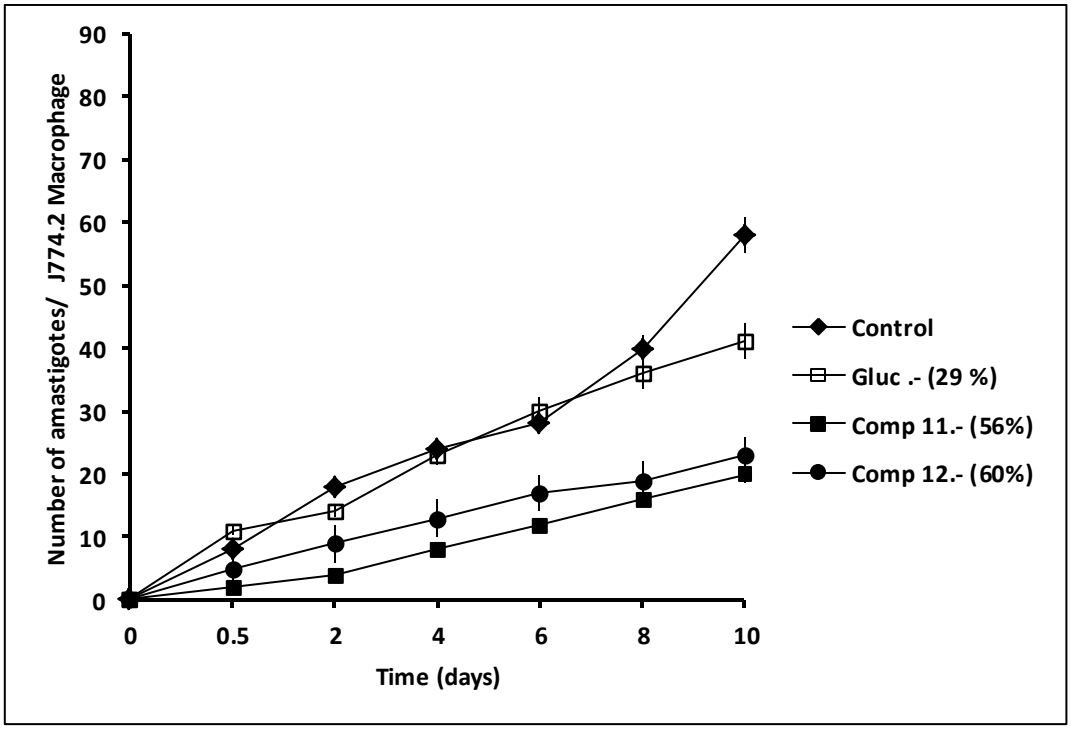
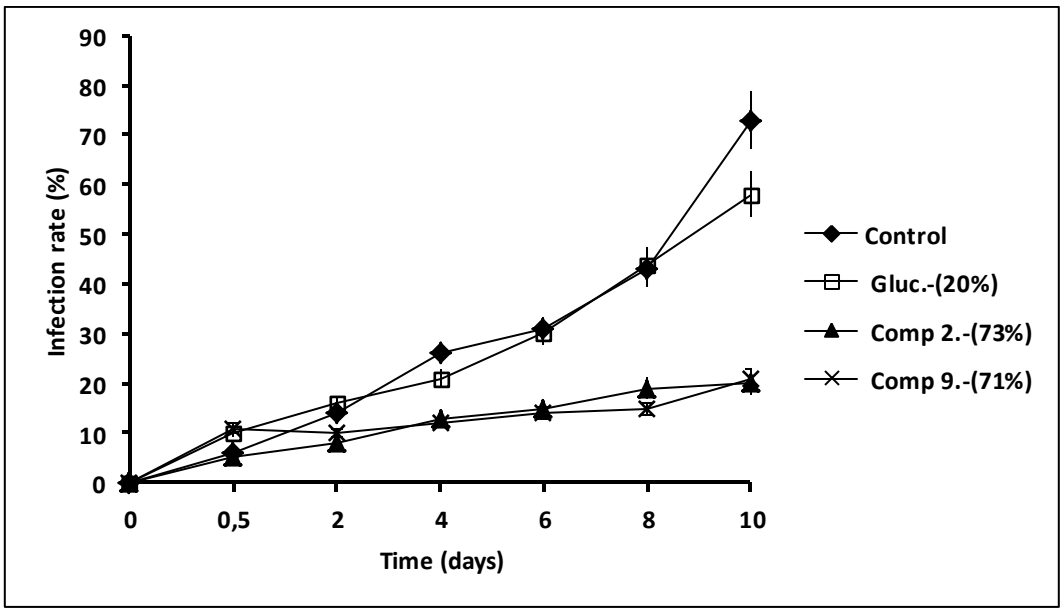
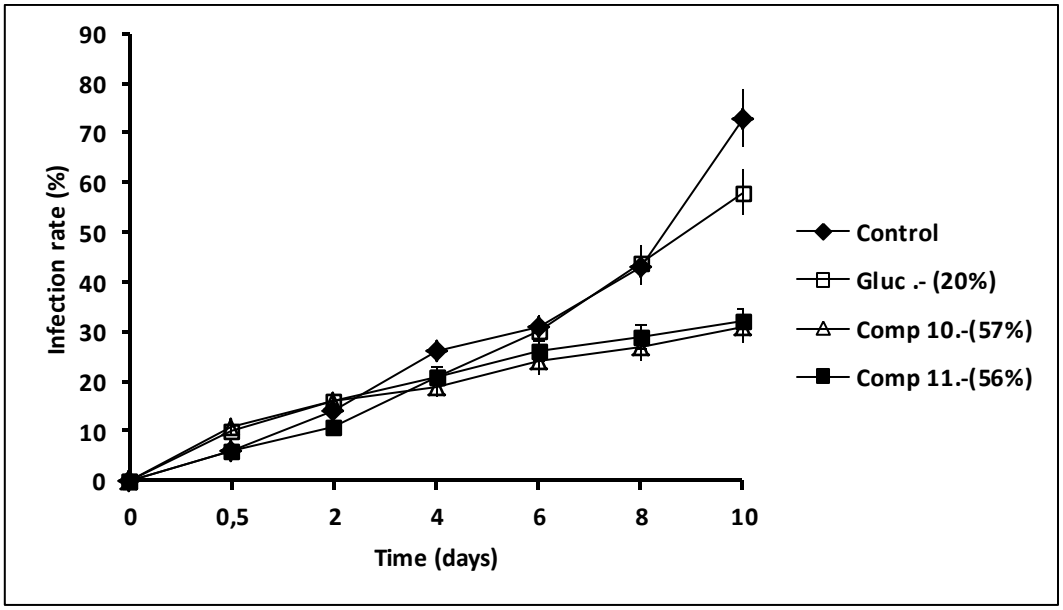
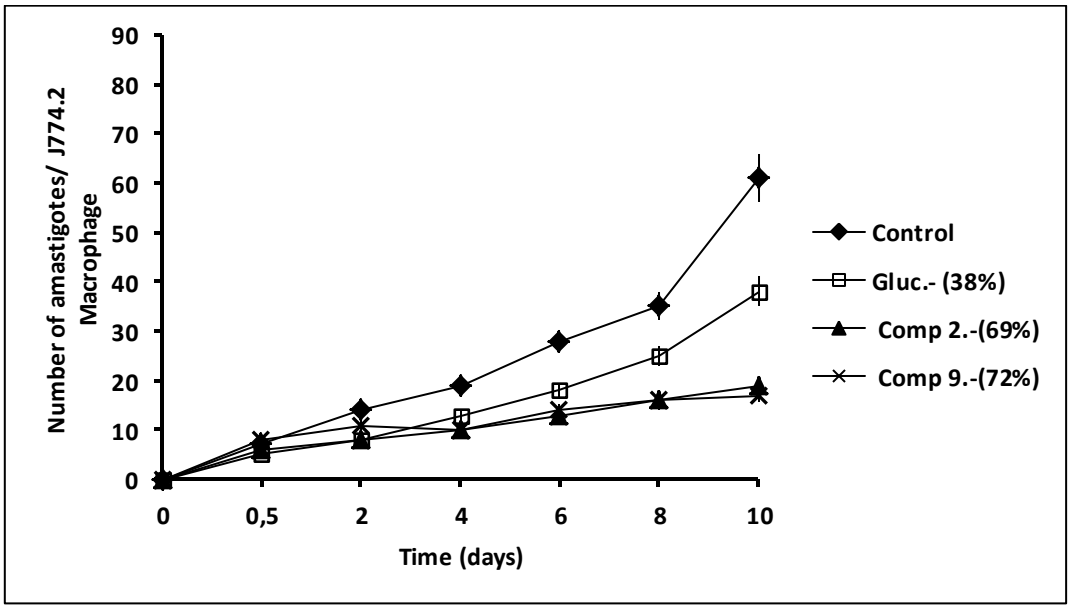


Figure 2. Infection rates and number of amastigotes per infected cell for *L. infantum*. Effect of 3-alkoxy-5-nitroindazole-derived ethylamine derivatives **1**, **2**, **10**, **11**, and **12** on the infection and growth rates and mean numbers of amastigotes per infected J774.2 macrophage cell (at IC_{25} concentration) of *L. infantum*. The inhibition percentage is indicated in parenthesis. Values are the means of three separate experiments. All compounds are statistically significant against glucantime at a P value < 0.05.




Infection rate (%)
90
80
70
60
50
40
30
20
10
0
0
0,5
2
4
6
8
10
Time (days)
Control
Gluc .- (20%)
Comp 10.-(57%)
Comp 11.-(56%)


Number of amastigotes/ J774.2 Macrophage
90
80
70
60
50
40
30
20
10
0
0
0,5
2
4
6
8
10
Time (days)
Control
Gluc.- (38%)
Comp 2.-(69%)
Comp 9.-(72%)

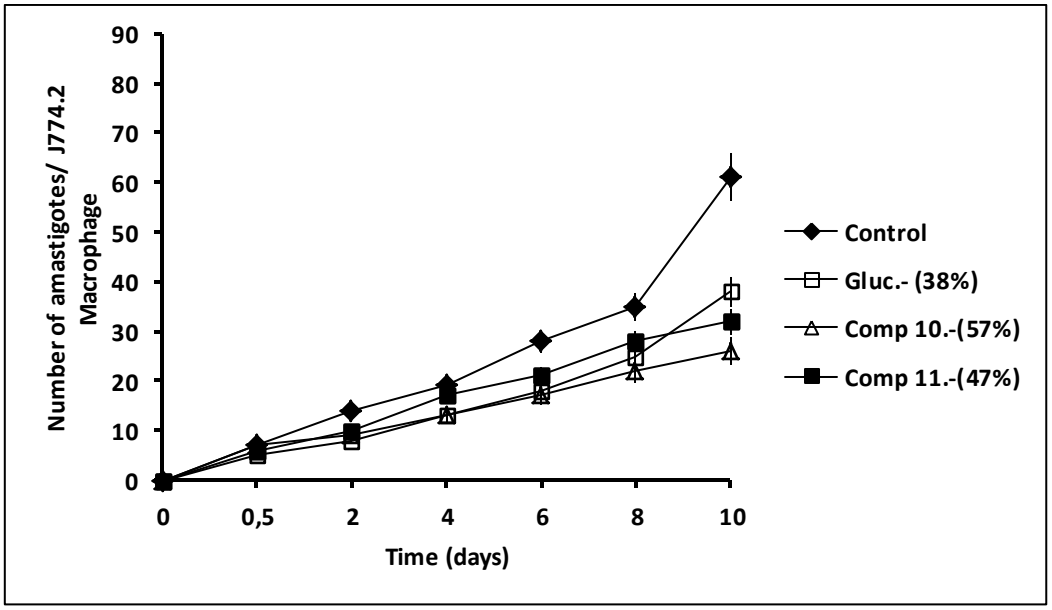
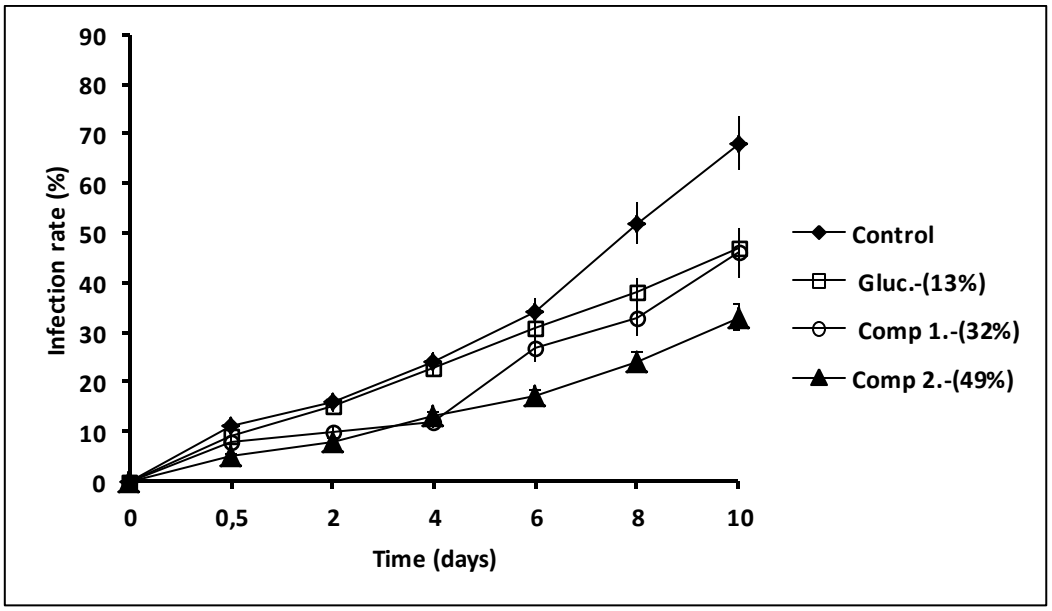
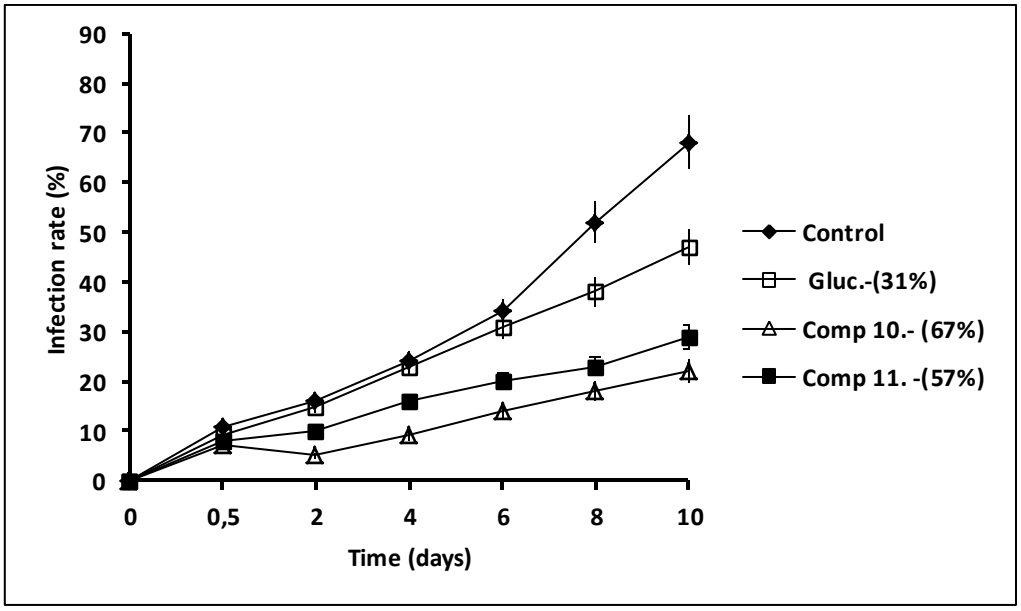
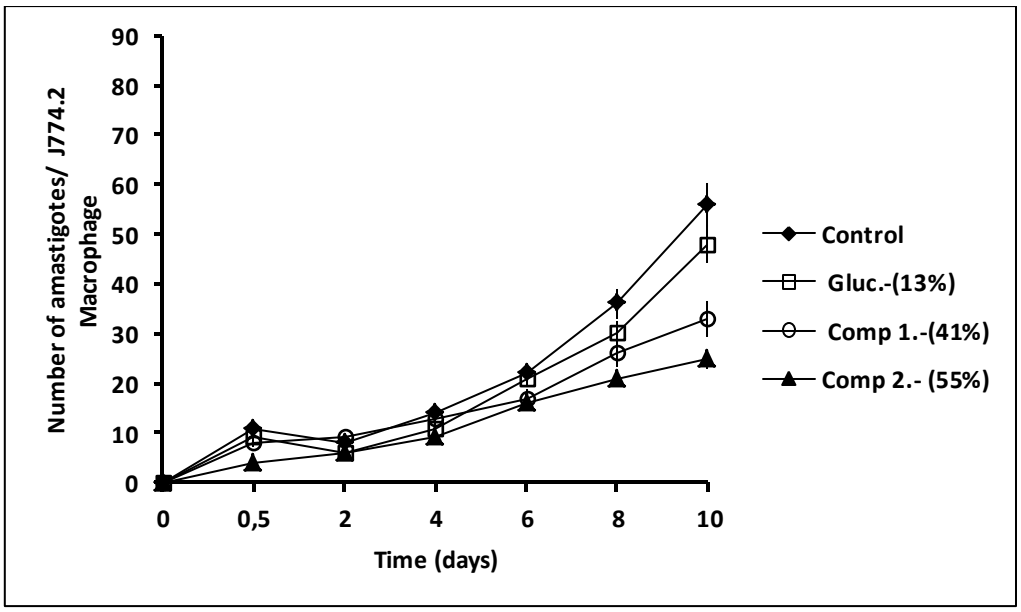


Figure 3. Effect of 3-alkoxy-5-nitroindazole-derived ethylamines derivatives **2**, **9**, **10**, and **11** on the infection and growth rates and mean numbers of amastigotes per infected J774.2 macrophage cell (at IC_{25} concentration) of *L. braziliensis*. Inhibition percentage is indicated in parenthesis. Values are the means of three separate experiments. All compounds are statistically significant against glucantime at a P value < 0.05.




Infection rate (%)
90
80
70
60
50
40
30
20
10
0
0
0,5
2
4
6
8
10
Time (days)
Control
Gluc.-(31%)
Comp 10.- (67%)
Comp 11. -(57%)


Number of amastigotes/ J774.2 Macrophage
90
80
70
60
50
40
30
20
10
0
0
0,5
2
4
6
8
10
Time (days)
Control
Gluc.-(13%)
Comp 1.-(41%)
Comp 2.- (55%)

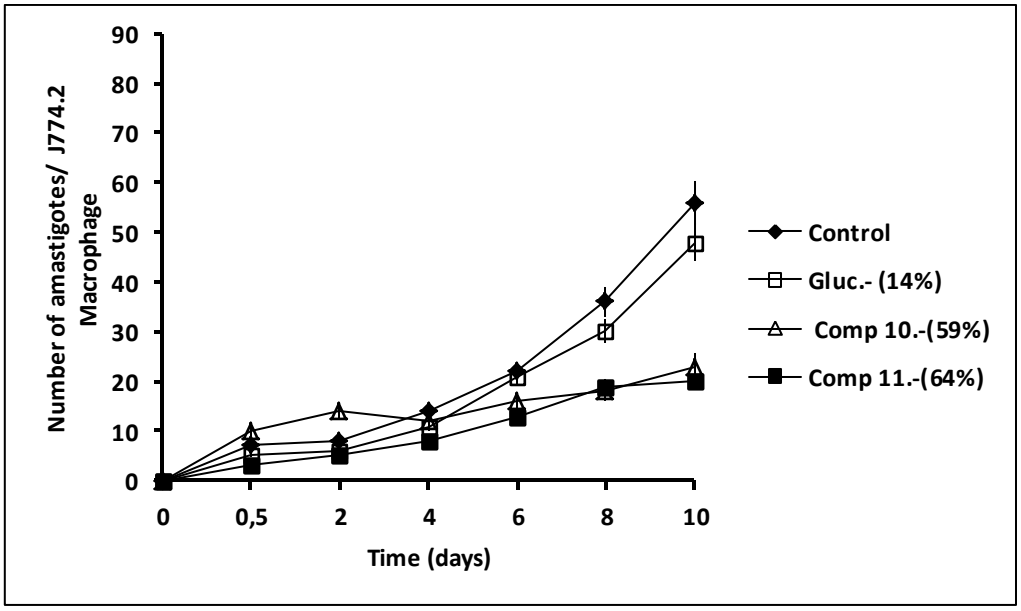


Figure 4. Effect of 3-alkoxy-5-nitroindazole-derived ethylamines derivatives **1**, **2**, **10**, and **11** on the infection and growth rates and mean numbers of amastigotes per infected J774.2 macrophage cell (at IC_{25} concentration) of *L. donovani.* The inhibition percentage is indicated in parenthesis. Values are the means of three separate experiments. All compounds are statistically significant against glucantime at a P value < 0.05.

The reductions in the rate of infection and the number of amastigotes per macrophage observed for the current compounds are similar or slightly lower than those observed for some 5-(indazol-1-y)-3-oxapentylamines previously studied against *L. infantum* and *L. braziliensis* (56–81% and 65–85%, respectively) (11).

Mechanism of action-SOD inhibition studies

Because Fe-SOD is critical for parasite survival, its activity inhibition caused by the compounds studied was assessed. Previous works have demonstrated that a vast majority of the drugs tested were effective against parasites by inhibiting Fe-SOD activity, while their inhibition of human Cu/Zn-SOD was not significant in comparison. (16, 21, 22) This was not the case in the present study. The experiment was carried out as usual and the drugs chosen proved to have a quite marginal effect on Fe-SOD, which in this case does not serve to explain the action mechanism. The compounds tested had no effect on *L. infantum* or *L. braziliensis* Fe-SOD. In the case of *L. donovani*, compound **2** had a slight inhibition (59%) as did compound **9** (69%) but these inhibitions occurred only when the parasites were treated with 100 μM of each

drug. Compound **10** was the most effective, as it completely inhibited the enzymatic activity when 50 μM concentration was reached; at lower concentrations, inhibition reached 25.4% with 12.5 μM and 77.8% with 25 μM.

For related antichagasic 5-nitroindazole derivatives, different alternative mechanisms, similar to those suggested for other antiprotozoal nitroheterocycles, have been proposed. All these possible mechanisms, discussed in a recent article (23), start with the intracellular reduction of the nitro group, followed by the generation of reactive oxygen species (ROS) and induction of oxidative stress in parasites, or by the production of highly reactive metabolites capable of reacting with essential biomolecules of parasites. In our case, the obtained results on the mode of action of these compounds are somewhat contradictory and we do not have conclusive results. In fact, a study of electrochemical and enzymatic reduction of antichagasic 5-nitroindazolinones suggested that these compounds could induce oxidative stress in the parasites, i.e., a mode of action similar to that initially accepted for nifurtimox (24); however, an additional study using 1,2-disubstituted indazolinones has shown that their action on *T. cruzi* trypomastigotes is due to damage induced at the mitochondrial level, and that oxidative stress is not involved (25).

On the other hand, interference with glycosomal or mitochondrial enzymes involved in the catabolism of *T. cruzi* (24) as well as the inhibition of trypanothione reductase has also been proposed for some antichagasic 5-nitroindazoles (26, 27, 28).

Metabolite production and excretion

Another approach used to gain insight into the mechanism of action of our drugs was to assess their effect on the parasite metabolism. Trypanosomatids are unable to oxidize glucose to carbon dioxide and water as vertebrates do; instead, they excrete part of the carbon skeleton in the form of different organic acids (29). There is an enzyme responsible of the production of each excreted compound of the metabolic pathway, so we can infer which enzyme has its activity altered because of the drug just by determining which metabolite registers higher or lower production than control.

In the case of *L. infantum*, metabolite production was reduced with respect to the control for every tested drug, except an exiguous increase in D-lactate production for compound **10**. The most pronounced alteration was succinate production when *L. infantum* was treated with compound **1**, which caused an increase of 243.1%. When the *L. braziliensis* metabolite alteration was assessed, we found that all metabolite

production was increased, especially pyruvate. For *L. donovani*, the same phenomenon again occurred, but the increase was much less notorious than in the previous case. An exception to this was that compound **1** reduced metabolite production instead of augmenting it, and the reduction was slight with respect to the control. Another minor reduction occurred in succinate production for compound **10**. Apart from this, the most remarkable metabolite alteration was found in *L. braziliensis*, which underwent an extraordinary surge in succinate production triggered by action of compound **11**, values being almost 600% higher than control.

Compound	Succinate	Pyruvate	Acetate	L-Alanine	D-Lactate
1	243,1	-32,4	-22,5	-18,2	-23,8
2	-15,3	-5,4	-12,2	-12	-11,3
10	-24,7	-8,1	-3,4	-0,3	0,6
11	-31,6	-24	-24,4	-21,3	-17,6
12	-20	-12,7	-13,1	-12,7	-14

Table 4. Metabolite-excretion chart of *L. infantum* treated with an IC_{25} dosage of the drugs. Results are expressed as percentages of augmentation or reduction on production and excretion of each metabolite with respect to the control.

Compound	Succinate	Pyruvate	Acetate	L-Alanine	D-Lactate
1	19,5	31,4	20,7	28,2	2,9
2	24,1	38,7	27,1	34,5	25,7
9	13	34,7	18	31	1,5
10	51,3	79	60,8	67,2	57,5
11	47,7	74,3	60,2	67,3	56,5
12	14	46,6	36,9	33,6	5,5

Table 5. Metabolite-excretion chart of *L. braziliensis* treated with an IC_{25} dosage of the drugs. Results are expressed as percentages of augmentation or reduction on production and excretion of each metabolite with respect to the control.

Compound	Succinate	Pyruvate	Acetate	L-Alanine	D-Lactate
1	-6,2	-0,4	-1,3	-0,7	-3,1
2	13,4	37,7	24,9	46,9	10,1
9	4,8	2,9	0,6	4,1	-0,8
10	-3,8	9,1	4,5	5,9	0,9
11	599,8	40,1	51,2	47,1	12,6
12	51,6	31,5	30,9	29,9	-4,4

Table 6. Metabolite-excretion chart of *L. donovani* treated with an IC_{25} dosage of the drugs. Results are expressed as percentages of augmentation or reduction on production and excretion of each metabolite with respect to the control.

Mitochondrial membrane potential alterations

The mitochondrial membrane potential of promastigotes treated with an IC_{25} dosage of the compounds was assessed, with the expectation that their antileishmania activity might be explained by altering the potential and thus triggering programmed cell death. However, only one alteration was detected (and a remarkable one) in the case of *L. donovani* when treated with compound **11**. For the other drugs and parasites, no difference was found between control and different treatments. The membrane depolarization can be seen as a shift to the left for the treatment curve with respect to the control curve.

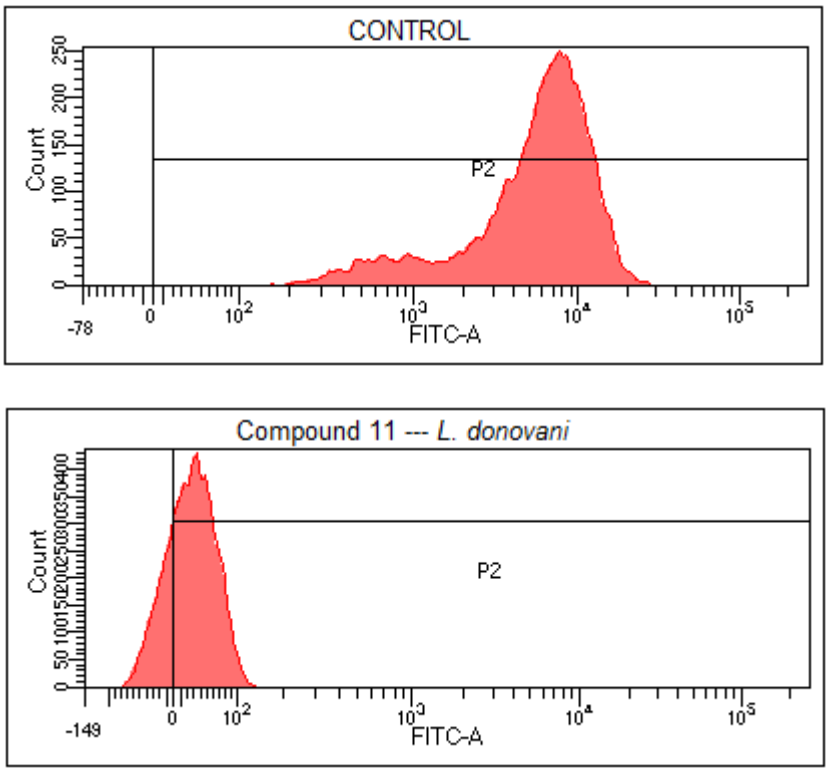


Figure 5. Mitochondrial-membrane potential graphs for control and for compound **11** against *L. donovani. FITC-A stands for Rho 123.*

This phenomenon is related with the previous metabolite excretion test, because, as stated previously, the most notable effect in *L. donovani* metabolite excretion was the extraordinary increase in succinate excretion when treated also with compound **11**. According to Barisón et al. (30) in their study of a metabolic switch of induced starvation in *T. cruzi* epimastigotes, another metabolic pathway occurs in the glycosomes and not in the mitochondria, for which the final product is succinate.

Bearing in mind how close *T. cruzi* and *Leishmania* are phylogenetically, we suggest that they could react in a similar way when these conditions appear.

Considering that mitochondrial membrane potential is nullified by the action of compound **11**, as the graph suggests, we might infer that the mitochondria are not working, which could force the cell to rely in this alternative, less efficient route to gain energy. This could explain the massive production and consequent excretion of this metabolite, making the results of NMR and Rhodamine assay support each other in the case of compound **11** against *L. donovani*. This might lead us to deduce that the promastigotes die of energetic collapse, as the mitochondria and all the metabolic events that occur within it cease to work.

Conclusions and future outlooks

As seen in previous studies, the activity of the 2-(5-nitroindazol-1-yl)ethylamines and related compounds was assessed in *T. cruzi* (12), where only two compounds showed noteworthy *in vitro* activity. In the present study, those drugs were found to be more effective against *Leishmania* species, as 6 compounds were active against at least one species (**1**, **9**, **12**), and three were active against all three of the species studied (**2**, **10**, **11**). Those 6 selected compounds proved to be considerably less toxic than the reference drug and were also effective in lowering the parasite's ability to infect and divide inside the host cell.

As the Fe-SOD is key for parasite survival inside the host cell, our group uses this enzyme to test the drug's ability inhibit this enzymatic activity, which is one of the most usual ways to explain why the drugs are effective (16, 21, 22). However, in this present study the drugs which showed leishmanicidal activity were not effective at inhibiting Fe-SOD activity, indicating that their action mechanism was something other than inhibition of Fe-SOD activity. Alternative possible mechanisms, similar to those proposed for related antichagasic 5-nitroindazoles, based on the intracellular reduction of the nitro group, are discussed.

Due to their *in vitro* leishmanicidal activity, we continued performing experiments with the 6 drugs chosen in order to advance our understanding of their action mechanism, testing their metabolite alteration via ^{1}H-NMR. This enabled us to detect different alterations in the parasite metabolic pathways, such as a general reduction of metabolite excretion in *L. infantum* (except succinate for compound **1**), an enhancement in metabolite excretion in *L. braziliensis*, and an slight increase for the drugs tested in *L. donovani*, except product **1**, which caused an exiguous reduction and

the extraordinary surge in succinate production triggered by compound **11**. This finding led us to deduce that the mitochondria were completely inhibited, and therefore we performed the rhodamine assay, which corroborated our suspicions for that case, as it turned out to be the only drug that caused a mitochondrial-membrane depolarization, causing cell death from energetic collapse (30).

The results confirm the value of 5-nitroindazole derivatives in the field of antileishmanial agents. In the current case, however, the structures of the most active compounds are rather heterogeneous, including a primary amine (**2**), a secondary amine (**1**), two 1-(2-bromoethyl)indazoles (**9** and **10**) and two 1-vinyl derivatives (**11** and **12**). Consequently, we cannot establish of a clear structure-activity relationship.

For the immediate future we have planned the identification of the products arising from the bioreduction of our 5-nitroindazoles, as well as the synthesis of fluorescent derivatives allowing the labelling of the possible organelles that constitute their primary target. We also believe in the convenience of carrying out *in vivo* studies with animal models of leishmaniasis in order to deepen the knowledge of these products and their therapeutic potential.

Acknowledgements

Authors appreciate the financial support from the Spanish Ministry of Science, Innovation and Universities (projects SAF2015-66690-R and RTI2018-093940-B-I00) and Spanish Ministry of Education and Science (project CSD 2010-00065).

R.M.E. is grateful for a FPU grant [FPU14/01537] from the Ministry of Education of Spain and B.A.V. is grateful for an initiation into research grant from FONDECYT 11150559.

References

[1]
Leishmaniasis (n.d.). https://www.who.int/news-room/fact-sheets/detail/leishmaniasis

[2]
M. Aguado, P. Espinosa, A. Romero-Maté, J.C. Tardío, S. Córdoba, J. Borbujo, Outbreak of cutaneous leishmaniasis in Fuenlabrada, Madrid, Actas Dermosifiliogr. 104 (2013) 334–342. doi:10.1016/j.adengl.2013.03.005.

[3]
N. García, I. Moreno, J. Alvarez, M.L. de la Cruz, A. Navarro, M. Pérez-Sancho, T. García-Seco, A. Rodríguez-Bertos, M.L. Conty, A. Toraño, A. Prieto, L. Domínguez, M. Domínguez, Evidence of Leishmania infantum infection in rabbits (Oryctolagus cuniculus) in a natural area in Madrid, Spain, Biomed. Res. Int. 2014 (2014) 318254. doi:10.1155/2014/318254.

[4]
D.M. Claborn, The biology and control of leishmaniasis vectors, J. Glob. Infect. Dis. 2 (2010) 127–134. doi:10.4103/0974-777X.62866.

[5]
M. Noguerol Álvarez, J.V. San Martín López, M. Aguado Lobo, P. Aparicio Azcárraga, Brote comunitario de leishmaniasis en la zona sur de la comunidad de Madrid, Aten. Primaria 44 (2012) 508–509. doi:10.1016/j.aprim.2012.04.003.

[6]
Reporte Epidemiológico: Córdoba, Argentina, nº 2141, 23 enero 2019
http://www.reporteepidemiologico.com/wp-content/uploads/2019/01/REC-2141.pdf

[7]
S.R.B. Uliana, C.T. Trinconi, A.C. Coelho, Chemotherapy of leishmaniasis: present challenges, Parasitology 145 (2018) 464–480. doi:10.1017/S0031182016002523.

[8]
E.S. Coimbra, L.M.R. Antinarelli, N.P. Silva, I.O. Souza, R.S. Meinel, M.N. Rocha, R.P.P. Soares, A.D. da Silva, Quinoline derivatives: Synthesis, leishmanicidal activity and involvement of mitochondrial oxidative stress as mechanism of action, Chem. Biol. Interact. 260 (2016) 50–57. doi:10.1016/j.cbi.2016.10.017.

[9]
K. Singh, G. Garg, V. Ali, Current therapeutics, their problems and thiol metabolism as potential drug targets in leishmaniasis, Curr. Drug Metab. 17 (2016) 897–919.

[10]
L. Boiani, A. Gerpe, V.J. Arán, S. Torres de Ortiz, E. Serna, N. Vera de Bilbao, L. Sanabria, G. Yaluff, H. Nakayama, A. Rojas de Arias, J.D. Maya, J.A. Morello, H.

Cerecetto, M. González, In vitro and in vivo antitrypanosomatid activity of 5-nitroindazoles, Eur. J. Med. Chem. 44 (2009) 1034–1040. doi:10.1016/j.ejmech.2008.06.024.

[11]
C. Marín, I. Ramírez-Macías, M.J. Rosales, B. Muro, F. Reviriego, P. Navarro, V.J. Arán, M. Sánchez-Moreno, In vitro leishmanicidal activity of 1,3-disubstituted 5-nitroindazoles, Acta Trop. 148 (2015) 170–178. doi:10.1016/j.actatropica.2015.04.028.

[12]
R. Martín-Escolano, B. Aguilera-Venegas, C. Marín, Á. Martín-Montes, J. Martín-Escolano, E. Medina-Carmona, V.J. Arán, M. Sánchez-Moreno, Synthesis and biological in vitro and in vivo evaluation of 2-(5-nitroindazol-1-yl)ethylamines and related compounds as potential therapeutic alternatives for Chagas disease, ChemMedChem 13 (2018) 2104–2118. doi:10.1002/cmdc.201800512.

[13]
P. González, C. Marín, I. Rodríguez-González, A.B. Hitos, M.J. Rosales, M. Reina, J.G. Díaz, A. González-Coloma, M. Sánchez-Moreno, In vitro activity of C20-diterpenoid alkaloid derivatives in promastigotes and intracellular amastigotes of Leishmania infantum, Int. J. Antimicrob. Agents 25 (2005) 136–141. doi:10.1016/j.ijantimicag.2004.08.010.

[14]
I.G. Kirkinezos, C.T. Moraes, Reactive oxygen species and mitochondrial diseases, Semin. Cell Dev. Biol. 12 (2001) 449–457. doi:10.1006/scdb.2001.0282.

[15]
S. Nwaka, D. Besson, B. Ramirez, L. Maes, A. Matheeussen, Q. Bickle, N.R. Mansour, F. Yousif, S. Townson, S. Gokool, F. Cho-Ngwa, M. Samje, S. Misra-Bhattacharya, P.K. Murthy, F. Fakorede, J.-M. Paris, C. Yeates, R. Ridley, W.C. Van Voorhis, T. Geary, Integrated dataset of screening hits against multiple neglected disease pathogens, PLoS Negl. Trop. Dis. 5 (2011) e1412. doi:10.1371/journal.pntd.0001412.

[16]
A. Martin-Montes, M. Santivañez-Veliz, E. Moreno-Viguri, R. Martín-Escolano, C. Jiménez-Montes, C. Lopez-Gonzalez, C. Marín, C. Sanmartín, R. Gutiérrez Sánchez,

M. Sánchez-Moreno, S. Pérez-Silanes, In vitro antileishmanial activity and iron superoxide dismutase inhibition of arylamine Mannich base derivatives, Parasitology 144 (2017) 1783–1790. doi:10.1017/S0031182017001123.

[17]
M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[18]
W.F. Beyer, I. Fridovich, Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions, Anal. Biochem. 161 (1987) 559–566.

[19]
C. Fernández-Becerra, M. Sánchez-Moreno, A. Osuna, F.R. Opperdoes, Comparative aspects of energy metabolism in plant trypanosomatids, J. Eukaryot. Microbiol. 44 (1997) 523–529. doi:10.1111/j.1550-7408.1997.tb05734.x.

[20]
J.M. Sandes, A. Fontes, C.G. Regis-da-Silva, M.C.A.B. de Castro, C.G. Lima-Junior, F.P.L. Silva, M.L.A.A. Vasconcellos, R.C.B.Q. Figueiredo, Trypanosoma cruzi cell death induced by the Morita-Baylis-Hillman adduct 3-hydroxy-2-methylene-3-(4-nitrophenylpropanenitrile), PLoS ONE 9 (2014) e93936. doi:10.1371/journal.pone.0093936.

[21]
Á. Martín-Montes, R. Ballesteros-Garrido, R. Martín-Escolano, C. Marín, R. Guitiérrez-Sánchez, B. Abarca, R. Ballesteros, M. Sanchez-Moreno, Synthesis and in vitro leishmanicidal activity of novel [1,2,3]triazolo[1,5- a]pyridine salts, RSC Advances. 7 (2017) 15715–15726. doi:10.1039/C7RA01070B.

[22]
Á. Martín-Montes, D. Plano, R. Martín-Escolano, V. Alcolea, M. Díaz, S. Pérez-Silanes, S. Espuelas, E. Moreno, C. Marín, R. Gutiérrez-Sánchez, C. Sanmartín, M. Sánchez-Moreno, Library of seleno-compounds as novel agents against Leishmania species, Antimicrob. Agents Chemother. 61 (2017). doi:10.1128/AAC.02546-16.

[23]
C. Fonseca-Berzal, A. Ibáñez-Escribano, F. Reviriego, J. Cumella, P. Morales, N. Jagerovic, J.J. Nogal-Ruiz, J.A. Escario, P.B. da Silva, M.N.C. Soeiro, A. Gómez-Barrio, V.J. Arán, Antichagasic and trichomonacidal activity of 1-substituted 2-benzyl-5-nitroindazolin-3-ones and 3-alkoxy-2-benzyl-5-nitro-2H-indazoles, Eur. J. Med. Chem. 115 (2016), 295–310. doi: 10.1016/j.ejmech.2016.03.036.

[24]
C. Folch-Cano, C. Olea-Azar, V.J. Arán, C. Diaz-Urrutia, ESR and electrochemical study of 1,2-disubstituted 5-nitroindazolin-3-ones and 2-substituted 3-alkoxy-5-nitro-2H-indazoles: reactivity and free radical production capacity in the presence of biological systems, Spectroc. Acta Pt. A Molec. Biomolec. Spectr. 75 (2010) 375–380. doi: 10.1016/j.saa.2009.10.044.

[25]
C. Fonseca-Berzal, C.F. da Silva, R.F.S. Menna-Barreto, M.M. Batista, J.A. Escario, V.J. Arán, A. Gómez-Barrio, M.N.C. Soeiro, Biological approaches to characterize the mode of action of two 5-nitroindazolinone prototypes on Trypanosoma cruzi bloodstream trypomastigotes, Parasitology 143 (2016), 1469–1478. doi: 10.1017/S0031182016001098.

[26]
B. Muro, F. Reviriego, P. Navarro, C. Marín, I. Ramírez-Macías, M.J. Rosales, M. Sánchez-Moreno, V.J. Arán, New perspectives on the synthesis and antichagasic activity of 3-alkoxy-1-alkyl-5-nitroindazoles, Eur. J. Med. Chem. 74 (2014) 124-134. doi: 10.1016/j.ejmech.2013.12.025.

[27]
C.A. Díaz-Urrutia, C.A. Olea-Azar, G.A. Zapata, M. Lapier, F. Mura, B. Aguilera-Venegas, V.J. Arán, R.A. López-Muñoz, J.D. Maya, Biological and chemical study of fused tri- and tetracyclic indazoles and analogues with important antiparasitic activity, Spectroc. Acta Pt. A Molec. Biomolec. Spectr. 95 (2012) 670–678. doi: 10.1016/j.saa.2012.04.076.

[28]
B. Aguilera-Venegas, C. Olea-Azar, V.J. Arán, H. Speisky, Indazoles: a new top seed structure in the search of efficient drugs against Trypanosoma cruzi, Future Med. Chem. 5 (2013) 1843–1859. doi: 10.4155/fmc.13.144.

[29]
J.F. Turrens, Oxidative stress and antioxidant defenses: a target for the treatment of diseases caused by parasitic protozoa, Mol. Aspects Med. 25 (2004) 211–220. doi:10.1016/j.mam.2004.02.021.

[30]
M.J. Barisón, L.N. Rapado, E.F. Merino, E.M. Furusho Pral, B. Suarez Mantilla, L. Marchese, C. Nowicki, A. M. Silber, and M.B. Cassera, Metabolomic profiling reveals a finely tuned, starvation-induced metabolic switch in Trypanosoma cruzi epimastigotes, J. Biol. Chem. (2017) 292 (21) 8964-8977.
doi: 10.1074/jbc.M117.778522.

Heterocyclic Diamines with Leishmanicidal Activity

Álvaro Martín-Montes[1], M. Paz Clares[2], Rubén Martín-Escolano[1], Estefanía Delgado-Pinar[2]Clotilde Marín[1], Enrique García-España[2], Manuel Sánchez-Moreno[1]

1. Departamento de Parasitología, Facultad de ciencias, Universidad de Granada, C/ Severo Ochoa s/n, 18071, Granada, España.
2. Instituto de Ciencia Molecular Universidad De Valencia, C/ Catedrático José Beltrán 2, 46980 Paterna (Valencia), Spain.

SUMMARY

Leishmaniasis is one of the world's most neglected diseases, and it has a worldwide prevalence of 12 million people. There are no effective human vaccines for its prevention, and treatment is hampered by outdated drugs. Therefore, research aiming to the development of new therapeutic tools to fight leishmaniasis remains a crucial goal today. With this purpose in mind, we present 10 new compounds made up by linking alkylated ethylenediamine units to pyridine or quinoline heterocycles with promising in vitro and in vivo efficacy against promastigote and amastigote forms of Leishmania infantum, Leishmania donovani and Leishmania braziliensis species. Three compounds (**2**, **4**, and **5**) showed a Selectivity Index much higher in the amastigote form than the reference drug glucantime. These four derivatives affected the parasite infectivity rates; the result was lower parasite infectivity rates than glucantime tested at an IC_{25} dose. In addition, these derivatives were substantially more active against the three Leishmania species tested than glucantime. The mechanism of action of these compounds has been studied, showing alterations in glucose catabolism and leading to greater levels of iron superoxide dismutase inhibition. These molecules could be potential candidates for leishmaniasis chemotherapy.

Introduction

Leishmaniasis is a widespread parasitic disease caused by protozoans of genus Leishmania and transmitted by the bite of phlebotomine sand flies. It is estimated that around 350 millions of people are at risk of infection, and WHO considers it as a Neglected Tropical Disease. Recently, due to environmental changes and migration is expanding to non-endemic areas [1]

Leishmaniasis has three different clinical forms: the most common and less dangerous is cutaneous leishmaniasis (CL), which causes skin lesions that heal spountaneously but leaves a lifelong scar. This clinical form is caused mainly by *L. infantum*. Mucocutaneous leishmaniasis (ML) is the most common form of leishmaniasis in Latin America, and can be debilitating when located in face, where it also causes deformations and tissue destruction one of the most common etiologic agents is *L. braziliensis*.

The most life-threatening form is Visceral leishmaniasis (VL) also known as Kala-azar. Symptoms include anaemia, fever and splenomegaly. If the treatment is not delivered on time, this form turns rapidly fatal. The main agent that causes VL is *L. donovani* in Europe, Africa, China and India [2, 3].

Current treatments are based on pentavalent antimonials and include drugs like miltefosine, glucantime, pentamidine and amphotericine B. These drugs have proven to be not as effective as they should, and also because of their high toxicity. For these reasons, there is a need to discover new drugs that should be more effective, affordable and less toxic [4].

In the search of new effective anti-leishmanial drugs able to broaden the narrow spectrum of compounds currently available, potential targets that perform essential functions for parasite survival are being investigated. Among them, antioxidant enzymes preventing oxidative stress that would otherwise lead to death of the parasite are in the spotlight. Within that group, trypanothione is currently receiving special consideration [5, 6].

However, another basic defense mechanism against oxidation is provided by the iron superoxide dismutase enzyme (Fe-SOD), which is located in the cytosol, the mitochondria and glycosomes of the parasite and plays an important role in the dismutation of harmful superoxide radicals into oxygen and hydrogen peroxide [7]. One further advantage of Fe-SOD as a drug target is that it is not present in humans, who use cooper/zinc and manganese superoxide dismutase (Cu,Zn and Mn-SOD) enzymes for the same purposes. Therefore, molecules able to inhibit selectively Fe-SOD could be promising candidates as leishmanicidal agents [8].

From a chemical point of view, we have recently observed that several polyamine compounds composed by an azamacrocyclic pyridine core incorporating hanging arms with different aromatic functionalities had interesting antiparasitic activities in relation to Chagas disease and Leishmaniasis. Molecular dynamic studies suggested that the active molecules interact blocking the access funnel of superoxide to get into the active center containing the metal ion. Moreover, such an interaction would disrupt the hydrogen bond network established around the water molecule coordinated to the iron atom (F. Olmo, M. P. Clares, C. Marín, J. González, M. Inclán, C. Soriano, K. Urbanová, R. Tejero, M. J. Rosales, R.L. Krauth-Siegel, M. Sánchez-Moreno, E. García-

España, Synthetic single and double aza-scorpiand macrocycles acting as inhibitors of the antioxidant enzymes iron superoxide dismutase and trypanothione reductase in Trypanosoma cruzi with promising results in a murine model, RSC Adv., 2014, 4, 65108–65120) altering basic parameters as the standard redox potential of the Fe^{III}/Fe^{II} couple [7]. Analysis of this modelling suggested that compounds having the same aromatic heterocycles linked to simple acyclic polyamines might also interact with the FeSOD enzyme in a similar fashion that would lead, therefore, to remarkable antiparasitic activities. Moreover, if operative this strategy would represent a clear advance in drug design because of the simplicity of the preparation that would allow obtaining the compounds in a gram-scale within one day work.

The mechanism of action of these compounds has been studied at metabolic levels by ^{1}H nuclear magnetic resonance (1 H NMR), and the study has been completed by testing their activity against Fe-SOD. These molecules could be potential candidates for Leishmania therapy [8, 10], because they show selectivity over Fe-SOD. Therefore, we decided to test these molecules against promastigote and amastigote forms of *Leishmania infantum*, *Leishmania donovani* and *Leishmania braziliensis* species.

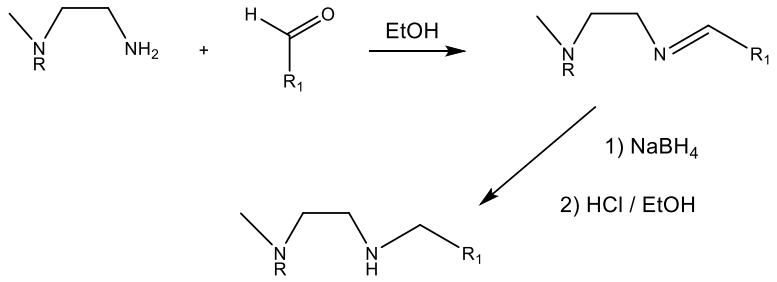
MATERIAL AND METHODS

Chemistry

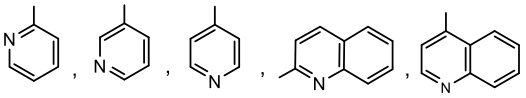
Synthesis

The ligands were synthesized by slightly modifying the procedure reported in refs. (J. A. R. Hartman, R. W. Vachet, J. H. Callahan, Gas, solution, and solid state coordination environments for the nickel(Ⅱ) complexes of a series of aminopyridine ligands of varying coordination number, Inorganica Chimica Acta 297 (2000) 79–87; A. Raja, V- Rajendira, P. U. Maheswari, R. Balamurugan, C. A. Kilner, M. A. Halcrow, M. Palaniandavar, Copper(Ⅱ) complexes of tridentatepyridylmethylethylenediamines: Role of ligand steric hindrance on DNA binding and cleavage, J. Inorg. Biochem. 99 (2005) 1717–1732, F. Groß, A. Müller-Hartmann, Heinrich Vahrenkamp, Zinc Complexes of Condensed Phosphates, 3[‡] Diphosphate-Zinc Complexes with Tridentate Coligands, Eur. J. Inorg. Chem. 2000, 236322370; H-D. Bian, J.-Y. Xu, W. Gu, S-P. Yan, D.-Z. Liao, Z.-H. Jiang, P. Cheng, Synthesis, structure and properties of terephthalate-bridgedcopper (Ⅱ) polymeric complex with zigzag chain, Inorg. Chem. Commun. 6 (2003) 573–576) for compounds L1 and L4. The synthesis consisted in the reaction of the appropriated carboxaldehyde with the corresponding diamine, in dry EtOH. The resulting mixture was stirred for 2 h at room temperature, and then, an excess of sodium borohydride was added portionwise. After 2 h, the solvent was evaporated to dryness. The residue was

treated with water and repeatedly extracted with CH_2Cl_2 (3 × 40 mL). The organic phase was collected and was dried with anhydrous MgSO4 and the solvent evaporated to dryness to give an oil. The oil was then taken in a minimum amount of EtOH and precipitated with HCl in dioxane to obtain its hydrochloride salt.



R= H, CH_3

R_1= 

Scheme 1: General procedure of synthesis of ligands.

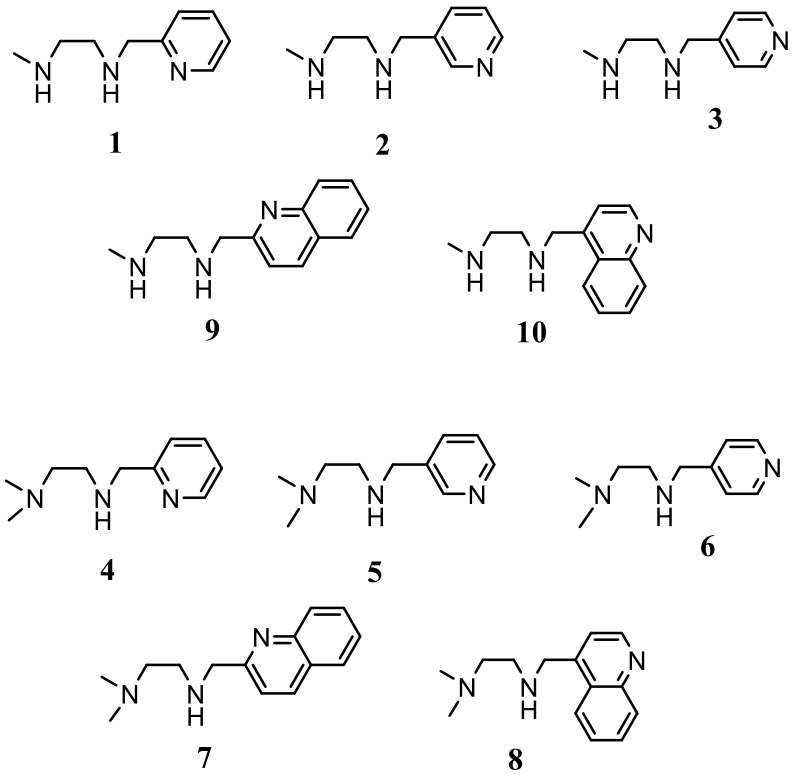


Figure 1. Drawing of the compounds

1-(2-pyridyl)-2,5-diazahexane. **(1).** $C_9H_{18}N_3Cl_3 \cdot 2H_2O$ (M.W. = 312,89 g/mol). Yield: 63,7 %. ^{1}H NMR (300 Hz, D_2O): δ_H (ppm): 2.78 (s, 3H), 3.48 - 3.51 (m, 2H), 3.59-3.64 (m, 2H), 4.67 (s, 2H), 7.92 - 7.96 (m, 1H), 8.015 (d, *J* = 8.1Hz, 1H), 8.46 (t, *J* = 8.0Hz, 1H), 8.795 (d, *J* = 5.6Hz, 1H) ^{13}C NMR (75 MHz, D_2O) δ: 146.99, 145.68, 143.96, 126.55, 126.36, 49.22, 44.49, 43.25, 33.21. Melting point: 165,6 ºC. Elemental Analysis: Calculate: C 34,79 %; H 7,13 %; N 13,52 % Experimental: C 35,05 %; H 6,74 %; N 14,72 %.

1-(3-pyridyl)-2,5-diazahexane. **(2).** $C_9H_{17}N_3Cl_2 \cdot 2H_2O$ (M. W.= 274,18 g/mol). Yield: 62,36 %. ^{1}H NMR (300 Hz, D_2O): δ_H (ppm): 2.81 (s, 3H), 3.43 (s, 4H), 4.34 (s, 2H), 7.68 (dd, J = 8.1Hz, J = 5.2Hz, 1H), 8.14 (d, J = 8.2Hz, 1H), 8.65 - 8.69 (m, 2H). ^{13}C NMR (75 MHz, D_2O) δ: 140.39, 125.17, 48.87, 45.03, 42.84, 33.09. Melting point: 239,4 ºC. Elemental Analysis: Calculate: C 39,42 %; H 7,71 %; N 15,32 % Experimental: C 39,35 %; H 4,83 %; N 14,30 %

1-(4-pyridyl)-2,5-diazahexane. **(3).** $C_9H_{18}N_3Cl_3$ (M. W. = 294,60 g/mol). Yield: 70,56 %. ^{1}H NMR (300 Hz, D_2O): δ_H (ppm): 2.81 (s, 3H), 3.49 - 3.54 (m, 2H), 3.62 - 3.67 (m, 2H), 4.68 (s, 2H),
8.17 (d, J = 6.2Hz, 2H), 8.89 (d, J = 6.2Hz, 2H) ^{13}C NMR (75 MHz, D_2O) δ: 151.02, 142.38, 127.14, 49.74, 44.37, 43.49, 33.22. Melting point: 220,5 ºC. Elemental Analysis: Calculate: C 53,49 %; H 6,59 %; N 14,39 % Experimental: C 53,49 %; H 6,59 %; N 14,39 %

1-(2-quinolyl)-2,5-diazahexane. **(9).** $C_{13}H_{19}N_3Cl_2 \cdot H_2O$ (M. W. = 291,82 g/mol). Yield: 52,76 %. ^{1}H NMR (300 Hz, D_2O): δ_H (ppm): 2.85 (s, 3H), 3.55 – 3.63 (m, 4H), 4.69 (s, 2H), 7.63 (d, J = 8.5Hz, 1H), 7.75 (t, J = 7.5Hz, 1H), 7.93 (t, J = 7.5Hz, 1H), 8.06 – 8.15 (m, 2H), 8.55 (d, J = 8.5Hz, 1H) ^{13}C NMR (75 MHz, D_2O) δ: 151.63, 145.57, 139.65, 131.24, 128.29, 127.80, 126.99, 120.09, 51.20, 44.83, 43.05, 33.10. Melting point: 215,8 ºC. Elemental Analysis: Calculate: C 36,94 %; H 6,88 %; N 14,35 % Experimental: C 37,80 %; H 6,72 %; N 14,03 %

1-(4-quinolyl)-2,5-diazahexane. **(10).** $C_{13}H_{20}N_3Cl_3 \cdot 2H_2O$ (M. W. = 360,71 g/mol). Yield: 64,34 %. ^{1}H NMR (300 Hz, D_2O): δ_H (ppm): 2.85 (s, 3H), 3.50 –

3.66 (m, 4H), 5.04 (s, 2H), 8.01 – 8.08 (m, 2H), 8.19 (t, J = 7.8Hz, 1H), 8.31 (d, J = 8.8Hz, 1H), 8.40 (d, J = 8.7Hz, 1H), 9.15 (d, J = 5.6Hz, 1H) ^{13}C NMR (75 MHz, D_2O) δ: 148.56, 144.57, 138.45, 134.99, 130.74, 126.66, 124.05, 122.16, 120.67, 47.50, 44.71, 43.80, 33.20.. Melting point: 214,8 ºC. Elemental Analysis: Calculate: C 43,44 %; H 6,73 %; N 11,69 % Experimental: C 43,29 %; H 6,70 %; N 11,65 %

1-(2-pyridyl)-5-methyl-2,5-diazahexane. **(4)** $C_{10}H_{20}N_3Cl_3 \cdot 0{,}5\ H_2O$ (M. W. = 297,65 g/mol). Yield: 65,3 %. ^{1}H NMR (300 Hz, D_2O): δ_H (ppm): 3.00 (s, 6H), 3.58 (s, 4H), 4.49 (s, 2H), 7.63 – 7.71 (m, 2H), 8.13 (t, J = 7.8Hz, 1H), 8.67 (d, J = 5.3Hz, 1H) ^{13}C NMR (75 MHz, D_2O) δ: 147.43, 145.53, 143.95, 126.46, 126.30, 52.78, 49.34, 43.31, 42.04.. Melting point: 213,7 ºC. Elemental Analysis: Calculate: C 40,35 %; H 7,11 %; N 14,12 % Experimental: C 40,73 %; H 7,54 %; N 14,41 %

1-(3-pyridyl)-5-methyl-2,5-diazahexane. **(5)** $C_{10}H_{20}N_3Cl_3$ (M. W. = 288,64 g/mol). Yield: 64,15 %. ^{1}H NMR (300 Hz, D_2O): δ_H (ppm): 3.01 (s, 6H), 3.57 – 3.68 (m, 4H), 4.57 (s, 2H), 8.09 (dd, J = 8.2Hz, J = 5.7Hz, 1H), 8.65 (d, J = 8.1Hz, 1H), 8.88 (d, J = 5.7Hz, 1H), 8.95 (s, 1H) ^{13}C NMR (75 MHz, D_2O) δ: 148.00, 142.90, 142.79, 130.77, 127.89, 52.32, 47.83, 43.40, 41.99. Melting point: 218,9 ºC. Elemental Analysis: Calculate: C 41,61 %; H 6,98 %; N 14,56 % Experimental: C 41,82 %; H 7,01 %; N 14,45 %

1-(4-pyridyl)-5-methyl-2,5-diazahexane. **(6)** $C_{10}H_{20}N_3Cl_3$ (M. W. = 288,64 g/mol). Yield: 67,8%. ^{1}H NMR (300 Hz, D_2O): δ_H (ppm): 3.02 (s, 6H), 3.59 – 3.70 (m, 4H), 4.64 (s, 2H), 8.16 (d, J = 6.71Hz, 2H), 8.90 (d, J = 6.8Hz, 2H) ^{13}C NMR (75 MHz, D_2O) δ 151.16, 142.20, 127.28, 52.44, 49.84, 43.40, 42.31. Melting point: 210,0 ºC. Elemental Analysis: Calculate: C 41,61 %; H 6,98 %; N 14,56 % Experimental: C 41,30 %; H 6,83 %; N 14,67 %

1-(2-quinolyl)-5-methyl-2,5-diazahexane. **(7)** $C_{14}H_{22}N_3Cl_3$ (M. W. = 338,70 g/mol). Yield: 66,25 %. ^{1}H NMR (300 Hz, D_2O): δ_H (ppm): 3.02 (s, 6H), 3.62 – 3.72 (m, 4H), 4.43 (s, 2H), 7.74 (d, J = 8.5Hz, 1H), 7.80 (t, J = 7.7Hz, 1H), 7.98 (t, J = 8.6Hz, 1H), 8.15 (t, J = 9.2Hz, 1H), 8.68 (d, J = 8.6Hz, 1H) ^{13}C NMR (75 MHz, D_2O) δ: 150.96, 143.66, 141.91, 132.49, 128.54, 128.01, 125.25, 120.46, 53.06, 50.58, 43.26, 42.01. Melting point: 211,1 ºC. Elemental Analysis:

Calculate: C 49,65 %; H 6,55 %; N 12,41 % Experimental: C 49,81 %; H 6,37 %; N 12,53 %

1-(4-quinolyl)-5-methyl-2,5-diazahexane. **(8)** $C_{14}H_{22}N_3Cl_3$ (M. W. = 338,70 g/mol). Yield: 66,62 %. ^{1}H NMR (300 Hz, D_2O): δ_H (ppm): 3.02 (s, 6H), 3.59 – 3.68 (m, 4H), 5.04 (s, 2H), 8.06 (t, J = 7.7Hz, 1H), 8.12 (d, J = 5.7Hz, 1H) 8.21 (t, J = 7.8Hz, 1H), 8.32 (d, J = 8.6Hz, 1H), 8.43 (d, J = 8.6Hz, 1H), 9.17 (d, J = 5.6Hz, 1H) ^{13}C NMR (75 MHz, D_2O) δ: 144.30, 138.02, 135.19, 130.85, 126.74, 124.13, 121.83, 120.71, 52.87, 47.63, 43.34, 42.60. Melting point: 211,1 ºC. Elemental Analysis: Calculate: C 49,65 %; H 6,5 5%; N 12,41 % Experimental: C 49,55 %; H 6,51 %; N 12,56 %

EMF Measurements. The potentiometric titrations were carried out at 298.1± 0.1 K using NaCl 0.15 M as supporting electrolyte. The experimental procedure (burette, potentiometer, cell, stirrer, microcomputer, etc.) has been fully described elsewhere. (E. García-España, M.-J. Ballester, F. Lloret, J. M. Moratal, J. Faus and A. Bianchi, *J. Chem. Soc., Dalton Trans.* 1988, 101−104) The acquisition of the emf data was performed with the computer program PASAT. (M. Fontanelli and M. Micheloni, Program for the automatic control of the microburette and the acquisition of the electromotive force readings (PASAT). Proceedings of the I Spanish-Italian Congress of Thermodynamics of Metal Complexes, Peñíscola, Castellón , Spain, 1990). The reference electrode was a Ag/AgCl electrode in saturated KCl solution. The glass electrode was calibrated as a hydrogen-ion concentration probe by titration of previously standardized amounts of HCl with CO_2-free NaOH solutions and the equivalent point determined by the Gran´s method((a) G. Gran, *Analyst* 1952, **77**, 661−671; (b) F. J. Rossotti and H. Rossotti, *J. Chem. Educ.* 1965, **42**, 375−378,) which gives the standard potential, Eº', and the ionic product of water (pKw=13.73(1)).

The computer program HYPERQUAD was used to calculate the protonation and stability constants (P. Gans, A. Sabatini and A. Vacca, *Talanta* 1996, **43**, 1739-1753). The pH range investigated was 2.5-11.0 and the concentration of the e ligands ranged from $1x10^{-3}$ to $5x10^{-3}$ M. The different titration curves for each system (at least two) were treated either as a single set or as separated

curves without significant variations in the values of the stability constants. Finally, the sets of data were merged together and treated simultaneously to give the final stability constants.

(I)Biological evaluation. Determination of the potential leishmanicidal activity and toxicity of new compounds.

Axenic cultures: The parasites were cultivated in axenic cultures using standard conditions for promastigote and amastigote forms of *L. infantum* (MCAN/ES/2001/UCM-10), *L. braziliensis* (MHOM/BR/1975/M2904) and *L. donovani* (MHOM/PE/84/LC26), according to the methodology described by [20].

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 of a female BALB/c mouse. The macrophages were grown in minimal essential medium (MEM) plus glutamine (2 mM) and 20% inactive FCS, with a humidified atmosphere of 95% air and 5% CO2 at 37 º °C. The cytotoxicity test on macrophages was performed by flow cytometric analysis according to a method previously described [21]. The percentage of viable cells was calculated with respect to the control culture. The IC50 was calculated using linear regression analysis from the Kc values of the concentrations employed.

In vitro activity assays

The compounds to be tested were first dissolved in dimethylsulfoxide (DMSO, Panreac, Barcelona, Spain) at a concentration of 0.1% and then assayed for toxicity and inhibitory effects on parasite and mammalian cells growth as previously described [20].

Extracellular forms (Promastigote assay: Effects of each compound against the promastigote forms at the different concentrations were tested at 72 h using a Neubauer haemocytometric chamber. The leishmanicidal effect was expressed as the IC50 value, i.e. the concentration required to result in 50% inhibition, calculated by linear regression analysis from the Kc values of the concentrations employed.

Intracellular forms (Amastigotes assay): J774.2 macrophage cells were grown and seeded at a density of 1×10^4 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultured for 2 days. The adherent macrophages were then infected with promastigotes of *L. infantum*, *L. braziliensis* and *L. donovani* at the stationary growth phase, at a ratio of 10:1 and maintained for 24 h at 37 º °C in air containing 5% CO_2. Non-phagocytosed

parasites were removed by washing, and the infected cultures were incubated with the testing compounds (concentrations ranging from 1 to 100 µM) and then cultured for 72 h in MEM plus glutamine (2 mM) and 20% inactive FCS. Compound activity was determined from the percentage reductions in amastigote number in treated versus untreated cultures in methanol-fixed and Giemsa-stained preparations. Values are the means of three separate determinations [22].

Infectivity assay:

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

(II) **Studies on the mechanism of action.**

Metabolite excretion: Cultures of *L. infantum*, *L. braziliensis* and *L. donovani* promastigotes (initial concentration 5×10^5 cells/mL) received the IC25 dose of each compound (except for control cultures). After incubation for 72 h at 28°C the cells were centrifuged at 400 g for 10 min. The supernatants were collected to determine the excreted metabolites by 1HNMR, and chemical shifts were expressed in ppm, using sodium 2,2-dimethyl-2-silapentane-5-sulphonate as the reference signal. The chemical displacements used to identify the respective metabolites were consistent with those described previously by some of the co-authors [23].

Fe-SOD enzymatic inhibition

Parasites were collected during the logarithmic growth phase by centrifugation (400 g for 10 min at room temperature). The pellet obtained after centrifugation, was resuspended in 3 mL of STE buffer (0.25 M sucrose, 25 mM Tris–HCl, 1 mM EDTA, pH 7.8) and the cells were lysed by three cycles of sonication for 30 s each at 60 W. The sonicated homogenate was centrifuged at 1500 g for 5 min at 4°C, and the pellet was washed three times in ice-cold STE buffer. This fraction was centrifuged (2500 g for 10 min at 4°C) and the supernatant was collected. Afterwards, the supernatant was subjected to ice-

cold ammonium sulphate precipitation between 35 and 85% salt concentration and the resulting precipitate was dissolved in 2.5 mL of distilled water and desalted by chromatography in Sephadex G-25 column (GE Healthcare Life Sciences®, PD 10 column) previously equilibrated with 2 mL of distilled water, taking it up to a final volume of 3.5 mL [1] [24]. The protein content was quantified using the Sigma Bradford test, which uses bovine serum albumin (BSA) as a standard [25]. Iron and copper−zinc superoxide dismutases activities were determined using a previously described method [26] that measures the reduction in nitroblue tetrazolium (NBT) by superoxide ions. According to the protocol, 845 µL of stock solution [3 mL of L-methionine (300 mg, 10 mL−l), 2 mL of NBT (1.41 mg, 10 mL−1) and 1.5 mL of Triton X-100 1% (v/v)] were added into each well, along with 30 µL of the parasite homogenate fraction were added to the mixtures containing the compounds. Then, the absorbance (A0) was measured at 560 nm in a UV spectrophotometer. Afterwards, each well was illuminated with UV light for 10 min under constant stirring and the absorbance (A1) was measured again. The human CuZn-SOD and substrates used in these assays were obtained from Sigma Chemical Co. The resulting data were analysed using the Newman-Keuls test.

Rodamine assays

Parasites were treated with the IC_{25} drug concentration for 72 hours. Conic bottom flasks with 5mL of IC_{25} concentration with MTL culture medium with $5x10^4$ parasites were prepared and stored at 27ºC. After 72 hours, they were centrifuged at 1500 rpm for 10 minutes. Pellets were then resuspended for washing with PBS. This was done three times. After the third wash, pellets were resuspended with rhodamine (Rho 123) 10 µg/mL in PBS and then the reaction took place during the following 20 minutes. After this time passed, it was annalyzed by flow citometry [27].

RESULTS AND DISCUSSION

Acid-Base Behaviour

The stepwise protonation constants of **1-3,9,10** were determined from potentiometric studies in 0.15 M NaCl at 298.1 K, and the results are collected in Table 1. The distribution diagram of the different protonated species formed by **these ligands** is collected in Fig. 2.

Table 1 Logarithms of the stepwise protonation constants for **Ligands** determined at 298.1 K in 0.15 M NaCl

Reaction	**1**	**2**	**3**	**9**	**10**
H+L ⇌ HL [a]	9.63 (1) [b]	9.653 (5)	9.503 (5)	9.68 (2)	9.512 (6)
HL+H ⇌ H_2L	5.62 (1)	5.687 (5)	5.713 (5)	5.41 (2)	5.297 (7)
H_2L+H⇌ H_3L	----	2.95 (2)	3.390 (6)	---	3.183 (8)
log β [c]	15.25 (1)	18.29 (3)	18.60 (1)	15.10 (3)	17.99 (1)

a) Charges omitted. b) Values in parenthesis are standard deviations in the last significant figure. c) $\beta = \Sigma K_{HjL}$

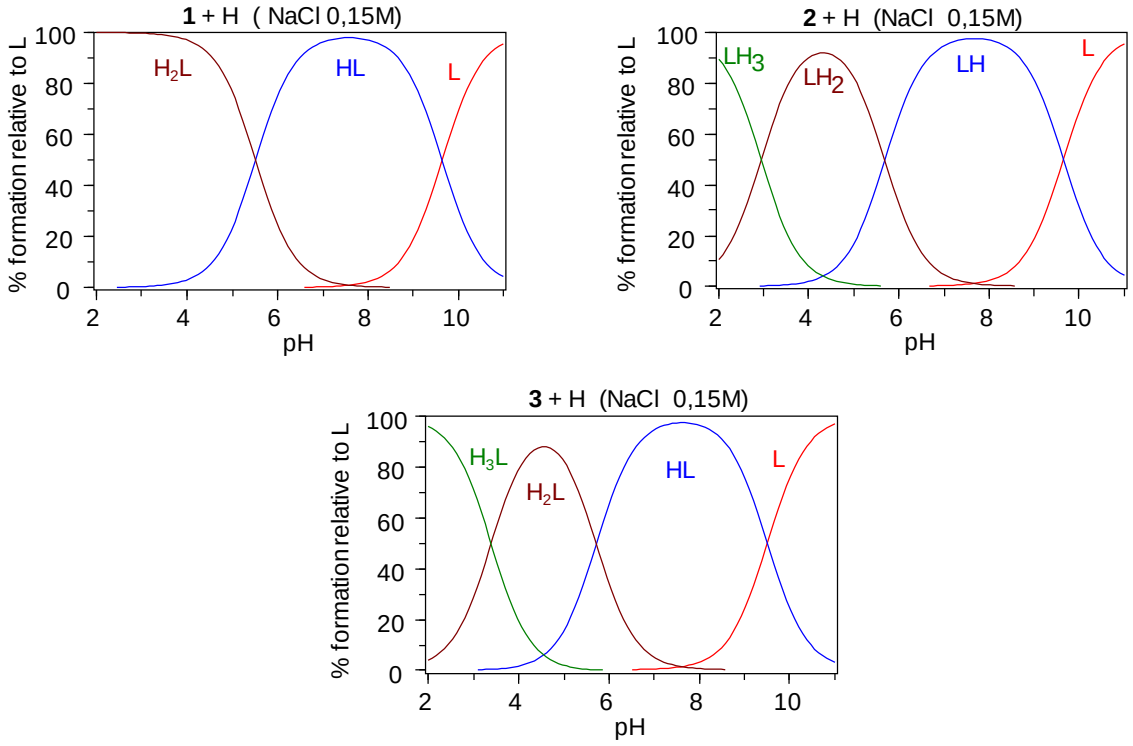


Figure 2. Distribution diagrams of the systems **1**-H^+, **2**-H^+ and **3**-H^+

The stepwise protonation constants of **4-8** were determined from potentiometric studies in 0.15 M NaCl at 298.1 K, and the results are collected in Table 2. The distribution diagram of the different protonated species formed by **these ligands** is collected in Fig. 3.

Table 2 Logarithms of the stepwise protonation constants for **Ligands** determined at 298.1 K in 0.15 M NaCl

Reacción	**4**	**5**	**6**	**7**	**8**
H+L ⇌ HL [a]	9.06 (1) [b]	9.18 (1)	8.865 (3)	9.17 (1)	10.08 (1)
HL+H ⇌ H_2L	5.58 (1)	5.63 (1)	5.407 (3)	5.45 (1)	5.59 (1)
H_2L+H⇌ H_3L	2.25 (2)	3.16 (1)	2.956 (6)	---	3.32 (1)
Log β [c]	16.88 (2)	17.98 (1)	17.22 (1)	14.63 (1)	18.99 (2)

a) Charges omitted. b) Values in parenthesis are standard deviations in the last significant figure. c) $\beta = \Sigma K_{HjL}$

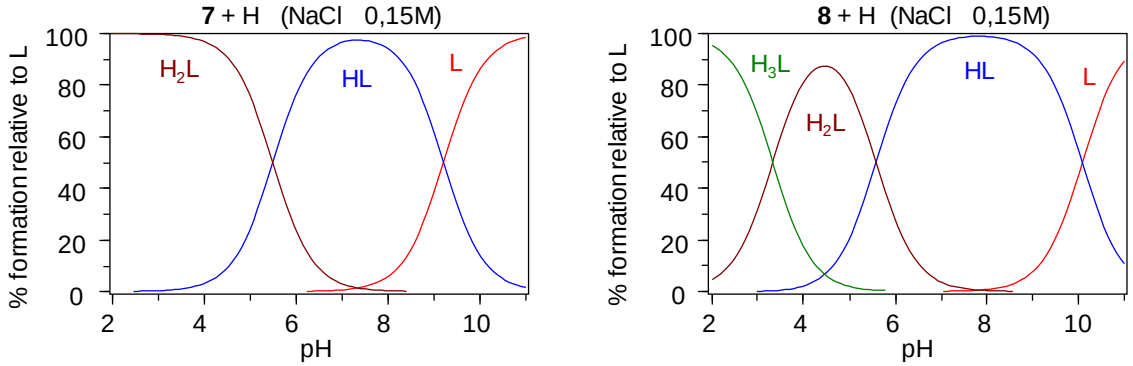


Figure 3. Distribution diagrams of the systems **7**-H^+, and **8**-H^+

^{1}H NMR

NMR is a valuable tool to establish protonation sequences in polyamine molecules. (REFS).The variation with pH of the ^{1}H signals provides information about the distribution of positive charges in the different amine groups of the molecule. Upon protonation of an amine group, the proton nuclei bound to its adjacent carbon experiment the largest downfield changes.

The pD values of the spectra in figure 4 have been selected taken into account the maximum formation percentages of the protonated species derived from the distribution diagrams in Figure 2. Ongoing from pH 10.5 to 7.5 in correspondence to the first protonation step of **2**, the largest downfield shift

shifts are observed for the signal of the methyl protons (H1) and the triplet signal of protons labeled as H2. This movements support that first protonation is mainly at nitrogen N1 instead than at N2. The displacements of the signals of protons H4, H2 and H3 below pH 7.5 give further support to the proposed average protonation sequence.

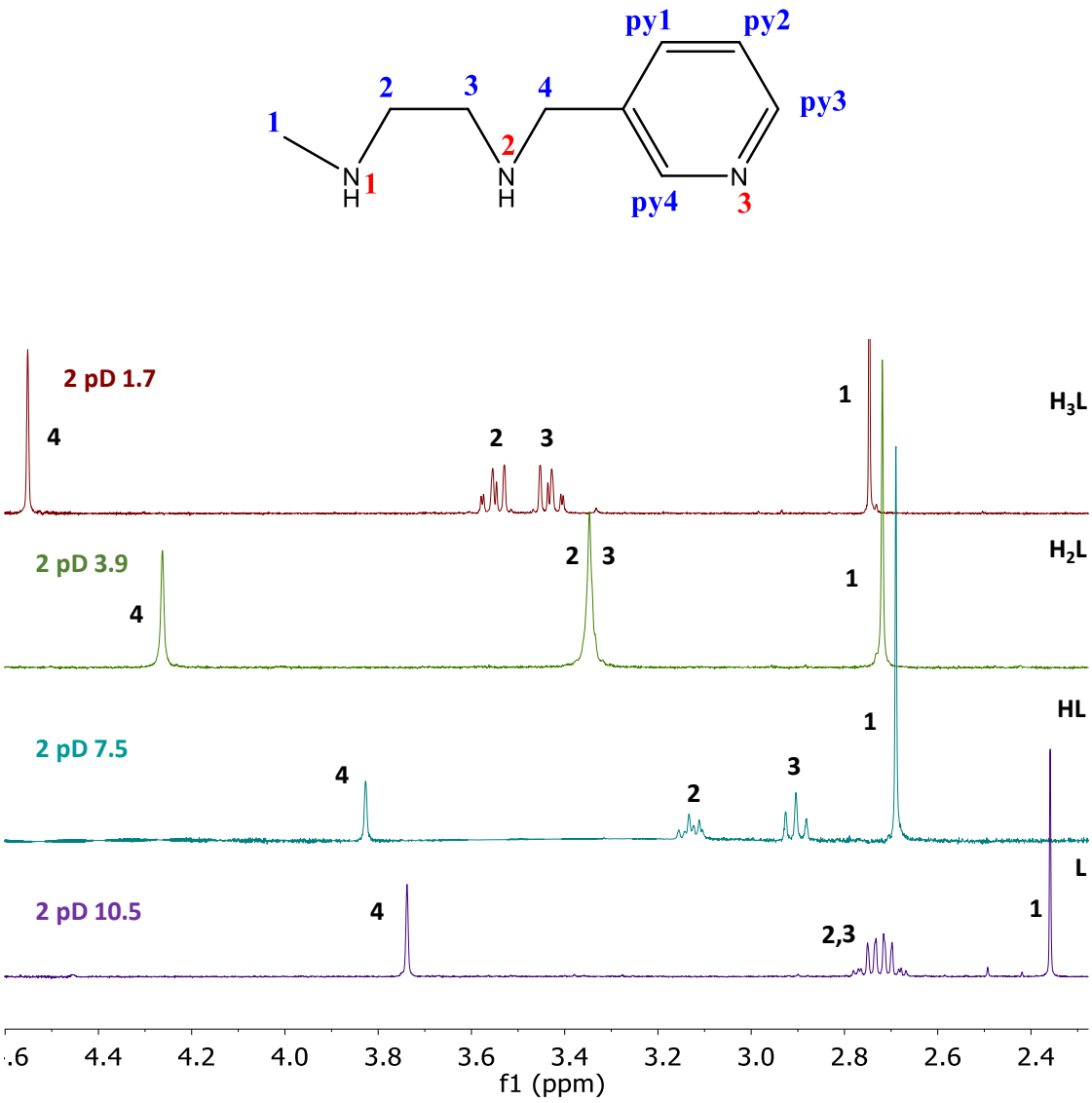


Figure 4. Variation with pH of the aliphatic region of the ^{1}H NMR spectra in D_2O of **2** (pD = 1.7, 3.9, 7.5, 10.5).

The analysis of the aromatic region of the NMR spectra shows that the greatest variation of the aromatic signals, in particular in the singlet signal attributable to py4, is produced below pH 3.9. This supports that third protonation occurs on the pyridine nitrogen.

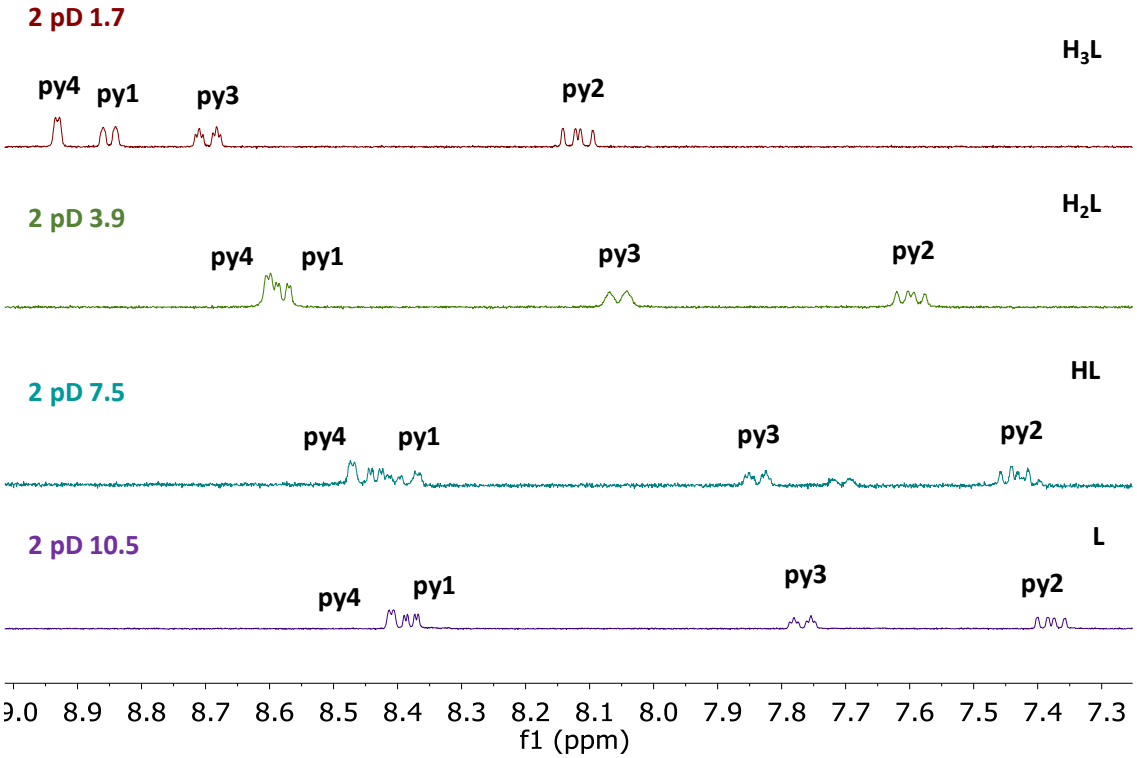


Figure 5. ^{1}H NMR spectra in D_2O of **2** in the aromatic zone registered at pD = 1.7, 3.9, 7.5 and 10.5.

We also explored the variation of the NMR signals of the analogous ligand having the dialkylated terminal **5** inferring similar conclusion (Fig 6).

Figure 6 shows that, also in this case, the signals shifting upfield most from pH 7.5 onwards are those of the proton nuclei closer to the tertiary amine group (H1,2 and H3) while the signal of protons H5 shifts mostly upfield below pH 7.5. Again the aromatic signals only bear upfield shifts at acidic pH values below 4.

All these NMR studies strongly suggest that, for both compounds, first protonation occurs predominantly on the tertiary nitrogen, while the secondary amine group closer to the pyridine (N3) wills the one protonating in second place. Finally, protonation of the pyridine group will occur in last place.

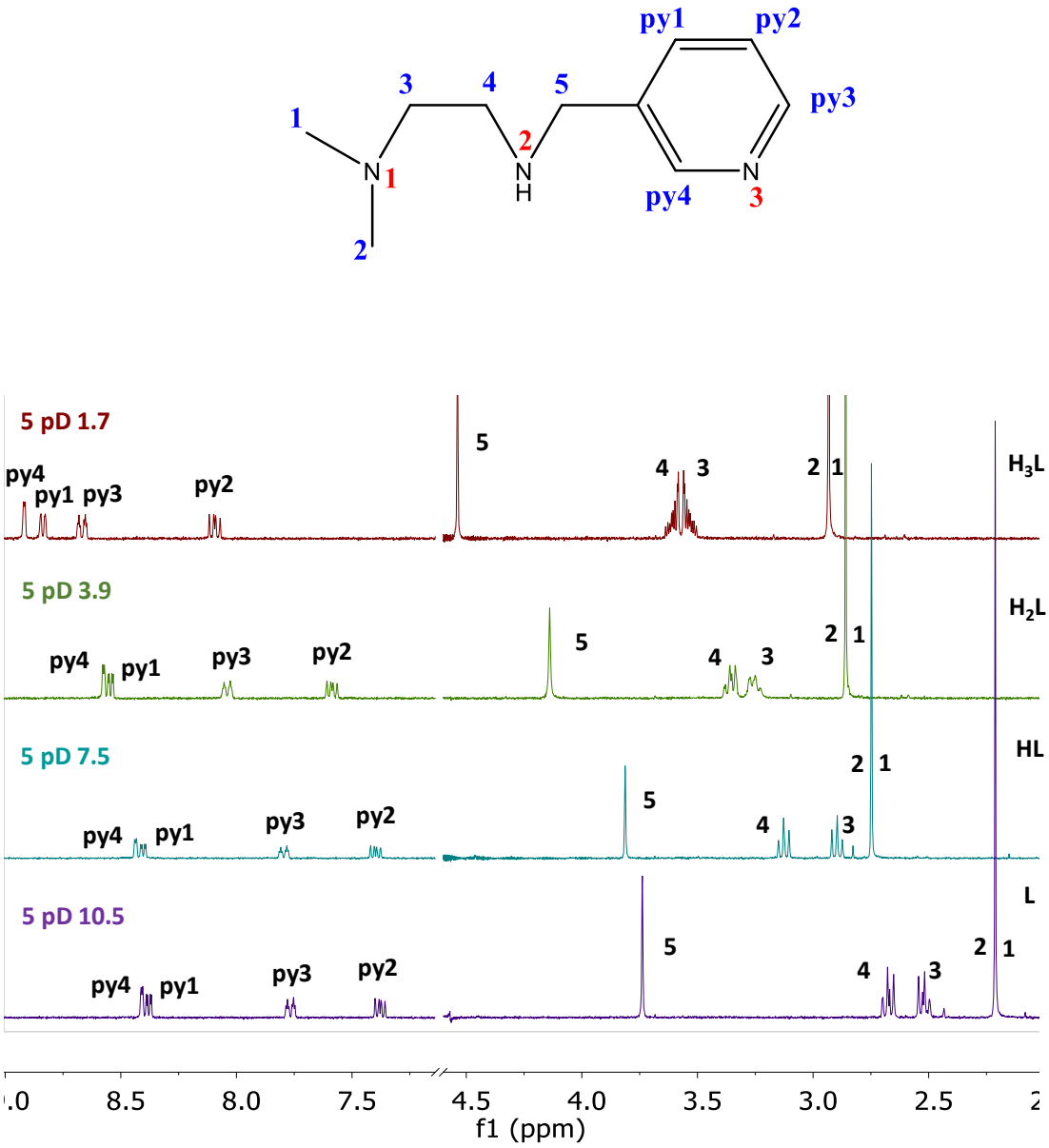


Figure 6. ^{1}H NMR spectra in D_2O of **5** recorded at pD = 1.7, 3.9, 7.5 and 10.5.

In vitro antileishmanial evaluation

In a first step we assayed the in vitro biological activity of compounds 1-10 (Table 3) against three significant species of *Leishmania*: *L. infantum*, *L. braziliensis* and *L. donovani* on both extra- and intracellular forms of the parasites. Extracellular forms are more commonly used due to the ease of working with them, but are less indicative of leishmanicidal activity. Using intracellular forms yields more accurate results, as promastigotes are converted to amastigotes in vertebrate host cells [20]. Intracellular assays were performed by infecting macrophage cells with promastigotes, which transformed into amastigotes within 1 day after infection. **Table 3** shows the IC_{50} values obtained

after 72 h of exposure when compounds 1-10 were tested on extra- and intracellular forms of *L. infantum, L. braziliensis* and *L. donovani*. Toxicity values against J774.2 macrophages after 72 h of culture were also calculated. Results obtained for the reference drug glucantime were included in all cases for comparison.

It could be observed that the leishmanicidal activities against the most indicative intracellular forms of the parasites were higher than those found for glucantime (comp **2**, **4** and **5**), whereas the effect on extracellular forms was erratic. Toxicity data found in mammalian cells was also noteworthy, in the case of compounds **1** and **8** we found out that toxicity values were very high and therefore were not considered for further studies, the other three mentioned compounds were found to be much less toxic for macrophages than the reference drug.

Thus, compounds **2**, **4**, and **5** were much less cytotoxic than glucantime. In order to obtain a more accurate picture of the features commented above, we show in **Table 4** the selectivity index values calculated from the data shown in **Table 3**, since they are very illustrative of the in vitro potential of the compounds tested with respect to the reference drug. The number of times that the SI of each compound exceeded the SI of glucantime is also shown in parenthesis. In this **Table 4** the differences between glucantime and tested compounds are clearly revealed. Selected compounds (**2**, **4**, and **5**) exhibited substantially better SI values than the reference drug in the three tested *Leishmania* species. Compound **2** showed a better activity than glucantime in both cellular forms of the three studied Leishmania species. Compound **5** was effective for both forms of *L. infantum*, but only effective for *L. braziliensis* extracellular forms and *L. donovani* intracellular forms.

The SI displayed in **Table 4** are also very illustrative about the difference in behaviour of different compounds, which recurs in the three species and in both extra and intracellular forms. In tests performed on *L. infantum* species, the SI exceeded that of the reference drug by 61- and 62-fold for the extra- and intracellular forms in the case of compound **2**, respectively, by 126- and 33-fold with the same compound on *L. braziliensis*, and 38 and 36-fold when it was tested on *L. donovani*.

Compounds were selected for further studies when most importantly when their SI values >30 in both cellular forms and if their IC_{50} concentrations were close to 10µM or lower, as they can be considered ideal candidates for further development as leishmanicidal drugs [28]. This requirement was satisfied by compounds **2**, **4** and **5**, with the exception that compound **5** only met the criteria in the case of *L. infantum*, being this the reason why the next studies carried out with this compound were only against *L. infantum*.

Compounds	Activity IC $_{50}$ (µM)[a]						Macrophage Toxicity IC_{50} (µM)[b]
	L. infantum		*L. braziliensis*		*L. donovani*		
	Promastigote Forms	Amastigote Forms	Promastigote Forms	Amastigote Forms	Promastigote Forms	Amastigote Forms	
Glucantime	18.0±3.1	24.2±2.6	18.0±3.1	24.2±2.6	18.0±3.1	24.2±2.6	15.2± 1.0
1	0.6±0.2	0.7±0.1	7.4±0.6	5.7±0.3	7.0±0.5	8.9±0.9	13.7± 3.1
2	6.4±0.6	8.4±0.5	4.1±0.2	15.8±0.5	11.7±0.8	14.4±0.1	310.3±11.5
3	12.6±2.1	13.9±1.0	14.3±1.0	27.9±1.2	18.4±0.8	5.1±0.3	222.7±17.3
4	28.6±2.5	33.6±1.4	37.3±0.9	30.8±1.3	34.8±1.5	38.8±1.4	1241.7±42.7
5	9.2±0.7	10.0±0.8	16.7±0.5	20.0±2.0	21.7±1.6	17.6±0.7	230.4±16.9
6	63.3±1.5	23.6±1.7	52.5±5.1	29.7±2.5	67.9±5.3	11.5±1.3	496.9±21.5
7	84.4±5.1	49.7±3.6	105.3±11.6	38.9±2.7	56.9±2.7	38.9±3.2	442.4±31.6
8	14.1±0.6	11.5±0.8	13.8±0.7	16.8±1.1	12.8±0.6	10.6±0.6	22.5±0.6
9	15.6±±0.8	14.3±0.6	28.0±0.3	26.5±0.9	27.5±0.4	31.9±1.1	353.9±31.7
10	12.5±1.1	18.7±1.5	18.4±1.1	21.7±2.3	24.1±0.6	26.8±2.1	164.3±10.0

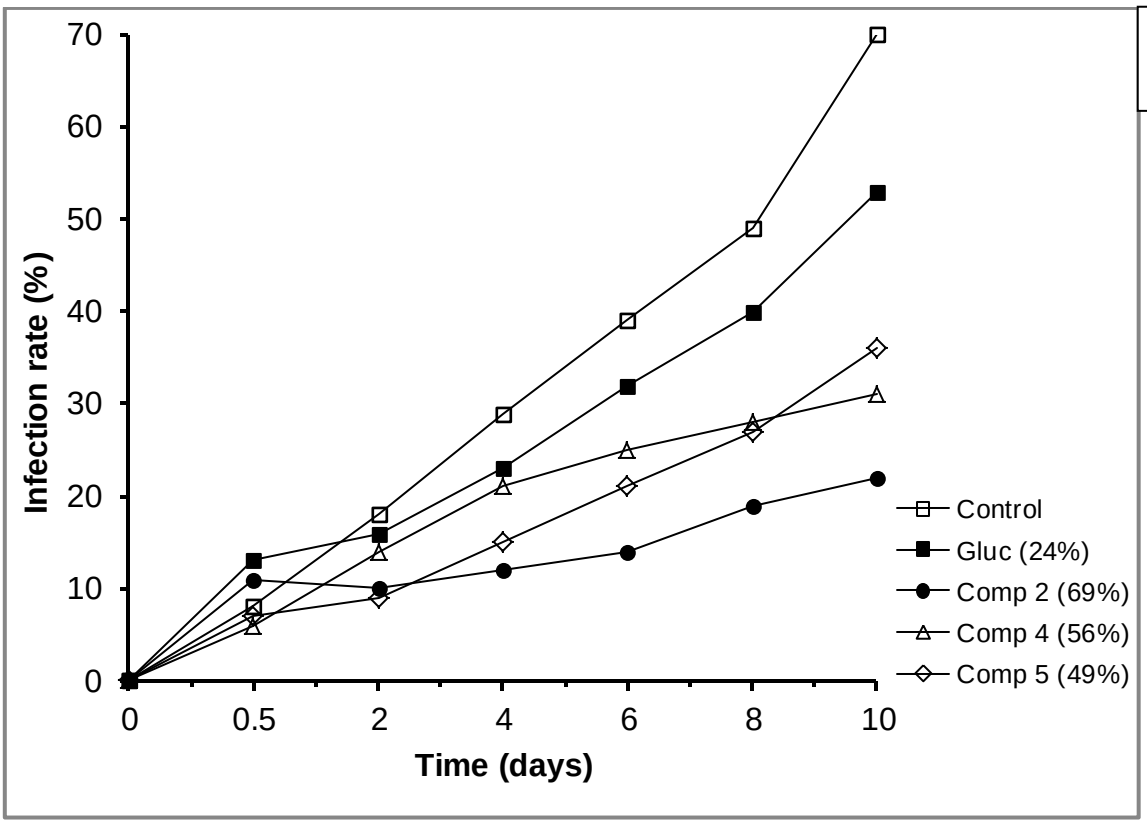
Table 3. A) Antileishmanial activity in extracellular and intracellular forms of the three studied species. B) Toxicity in macrophage cells.

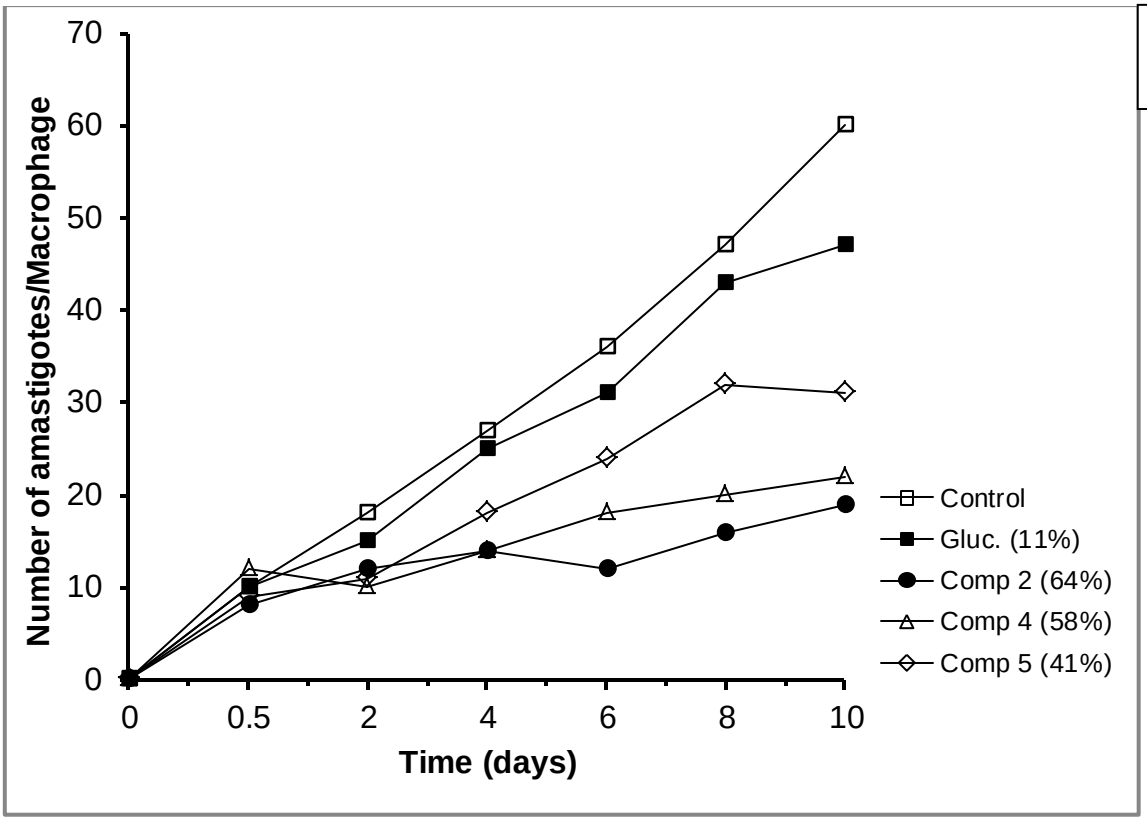
Compounds	Selectivity Index					
	L. infantum		L. braziliensis		L. donovani	
	Promastigote Forms	Amastigote Forms	Promastigote Forms	Amastigote Forms	Promastigote Forms	Amastigote Forms
Glucantime	0.8	0.6	0.6	0.6	0.7	0.6
1	23 (28)	20 (33)	2 (3)	2 (4)	2 (3)	1 (3)
2	48 (61)	37 (62)	76 (126)	20 (33)	26 (38)	21 (36)
3	18 (22)	16 (27)	16 (26)	8 (13)	12 (17)	44 (73)
4	43 (54)	37 (62)	33 (55)	40 (67)	36 (51)	32 (53)
5	25 (42)	23 (38)	14 (23)	11 (19)	11 (15)	13 (22)
6	8 (10)	21 (35)	9 (16)	17 (28)	7 (10)	43 (72)
7	5 (7)	9 (15)	4 (7)	11 (19)	8 (11)	11 (19)
8	2 (2)	2 (3)	2 (3)	1 (2)	2 (2)	2 (3)
9	23 (28)	25 (41)	13 (21)	13 (22)	13 (19)	11 (18)
10	13 (16)	9 (15)	9 (15)	8 (13)	7 (10)	6 (10)

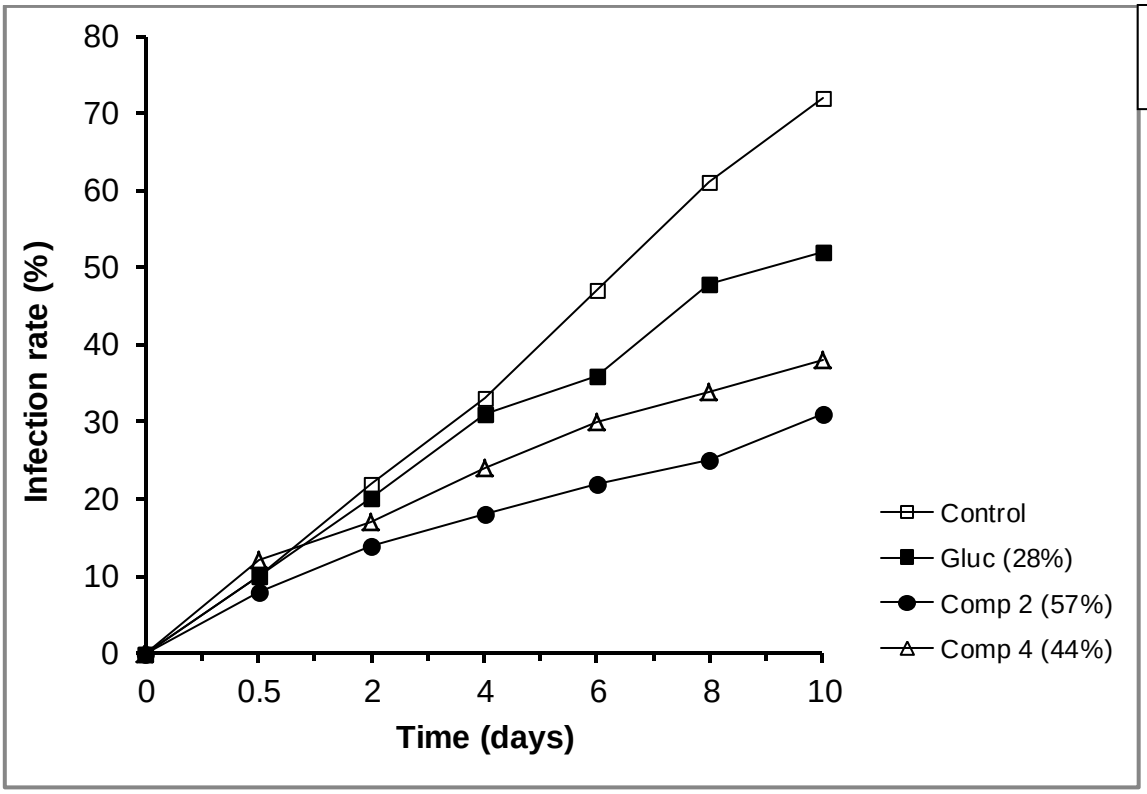
Table 4. Selectivity index. Result of IC_{50} of macrophages divided by IC_{50} of parasite cells (intracellular and extracellular forms) number in brackets shows the number of times the compound is more active than the reference drug.

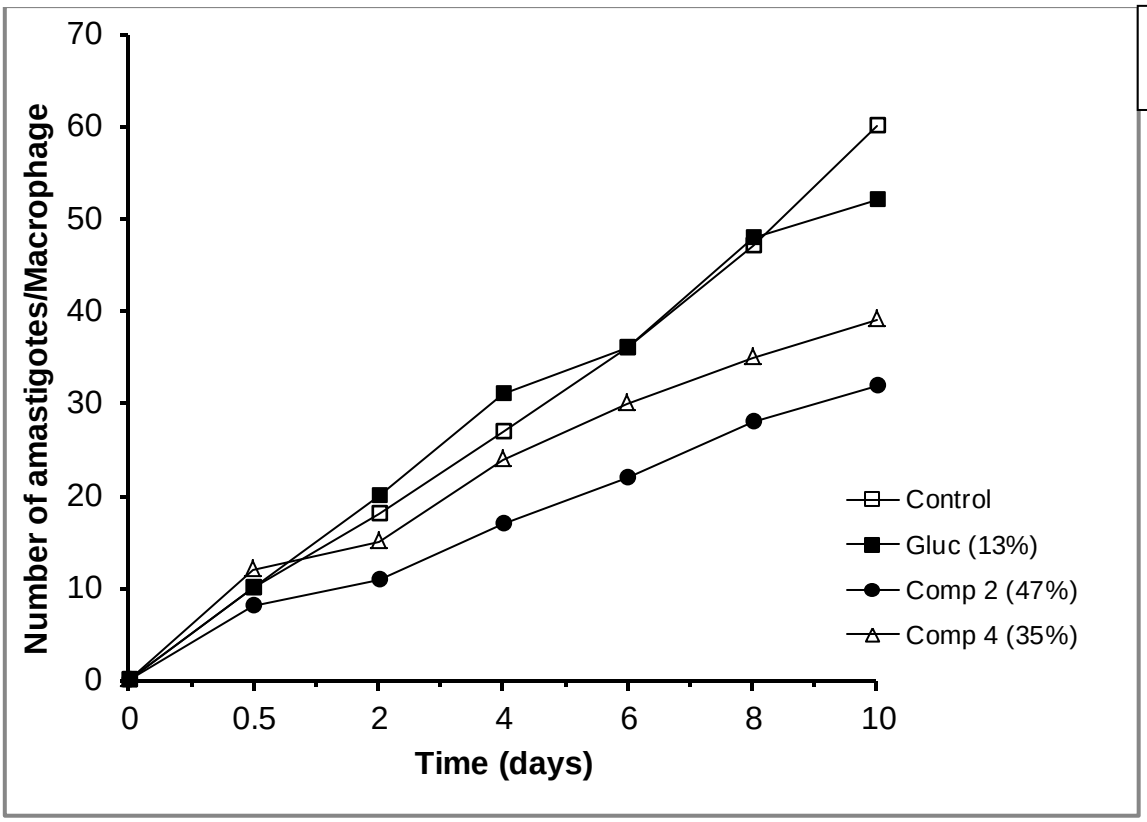
Infectivity assay

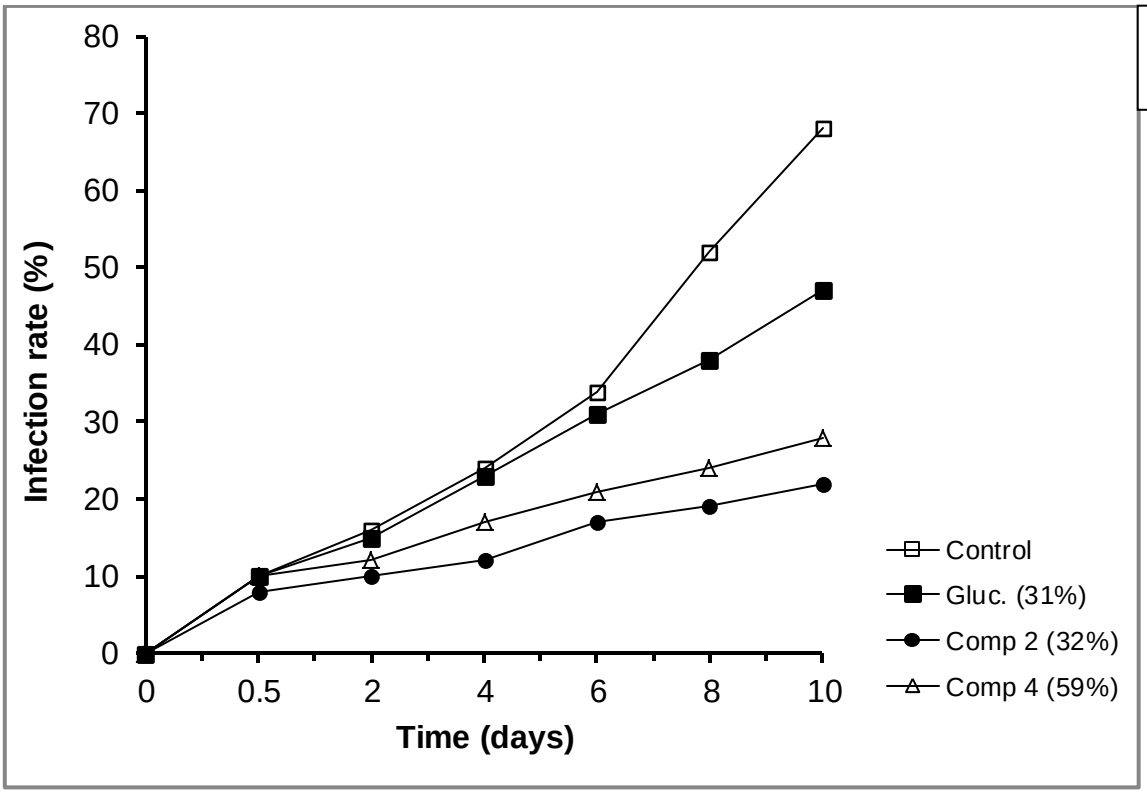
In order to gain a better insight into the activities of the lead compounds **2**, **4** and **5**, their effect on the infectivity and intracellular replication of amastigotes was subsequently determined. Macrophage cells were grown and infected with promastigotes in the stationary phase. Parasites invaded cells and underwent morphological conversion to amastigotes within 1 day after infection. On day 10, the rate of host cell infection reached its maximum (control experiment). We used the IC_{25} of each product as the test dosage. Figure 1 shows the effect of studied derivatives on the infection and growth rates of the three *Leishmania* species. A measure of the average number of amastigotes per infected macrophage led to similar conclusions: in the case of *L. infantum* (Fig. 4A, 4B), all compounds were more effective than glucantime. Amastigote numbers obtained on *L. braziliensis* (Fig. 4C, 4D) also showed that all compounds **2** and **4** were clearly more effective than glucantime under the tested conditions, a similar situation takes places for compounds **2** and **4** in the case of *L. donovani* (Fig 4E, 4F).


A
Infection rate (%)
70
60
50
40
30
20
10
0
0
0.5
2
4
6
8
10
Time (days)
Control
Gluc (24%)
Comp 2 (69%)
Comp 4 (56%)
Comp 5 (49%)


B
Number of amastigotes/Macrophage
70
60
50
40
30
20
10
0
0
0.5
2
4
6
8
10
Time (days)
Control
Gluc. (11%)
Comp 2 (64%)
Comp 4 (58%)
Comp 5 (41%)


C
Infection rate (%)
80
70
60
50
40
30
20
10
0
0
0.5
2
4
6
8
10
Time (days)
Control
Gluc (28%)
Comp 2 (57%)
Comp 4 (44%)


D
Number of amastigotes/Macrophage
70
60
50
40
30
20
10
0
0
0.5
2
4
6
8
10
Time (days)
Control
Gluc (13%)
Comp 2 (47%)
Comp 4 (35%)



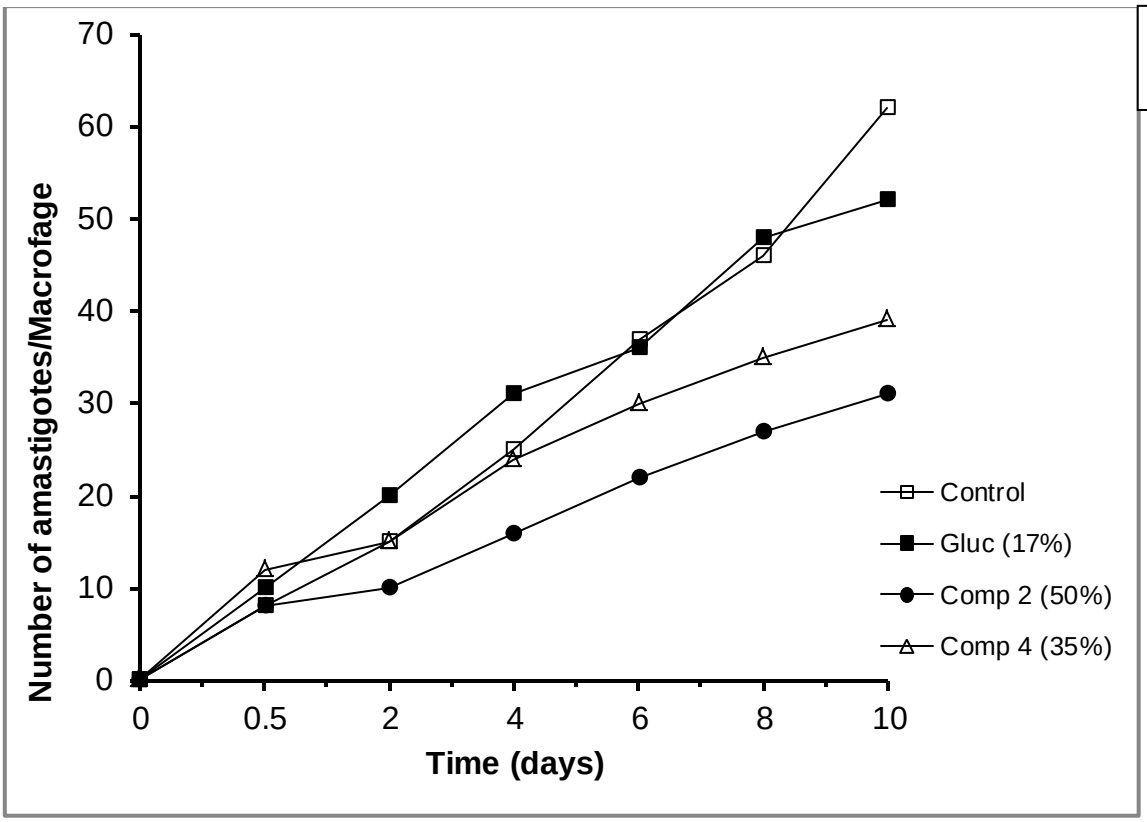


FIG 4 Effects of derivatives **2**, **4** and **5** on the rates of infection (A) and number of amastigotes/ infected cell (B) of *L. infantum*, and effects of derivatives 2 and 4 in rates of infection (C, E) and in number of amastigotes/infected cell (D, F) of *L. braziliensis* and *L. donovani* respectively, when the compounds were tested at the IC_{25}. Values are the means from three separate experiments.

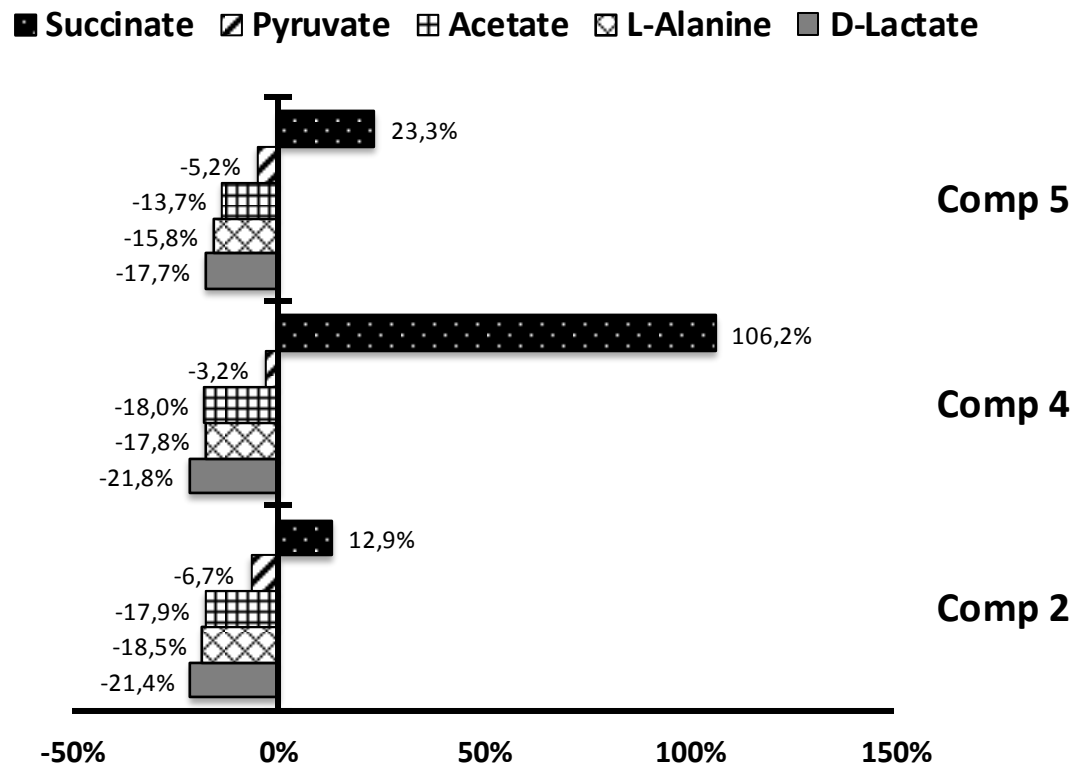
Metabolite excretion effect

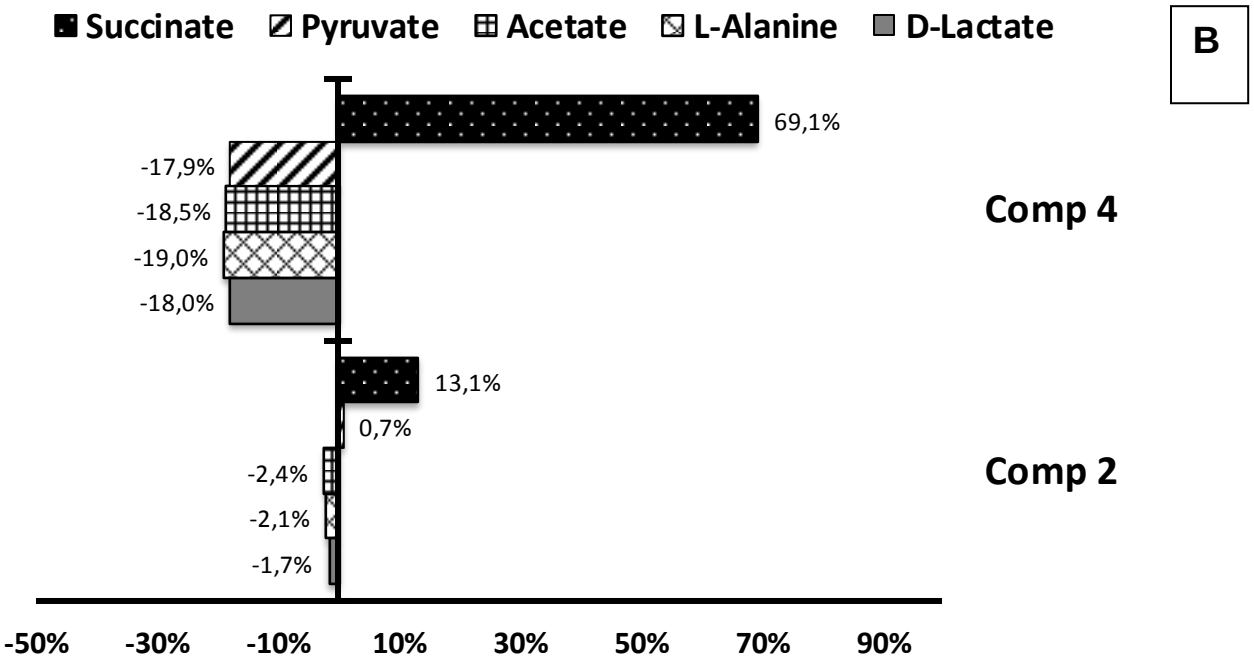
Since trypanosomatids are unable to completely degrade glucose to CO_2 under aerobic conditions, they excrete much of the hexose skeleton into the medium as partially oxidized fragments in the form of fermented metabolites. The nature and percentages of those excretion products depend on the pathway used for glucose metabolism by each species[29]. The final products of glucose catabolism in Leishmania are usually CO_2, succinate, acetate, D-lactate, L-alanine and, in some cases, ethanol[30]. Among them, succinate is the most relevant, because its main role is to maintain the glycosomal redox balance, allowing the reoxidation of NADH produced in the glycolytic pathway. Succinic fermentation requires only half of the phosphoenolpyruvate produced to maintain the NAD+/NADH balance, and the remaining pyruvate is converted inside the mitochondrion and the cytosol into acetate, D-lactate, L-alanine or ethanol, according to the degradation pathway followed by a specific species [31,32].

In our studies, we registered the ^{1}H NMR spectra of promastigotes from the *L. infantum*, *L. braziliensis* and *L. donovani* species after treatment with compounds with significant leishmanicidal activity, at a IC_{25} concentration, and the final excretion products were identified qualitatively and quantitatively.

Succinate production is increased in all the cases. Also, a slight reduction in the other catabolites excretion takes place in almost every case, except in *L.donovani*. These changes can be explained by an enzymatic function alteration due to compound's action. (Figure 5)

Regarding the studies to elucidate the possible mechanism of action, the studied compounds produce greater glucose metabolism alteration because they increase succinate excretion (Fig. 2). Detection of large amounts of succinate as a major end product is an usual feature, because it relies on glycosomal redox balance, enabling re-oxidation of the NADH produced in the glycolytic pathways. It is interesting to mention that the increase in succinate with these compounds indicates catabolic changes that could be related to mitochondria malfunction, due to the redox stress produced by inhibition of the mitochondrion-resident Fe-SOD enzyme [23].


Succinate
Pyruvate
Acetate
L-Alanine
D-Lactate
A
23,3%
-5,2%
-13,7%
-15,8%
-17,7%
Comp 5
106,2%
-3,2%
-18,0%
-17,8%
-21,8%
Comp 4
12,9%
-6,7%
-17,9%
-18,5%
-21,4%
Comp 2
-50%
0%
50%
100%
150%


Succinate
Pyruvate
Acetate
L-Alanine
D-Lactate
B
69,1%
-17,9%
-18,5%
-19,0%
-18,0%
Comp 4
13,1%
0,7%
-2,4%
-2,1%
-1,7%
Comp 2
-50%
-30%
-10%
10%
30%
50%
70%
90%

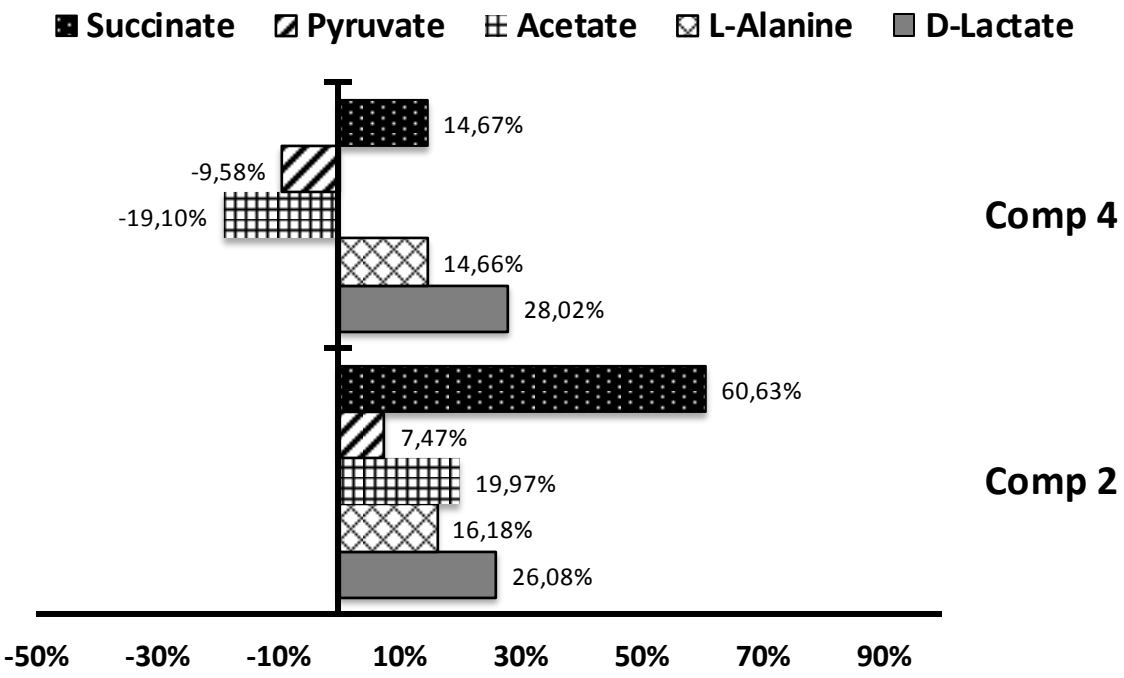


Fig 5 Variation in the area of the peaks (in percentage) corresponding to metabolites excreted by *L.infantum (A), L. braziliensis (B) and L. donovani (C)* forms in the presence of compounds 2 , 4 and 5 at their IC_{25} compared to the area for a control sample, determined by ^{1}H NMR.

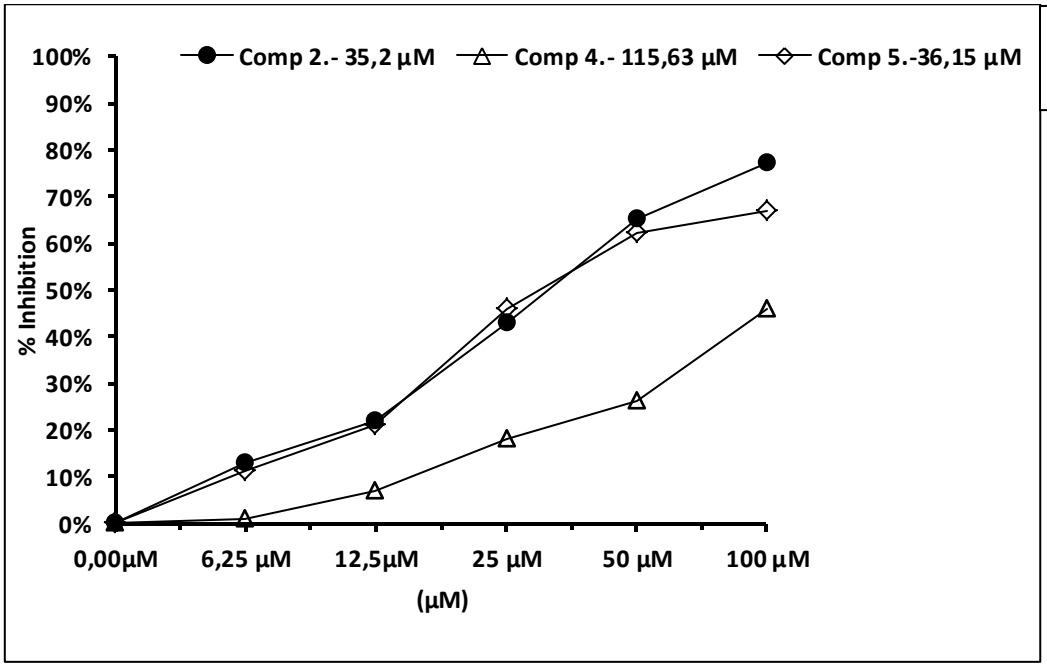
SOD enzymatic inhibition in Leishmania parasites and in human erythrocytes

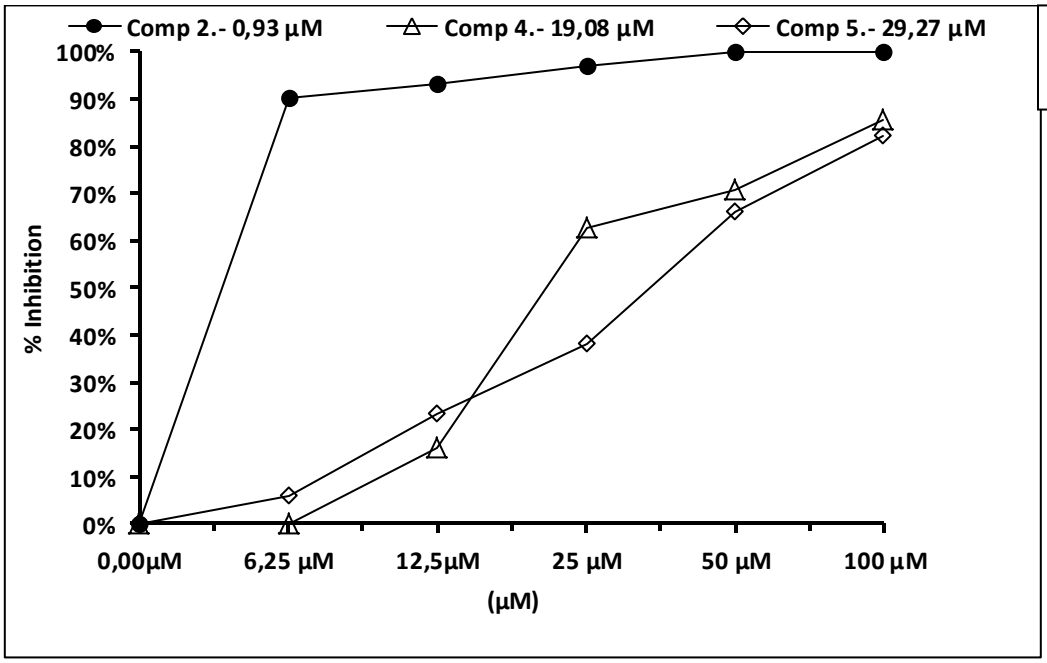
Considering the obtained results, we decided to test the effects of these compounds on Fe-SOD isolated from L. infantum, L. braziliensis and L. donovani over a range of concentrations, from 12.5 to 100 µM. We used promastigote forms of both species, which excrete Fe-SOD when cultured in a medium lacking inactive FBS [21]. The inhibition data obtained are shown in Fig. 6(B–D), and the corresponding IC50 values are included for easier evaluation of the displayed graphs; for comparison, Fig. 6A shows the effects of the same compounds on CuZn-SOD obtained from human erythrocytes.

Regarding the SOD enzymatic inhibition in the Leishmania parasites and in human erythrocytes (Fig. 6), the most remarkable result was the inhibitory effect on Fe-SOD found for the highly antileishmanial compounds **2**, **4**, and **5**, specially compound **2** inhibition of *L. infantum and L. donovani*, that reached a high inhibition rate even at low concentrations whereas its inhibition of human CuZnSOD was clearly lower.

In addition, these compounds led to greater levels of Fe-SOD inhibition. All these data appear to confirm some type of relation between the antileishmanial

activity and the Fe-SOD inhibition, coinciding with the results described in previous work [23, 29].





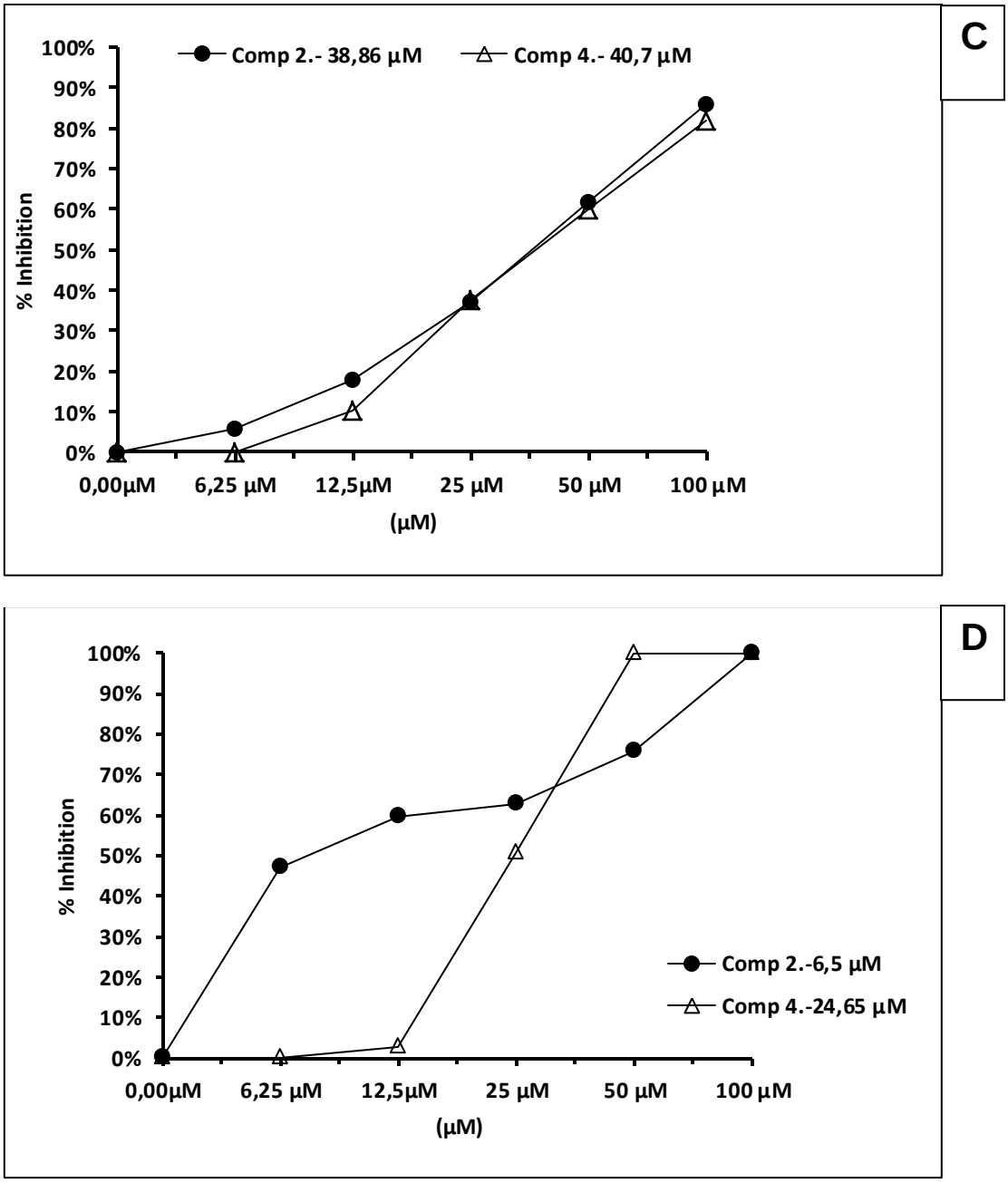


FIG 6 (A) *In vitro* inhibition (in percent) of CuZn-SOD from human erythrocytes by the differences between the activities of the control homogenate and homogenates incubated with the indicated compounds were obtained according to the Newman-Keuls test. *In vitro* inhibition (in percent) of Fe-SOD from *L. infantum* (B), *L. braziliensis* (C) and *L. donovani* (D) promastigote forms by the 2, 4 and 5 derivatives. The concentrations in the keys are IC_{50}, which are the concentrations required to give 50% inhibition and which were calculated by linear regression analysis from the values of the equilibrium constants (*Kc*) at the concentrations employed (12.5 to 100 µM). The values are averages from three different determinations.

Rhodamine assays

In Fig. 4 different readings for rhodamine intensity are shown. In every graphic the shaded interval is the one for control, the others are labeled to their compound number.

Flow cytometry analysis of treated cells labeled with Rho 123 indicated a slight loss of the mitochondrial membrane potential. This could be appreciated by a decrease of the Rho 123 fluorescence intensity.

In order for the mitochondria to work properly, this potential should be as high as possible. We carry out this test in order to determine if the compounds interfere with the mitochondrial membrane function, which could be seen as a lower-than-control rhodamine intensity (as seen in the graphic X axis). In the case that the compound has an effect in the mitochondrial membrane, the intensity would be lower. This only occurs in the case of compounds 2 and 4 for *Leishmania donovani*. No significant membrane alterations were observed in the case of *the other two species.*

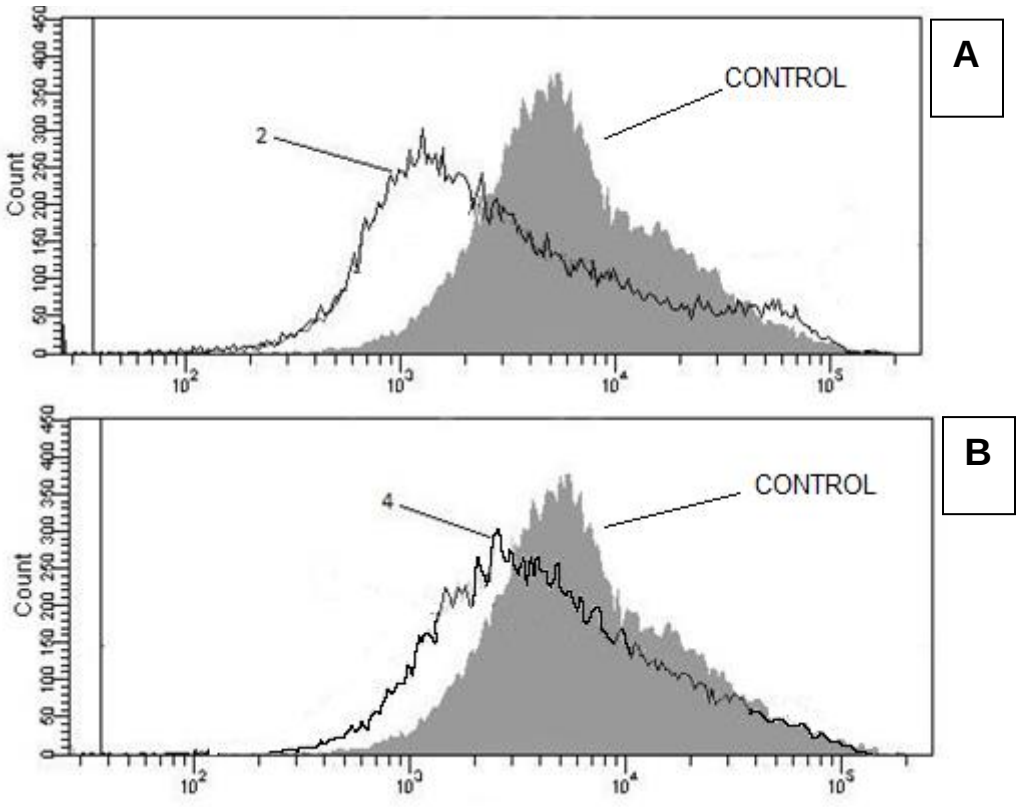


Fig 7. Overlay flow citometric histograms of the effects of **2** and **4** compounds tested at IC_{25} dosage on the mitochondrial membrane potential. Treated cells were labeled with Rho 123 (FL1-H). (A) Overlay of control and compound **2** (A) and **4** (B) and control peaks. The depolarization can be observed as a displacement to the left of the treated peak with respect to the control.

Discussion

Our results suggest that the alterations in mitochondrial membrane potential, induced by drug treatment, cannot explain the leishmanicidal effects for this new family of compounds, as they have no effect on two species and the only alterations that can be observed are too slight to be determinant, which leads to think that this phenomenon helps the enhancement of leishmanicidal activity on *L. donovani,* but it's not as important as the inhibitory effect on the FeSOD activity, which is clearly more cumbersome in comparison. It is also noteworthy that compound **4** can alter the metabolic route significantly in *L. infantum* and *L. braziliensis,* presumably by blocking the metabolism in the early stages of the glycolisis, as the overproduction and excretion of succinate and the depletion of the other metabolites suggest. The same effect can be seen in compound **5**, that has only been effective in *L. infantum*, but it is not as determinant as compound **4** is when altering metabolism. Curiously, this compound has a different effect in *L. donovani*, where enhances production of succinate, L-alanine and D-lactate, but in less quantity than in the other two parasites. This maybe could be explained by the mitochondrial membrane depolarization that can be observed only in this parasite.
In the case of compound **2,** it is more logical that its activity could be explained by its effect on the FeSOD, as there is almost no alteration on the metabolism, while is the most active compound when inhibiting the FeSOD activity, even at low concentrations.

References

[1] WHO | The Weekly Epidemiological Record (WER), No.22, 91, (2016) 285-296

[2] F. Chappuis, S. Sundar, A. Hailu, H. Ghalib, S. Rijal, R.W. Peeling, J. Alvar, M. Boelaert, Visceral leishmaniasis: what are the needs for diagnosis, treatment and control?, Nat. Rev. Microbiol. 5 (2007) 873–882. doi:10.1038/nrmicro1748.

[3] J. Lukes, I.L. Mauricio, G. Schönian, J.-C. Dujardin, K. Soteriadou, J.-P. Dedet, K. Kuhls, K.W.Q. Tintaya, M. Jirků, E. Chocholová, C. Haralambous, F. Pratlong, M. Oborník, A. Horák, F.J. Ayala, M.A. Miles, Evolutionary and geographical history of the Leishmania donovani complex with a revision of current taxonomy, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 9375–9380. doi:10.1073/pnas.0703678104.

[4] D. Menezes, J.P. Bezerra, C.E.S. Guedes, A.L. de O.A. Petersen, D.B.M. Fraga, P.S.T. Veras, Advances in Development of New Treatment for Leishmaniasis, BioMed Research International. (2015). doi:10.1155/2015/815023.

[5] L. Flohé, in P.M. Selzer, Antiparasitic and Antibacterial Drug Discovery: From Molecular Targets to Drug Candidates, John Wiley & Sons, 2009.

[6] B. Manta, M. Comini, A. Medeiros, M. Hugo, M. Trujillo, R. Radi, Trypanothione: a unique bis-glutathionyl derivative in trypanosomatids, Biochim. Biophys. Acta. 1830 (2013) 3199–3216. doi:10.1016/j.bbagen.2013.01.013.

[7] A.-F. Miller, Superoxide dismutases: active sites that save, but a protein that kills, Curr Opin Chem Biol. 8 (2004) 162–168. doi:10.1016/j.cbpa.2004.02.011.

[8] J.F. Turrens, Oxidative stress and antioxidant defenses: a target for the treatment of diseases caused by parasitic protozoa, Mol. Aspects Med. 25 (2004) 211–220. doi:10.1016/j.mam.2004.02.021.

[9] F. Olmo, M. P. Clares, C. Marín, J. González, M. Inclán, C. Soriano, K. Urbanová, R. Tejero, M. J. Rosales, R.L. Krauth-Siegel, M. Sánchez-Moreno, E. García-España, Synthetic single and double aza-scorpiand macrocycles acting as inhibitors of the antioxidant enzymes iron superoxide dismutase and trypanothione reductase in Trypanosoma cruzi with promising results in a murine model, RSC Adv., 2014, 4, 65108–65120

[10] M. Sánchez-Moreno, F. Gómez-Contreras, P. Navarro, C. Marín, I. Ramírez-Macías, M.J. Rosales, L. Campayo, C. Cano, A.M. Sanz, M.J.R. Yunta, Imidazole-containing phthalazine derivatives inhibit Fe-SOD performance in Leishmaniaspecies and are active in vitro against visceral and mucosal leishmaniasis, Parasitology. 142 (2015) 1115–1129. doi:10.1017/S0031182015000219.

[11] (J. A. R. Hartman, R. W. Vachet, J. H. Callahan, Gas, solution, and solid state coordination environments for the nickel(II) complexes of a series of aminopyridine ligands of varying coordination number, Inorganica Chimica Acta 297 (2000) 79–87

[12] A. Raja, V- Rajendira, P. U. Maheswari, R. Balamurugan, C. A. Kilner, M. A. Halcrow, M. Palaniandavar, Copper(II) complexes of tridentatepyridylmethylethylenediamines: Role of ligand steric hindrance on DNA binding and cleavage, J. Inorga. Biochem. 99 (2005) 1717–1732,

[13] F. Groß, A. Müller-Hartmann, Heinrich Vahrenkamp, Zinc Complexes of Condensed Phosphates, 3[‡] Diphosphate-Zinc Complexes with Tridentate Coligands, Eur. J. Inorg. Chem. 2000, 236322370

[14] H-D. Bian, J.-Y. Xu, W. Gu, S-P. Yan, D.-Z. Liao, Z.-H. Jiang, P. Cheng, Synthesis, structure and properties of terephthalate-bridgedcopper (II) polymeric complex with zigzag chain, Inorg. Chem. Commun. 6 (2003) 573–576

[15] E. García-España, M.-J. Ballester, F. Lloret, J. M. Moratal, J. Faus and A. Bianchi, *J. Chem. Soc., Dalton Trans.* 1988, 101−104

[16] M. Fontanelli and M. Micheloni, Program for the automatic control of the microburette and the acquisition of the electromotive force readings (PASAT). Proceedings of the I Spanish-Italian Congress of Thermodynamics of Metal Complexes, Peñíscola, Castellón , Spain, 1990

[17](a) G. Gran, *Analyst* 1952, **77**, 661−671;

[18](b) F. J. Rossotti and H. Rossotti, *J. Chem. Educ.* 1965, **42**, 375−378

[19] P. Gans, A. Sabatini and A. Vacca, *Talanta* 1996, **43**, 1739-1753

[20] P. González, C. Marín, I. Rodríguez-González, A.B. Hitos, M.J. Rosales, M. Reina, J.G. Díaz, A. González-Coloma, M. Sánchez-Moreno, In vitro activity of C20-diterpenoid alkaloid derivatives in promastigotes and intracellular amastigotes of Leishmania infantum, Int. J. Antimicrob. Agents. 25 (2005) 136–141. doi:10.1016/j.ijantimicag.2004.08.010.

[21] I.G. Kirkinezos, C.T. Moraes, Reactive oxygen species and mitochondrial diseases, Semin. Cell Dev. Biol. 12 (2001) 449–457. doi:10.1006/scdb.2001.0282.

[22] M. Sánchez-Moreno, F. Gómez-Contreras, P. Navarro, C. Marín, I. Ramírez-Macías, F. Olmo, A.M. Sanz, L. Campayo, C. Cano, M.J.R. Yunta, In vitro leishmanicidal activity of imidazole- or pyrazole-based benzo[g]phthalazine derivatives against Leishmania infantum and Leishmania braziliensis species, J Antimicrob Chemother. 67 (2012) 387–397. doi:10.1093/jac/dkr480.

[23] C. Marín, I. Ramírez-Macías, A. López-Céspedes, F. Olmo, N. Villegas, J.G. Díaz, M.J. Rosales, R. Gutiérrez-Sánchez, M. Sánchez-Moreno, In Vitro and in Vivo Trypanocidal Activity of Flavonoids from Delphinium staphisagria against Chagas Disease, J. Nat. Prod. 74 (2011) 744–750. doi:10.1021/np1008043.

[24] C. Fernández-Becerra, M. Sánchez-Moreno, A. Osuna, F.R. Opperdoes, Comparative Aspects of Energy Metabolism in Plant Trypanosomatids, Journal of Eukaryotic Microbiology. 44 (1997) 523–529. doi:10.1111/j.1550-7408.1997.tb05734.x.

[25] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[26] W.F. Beyer, I. Fridovich, Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions, Anal. Biochem. 161 (1987) 559–566.

[27] J.M. Sandes, A. Fontes, C.G. Regis-da-Silva, M.C.A.B. de Castro, C.G. Lima-Junior, F.P.L. Silva, M.L.A.A. Vasconcellos, R.C.B.Q. Figueiredo,

Trypanosoma cruzi cell death induced by the Morita-Baylis-Hillman adduct 3-Hydroxy-2-methylene-3-(4-nitrophenyl-propanenitrile), PLoS ONE. 9 (2014) e93936. doi:10.1371/journal.pone.0093936.

[28] S. Nwaka, A. Hudson, Innovative lead discovery strategies for tropical diseases, Nat Rev Drug Discov. 5 (2006) 941–955. doi:10.1038/nrd2144.

[29] M.L. Ginger, Trypanosomatid biology and euglenozoan evolution: new insights and shifting paradigms revealed through genomes equencing, Protist.156(2005)377392.doi:10.1016/j.protis.2005.10.001.

[30] J.J. Cazzulo, Aerobic fermentation of glucose by trypanosomatids, FASEB J. 6 (1992) 3153–3161.

[31] F. Bringaud, L. Rivière, V. Coustou, Energy metabolism of trypanosomatids: adaptation to available carbon sources, Mol. Biochem. Parasitol. 149 (2006) 1–9. doi:10.1016/j.molbiopara.2006.03.017.

[32] P.A.M. Michels, F. Bringaud, M. Herman, V. Hannaert, Metabolic functions of glycosomes in trypanosomatids, Biochim. Biophys. Acta. 1763 (2006) 1463–1477. doi:10.1016/j.bbamcr.2006.08.019.

Legends

Scheme 1 General procedure of synthesis of ligands

Figure 1. Variation with pH of the aliphatic region of the ^{1}H NMR spectra in D_2O of L2 (pD = 1.7, 3.9, 7.5, 10.5). The analysis of the aromatic region of the NMR spectra shows that the greatest variation of the aromatic signals, in particular in the singlet signal attributable to py4, is produced below pH 3.9. This supports that third protonation occurs on the pyridine nitrogen.

Fig 2. ^{1}H NMR spectra in D_2O of L2 in the aromatic zone registered at pD = 1.7, 3.9, 7.5, and 10.5.

Fig 3. ^{1}H NMR spectra in D_2O of L5 recorded at pD = 1.7, 3.9, 7.5, y 10.5.

Fig 4 Effects of derivatives 2, 4 and 5 on the rates of infection (A) and number of amastigotes/ infected cell (B) of *L. infantum*, and effects of derivatives 2 and 4 in rates of infection (C, E) and in number of amastigotes/infected cell (D, F) of *L. braziliensis* and *L. donovani* respectively, when the compounds were tested at the IC_{25}. Values are the means from three separate experiments.

Fig 5 Variation in the area of the peaks (in percentage) corresponding to metabolites excreted by *L.infantum (A), L. braziliensis (B) and L. donovani (C)*

forms in the presence of compounds 2 , 4 and 5 at their IC_{25} compared to the area for a control sample, determined by ^{1}H NMR.

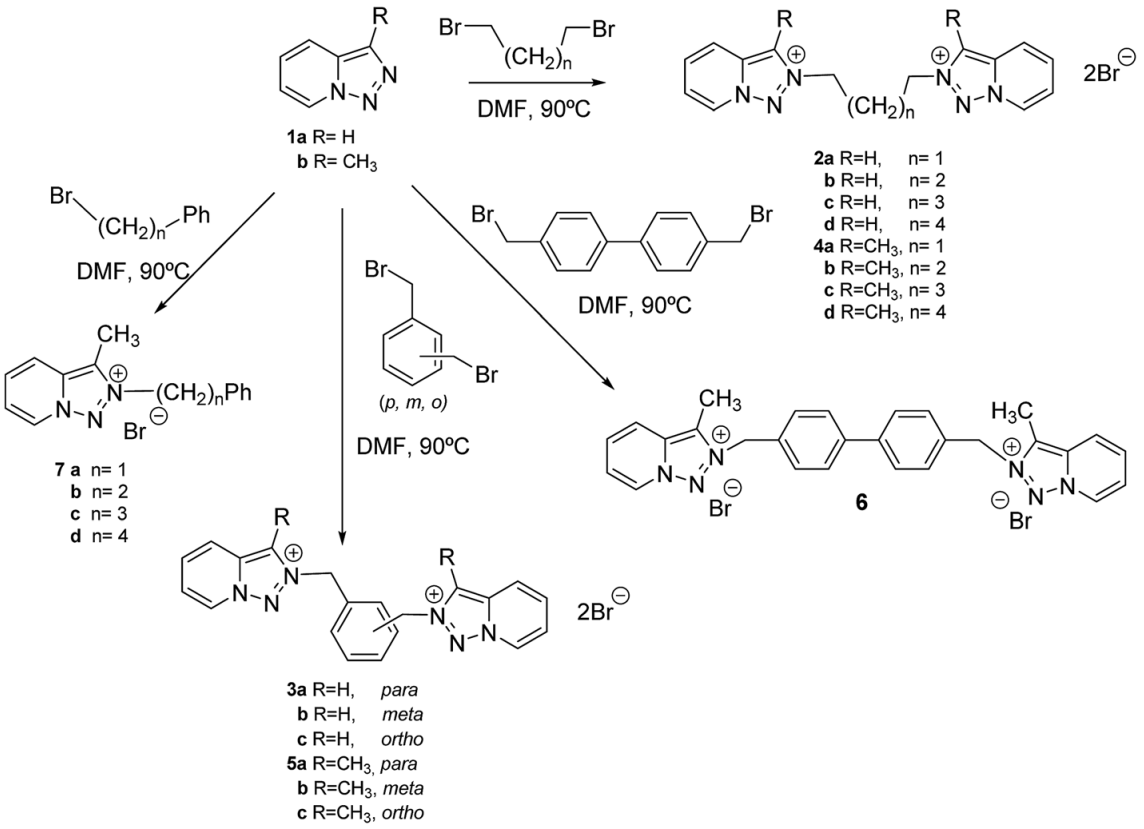
Fig 6 (A) *In vitro* inhibition (in percent) of CuZn-SOD from human erythrocytes by the differences between the activities of the control homogenate and homogenates incubated with the indicated compounds were obtained according to the Newman-Keuls test. *In vitro* inhibition (in percent) of Fe-SOD from *L. infantum* (B), *L. braziliensis* (C) and *L. donovani* (D) promastigote forms by the 2, 4 and 5 derivatives. The concentrations in the keys are IC_{50}, which are the concentrations required to give 50% inhibition and which were calculated by linear regression analysis from the values of the equilibrium constants (*Kc*) at the concentrations employed (12.5 to 100 µM). The values are averages from three different determinations.

Fig 7 Overlay flow citometric histograms of the effects of **2** and **4** compounds tested at IC_{25} dosage on the mitochondrial membrane potential. Treated cells were labeled with Rho 123 (FL1-H). (A) Overlay of control and compound **2** (A) and **4** (B) and control peaks. The depolarization can be observed as a displacement to the left of the treated peak with respect to the control.

6. Discussion

A total of 5 compound families have been studied following the methodology described previously. Some products of each one yielded interesting results and could be studied furtherly to see if they can constitute new treatments for leishmaniasis.

- There were 5 triazolo pyridine disalts from the initial 21 that were active against *Leishmania*. (Compounds **2b**, **4a**, **4c**, **6**, **7d**). [28]

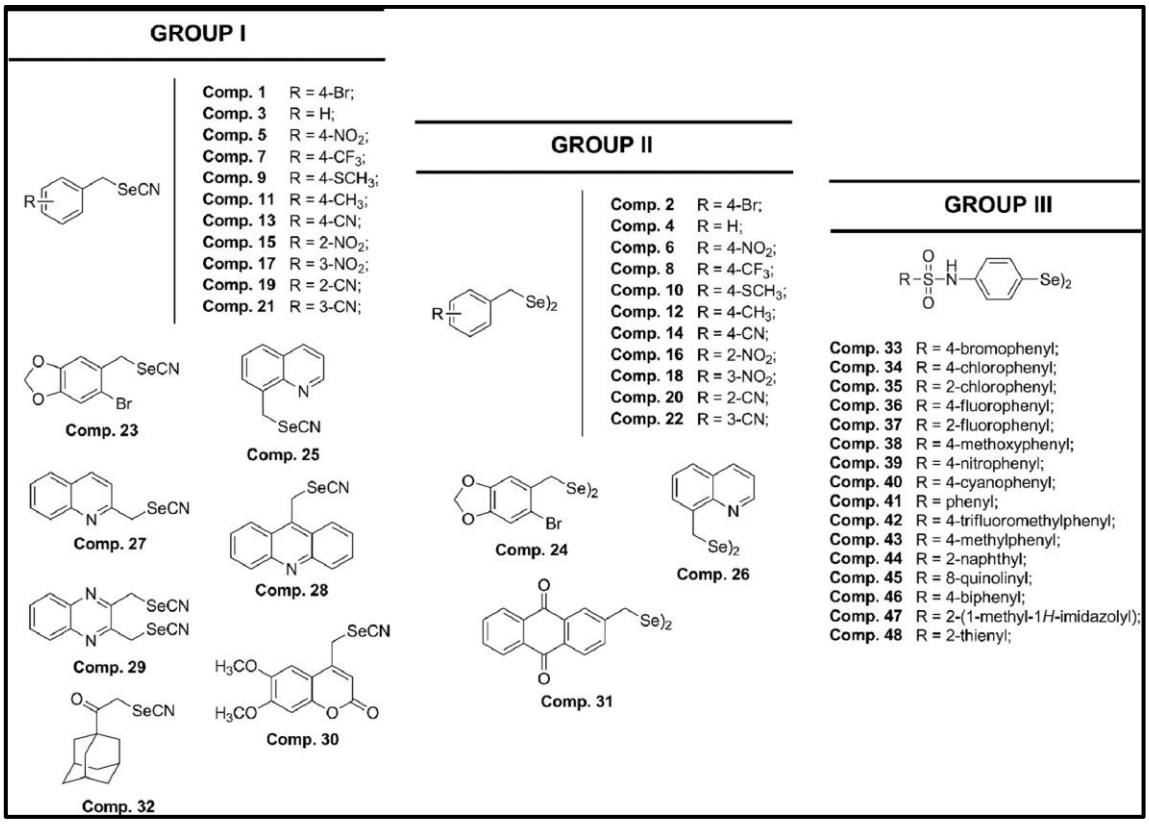


- In the case of the arylamine Mannich base derivatives, 6 out of 20 were active: **3**, **4**, **6**, **7**, **10** and **17**. [29]

Code	Ar_1	amine	Ar_2
1			*p*-trifluoromethylphenyl
2			*p*-chlorophenyl
3			*p*-nitrophenyl
4			*p*-trifluoromethylphenyl
5			*p*-chlorophenyl
6			*p*-nitrophenyl
7			*p*-fluorophenyl
8			Benzyl
9			3-fluorobenzyl
10			4-nitro-2-trifluromethylphenyl
11			3-trifluoromethylphenyl
12			*p*-chlorophenyl
13			4-chloro-2-methoxyphenyl
14			*p*-trifluoromethylphenyl
15			*p*-nitrophenyl
16			*p*-fluorophenyl
17			*p*-trifluoromethylphenyl
18			*p*-chlorophenyl
19			*p*-nitrophenyl
20			*p*-fluorophenyl

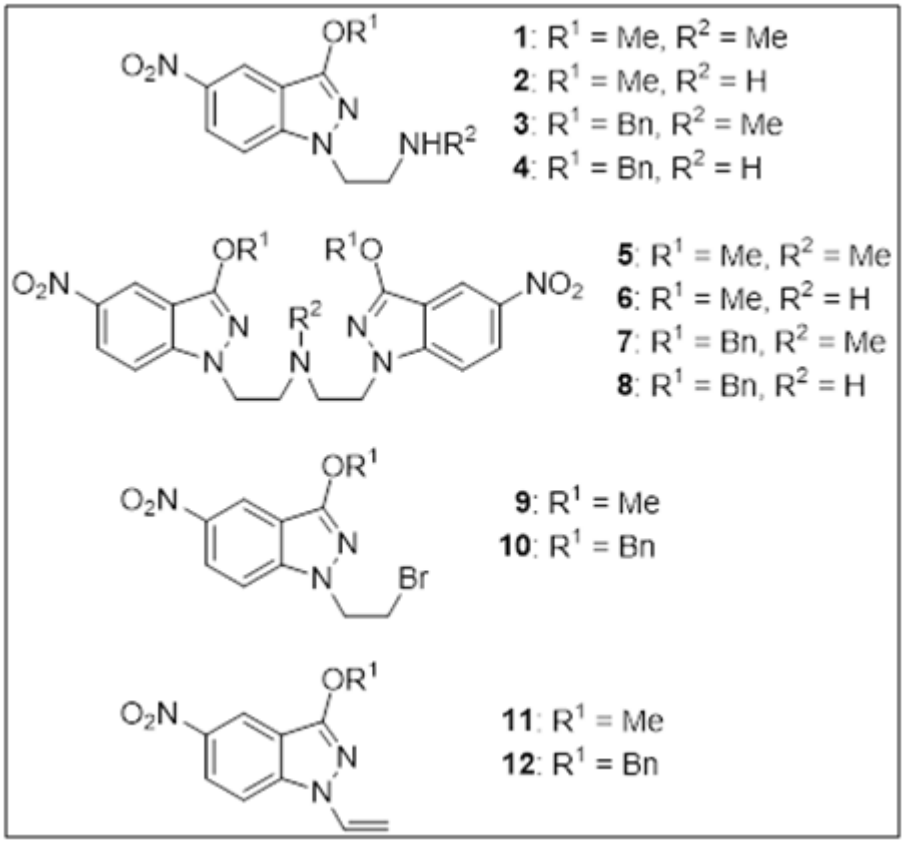
Chemical structures as shown in [30]

- The same number of seleno-compounds showed antileishmanial activity, from the initial 48 roster: **8**, **10**, **11**, **15**, **45** and **48**. [31]

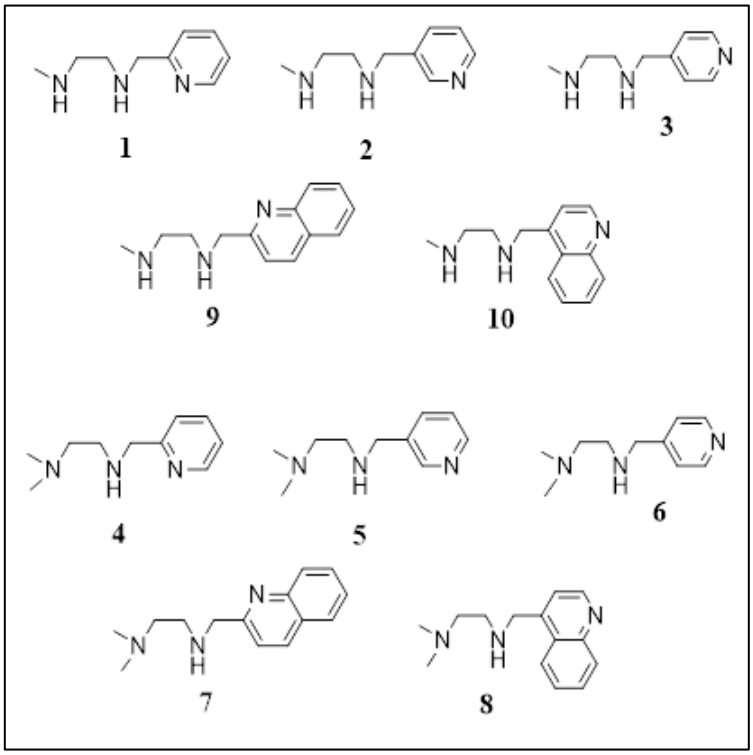


- Again, 6 ethylamines and related compounds out of 12 were active: **1**, **2**, **9**, **10**, **11** and **12**. [32]

DISCUSSION



- Finally, 3 out of 10 small diamines (**2**, **4** and **5**) are active. [33]



The followed screening strategy has been under use for many years and a vast number of chemical families have been tested with it. During the research made for this work, it can be observed that some changes have been made to the experiments. Those modifications obey to the aim of refine and adapt those experiments to the current scientific criteria. As it is needed to be done with current treatments for Leishmaniasis, so it needs to be done with the used techniques, as it is imperative to develop new, effective, accurate, quicker and more affordable experiments that enable the attainment of data.

As it can be seen, normally, only few compounds of each family are effective, and it's to be assumed that this number will narrow when those that have been effective in the *in vitro* experiments are tested in further experiments. For this reason, it will always be needed to continue developing new compounds that will be tested using this screening strategy, as only few candidates will come up of each new family.

As a general conclusion, the studied compounds (except the ethylamine family) are effective because of their ability to reduce the parasite FeSOD activity, which is key to their survival inside the host cell, where the oxidative burst takes place as a primary defense against infection, creating reactive oxigen species (ROS).

This is usually the main explanation for the drug candidate effectiveness, although it is not the only one. Throughout this research work there are some drugs that along the effect on FeSOD activity, have other effects that take place on the parasite increasing the antiparasitary activity, such as metabolic and structural, which prove that those candidates have several targets and that the approach made in our laboratory only focus in some of them, but that doesn't necessarily means that they're the only ones, it is quite possible that there are other effects out of our scope, and those effects could be discovered with other equipment and techniques.

It is also worth to point out that we describe the effects, but not the exact causes of how the candidates trigger the alterations on the parasites, as this would again require deeper studies.

That aside, different experiments can lead to the same conclusions, as happens in the case of ethylamines, where the ^{1}H NMR experiment showed that the metabolic route was being altered and the rhodamine staining and cytometry assay showed that there was a notorious mitochondrial membrane depolarization when *L. donovani* was treated with compound **11**. Those results put together suggest that the glycolytic pathway shift is the result of the depolarization.

In the case of Triazolo pyridine salts, they owe their activity to a great Fe-SOD inhibition, aided with glycolytic pathway alterations. Two factors make this family outstand: their high solubility in water and a very low cytotoxicity towards mammal cells, which made their Selectivity Index way better than the reference drug.

Similarly occurs with the arylamine Mannich base derivatives, these drugs also present low toxicity values, and a strong inhibition of Fe-SOD. They were specially active against *L. donovani* and *L. infantum*, species that belong to the *L. donovani* complex and can cause VL. These candidates show promising results for treatments against the most severe clinical form of the disease.

With respect to the seleno-compounds, the same phenomena are observed: they're active due to the decrease of Fe-SOD activity and metabolic alterations. As stated in the article, it's presumable that they cause these effects because of their small size, that enable them to enter the cristae on the inner mitochondrial membrane, where the glycolysis takes place and where some amounts of Fe-SOD are found.

Again, Fe-SOD activity is reduced when diamines act. It is also worth to mention that number **4** is able to block glycolysis in its early stages in *L. infantum* and *L. braziliensis*.

However, although a general tendency that antileishmanial drug candidates are effective because they primarily reduce significantly the parasitic Fe-SOD activity and alter to some extent the glycolytic pathway, this is not always the case, as happens in

the case of ethylamines, that trigger metabolic alterations like the other families, but they're not potent Fe-SOD activity inhibitors as the others, however, this fact don't make them lesser antileishmanial drugs, as they show a decent activity and could constitute new agents.

To sum up, low toxicity values will always increase product effectiveness as an antiparasitic drug, and a Fe-SOD activity inhibition will result in highly selective compounds, specially if accompanied by a set of metabolic alterations.

7. References

1. Regional committee for Europe. Sixty-second session, 2012; (b) OMS 2012 Technical Report 975. Geneva: WHO; (c) NEGLECTED DISEASE RESEARCH AND DEVELOP-MENT: THE PUBLIC DIVIDE. G-Finder report. 2013

2. Santos CR, Tuon FF, Cieslinski J, de Souza RM, Imamura R, Amato VS (2019) Comparative study on liposomal amphotericin B and other therapies in the treatment of mucosal leishmaniasis: A 15-year retrospective cohort study. PLoS ONE 14(6): e0218786. https://doi.org/10.1371/journal.pone.0218786

3. Reporte Epidemiológico: Córdoba, Argentina, nº 2142, 25 enero 2019 [http://www.reporteepidemiologico.com/wp-content/uploads/2019/01/REC-2142.pdf;

4. Reporte Epidemiológico: Córdoba, Argentina, nº 2168, 29 marzo 2019 http://www.reporteepidemiologico.com/wp-content/uploads/2019/03/REC-2168.pdf]

5. D.M. Claborn, The biology and control of leishmaniasis vectors, Journal of Global Infectious Diseases. 2 (2010) 127–134. doi:10.4103/0974-777X.62866.

6. R. Lainson, L. Ryan, J.J. Shaw, Infective stages of Leishmania in the sandfly vector and some observations on the mechanism of transmission, Memorias do Instuto Oswaldo Cruz. 82 (1987) 421–424. doi:10.1590/s0074-02761987000300015.

7. WHO TRS Series, #949, 2010

8. W. Trager, The development of Leishmania donovani in vitro at 37 degree C; effects of the kind of serum, Journal of Experimental Medicine 97 (1953) 177–188. doi:10.1084/jem.97.2.177.

9. S. Crowther, J.D. Fulton, L.P. Joyner, The metabolism of Leishmania donovani in culture, Biochemical Journal. 56 (1954) 182–185. doi:10.1042/bj0560182.

10. K.P. Chang, D.M. Dwyer, Leishmania donovani. Hamster macrophage interactions in vitro: cell entry, intracellular survival, and multiplication of amastigotes, Journal of Experimental Medicine 147 (1978) 515–530. doi:10.1084/jem.147.2.515.

11. S. Kamhawi, Phlebotomine sand flies and Leishmania parasites: friends or foes?, Trends in Parasitology. 22 (2006) 439–445. doi:10.1016/j.pt.2006.06.012.

12. https://www.cdc.gov/parasites/leishmaniasis/biology.html

13. A.M. Montalvo, J. Fraga, L. Monzote, M. García, L. Fonseca. Leishmaniasis diagnosis: going from microscopic observation of parasite to DNA detection. Revista Cubana de Medicina Tropical. 64(2) (2012) 108-131.

14. Azmi K, Nasereddin A, Ereqat S, Schnur L, Schonian G, Abdeen Z. Methods incorporating a polymerase chain reaction and restriction fragment length polymorphism and their use as a `gold standard' in diagnosing Old World cutaneous leishmaniasis. Diagnostic Microbiology and Infectious Disease. 71 (2011) 151-155.

15. A.M. Croft, N.A. Taylor, K.E. Rodenhurst. Sandflies and leishmaniasis, Lancet. 367 (2006) 112. doi:10.1016/S0140-6736(06)67960-9.

16. G. Baneth, S.E. Shaw. Chemotherapy of canine leishmaniosis, Veterinary Parasitology. 106 (2002) 315–324. doi:10.1016/s0304-4017(02)00115-2.

17. (Bloom & Ryan, Hunter's tropical Medicine and Emerging Infectious Disease (Ninth Edition, 2012)

18. S. Sundar, H. Mehta, A.V. Suresh, S.P. Singh, M. Rai, H.W. Murray, Amphotericin B treatment for Indian visceral leishmaniasis: conventional versus lipid formulations, Clinical Infectious Diseases. 38 (2004) 377–383. doi:10.1086/380971.

19. E.M. Moore, D.N. Lockwood, Treatment of visceral leishmaniasis, Journal of Global Infectious Diseases. 2 (2010) 151–158. doi:10.4103/0974-777X.62883.

20. (S. Nwaka, D. Besson, B. Ramirez, L. Maes, A. Matheeussen, Q. Bickle, N.R. Mansour, F. Yousif, S. Townson, S. Gokool, F. Cho-Ngwa, M. Samje, S. Misra-Bhattacharya, P.K. Murthy, F. Fakorede, J.-M. Paris, C. Yeates, R. Ridley, W.C. Van Voorhis, T. Geary, Integrated dataset of screening hits against multiple neglected disease pathogens, PLoS Neglected Tropical Diseases. 5 (2011) e1412. doi:10.1371/journal.pntd.0001412.)

21. M. Sánchez-Moreno, C. Marín, P. Navarro, L. Lamarque, E. García-España, C. Miranda, O. Huertas, F. Olmo, F. Gómez-Contreras, J. Pitarch and F. Arrebola, Journal of Medicinal Chemistry. 55 (2012), 4231–4243.

22. M. Sánchez-Moreno, A. M. Sanz, F. Gómez-Contreras, P. Navarro, C. Mar´ın, I. Ram´ırez-Macias, M. J. Rosales, F. Olmo, I. Garcia-Aranda, L. Campayo, C. Cano, F. Arrebola and M. J. R. Yunta, Journal of Medicinal Chemistry. 54 (2011), 970–979.

23. P. González, C. Marín, I. Rodríguez-González, A.B. Hitos, M.J. Rosales, M. Reina, J.G. Díaz, A. González-Coloma, M. Sánchez-Moreno, In vitro activity of C20-diterpenoid alkaloid derivatives in promastigotes and intracellular amastigotes of

Leishmania infantum, International Journal of Antimicrobial Agents. 25 (2005) 136–141. doi:10.1016/j.ijantimicag.2004.08.010.

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

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

26. J.F. Turrens. Oxidative stress and antioxidant defenses: a target for the treatment of diseases caused by parasitic protozoa. Molecular Aspects of Medicine. 25 (2004) 211–220. doi:10.1016/j.mam.2004.02.021)

27. J.M. Sandes, A. Fontes, C.G. Regis-da-Silva, M.C.A.B. de Castro, C.G. Lima-Junior, F.P.L. Silva, M.L.A.A. Vasconcellos, R.C.B.Q. Figueiredo. Trypanosoma cruzi cell death induced by the Morita-Baylis-Hillman adduct 3-Hydroxy-2-methylene-3-(4-nitrophenylpropanenitrile). PLoS ONE. 9 (2014) e93936. doi:10.1371/journal.pone.0093936.).

28. Á. Martín-Montes, R. Ballesteros-Garrido, R. Martín-Escolano, C. Marín, R. Guitiérrez-Sánchez, B. Abarca, R. Ballesteros, M. Sanchez-Moreno. Synthesis and in vitro leishmanicidal activity of novel [1,2,3]triazolo[1,5-a]pyridine salts. RSC Advances. 7 (2017) 15715–15726. doi:10.1039/C7RA01070B.

29. A. Martin-Montes, M. Santivañez-Veliz, E. Moreno-Viguri, R. Martín-Escolano, C. Jiménez-Montes, C. Lopez-Gonzalez, C. Marín, C. Sanmartín, R. Gutiérrez

Sánchez, M. Sánchez-Moreno, S. Pérez-Silanes, In vitro antileishmanial activity and iron superoxide dismutase inhibition of arylamine Mannich base derivatives, Parasitology. 144 (2017) 1783–1790. doi:10.1017/S0031182017001123.

30. E. Moreno-Viguri, C. Jiménez-Montes, R. Martín-Escolano, M. Santivañez-Veliz, A. Martin-Montes, A. Azqueta, M. Jimenez-Lopez, S. Zamora Ledesma, N. Cirauqui, A. López de Ceráin, C. Marín, M. Sánchez-Moreno, S. Pérez-Silanes. Correction to In Vitro and in Vivo Anti-Trypanosoma cruzi Activity of New Arylamine Mannich Base-Type Derivatives, Journal of Medicinal Chemistry. 60 (2017) 2604. doi:10.1021/acs.jmedchem.7b00263.

31. Á. Martín-Montes, D. Plano, R. Martín-Escolano, V. Alcolea, M. Díaz, S. Pérez-Silanes, S. Espuelas, E. Moreno, C. Marín, R. Gutiérrez-Sánchez, C. Sanmartín, M. Sánchez-Moreno, Library of Seleno-Compounds as Novel Agents against Leishmania Species, Antimicrobial Agents Chemotherapy. 61 (2017) e02546-16. doi:10.1128/AAC.02546-16.

32. A. Martín-Montes, B. Aguilera-Venegas, R. M. Morales-Martín, R. Martín-Escolano, S.r Zamora-Ledesma, C. Marín, V. J. Arán, M. Sánchez-Moreno. In vitro assessment of 3-alkoxy-5-nitroindazole-derived ethylamines and related compounds as potential antileishmanial drugs. Bioorganic Chemistry. 92 (2019) doi:10.1016/j.bioorg.2019.103274

33. Heterocyclic diamines with Leishmanicidal activity. *NON PUBLISHED, UNDER PATENT PROCESS.*