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In vitro effects of bis(N-[4-(hydroxyphenyl)methyl]-2-pyridinemethamine) zinc perchlorate monohydrate 4 on the physiology and interaction process of *Leishmania amazonensis*

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ABSTRACT

Leishmaniasis is one of the most relevant neglected tropical diseases in the world, affecting 14 million people. Despite the high morbidity, mortality and socio-economic impact, few therapeutic options are available for this disease. To make matters worse, the available molecules have several limitations such as limited efficacy, high cost, side effects and increased resistance. In this context, our group previously synthesized new compounds with anti-leishmania potential being the bis(*N*-[4-(hydroxyphenyl)methyl]-2-pyridinemethamine)zinc perchlorate monohydrate **4** (complex **4**) the most promising one. Therefore, this present work revealed some morphological and physiological changes promoted by complex **4** on *Leishmania amazonensis* promastigotes as well as it was evidenced its potential against intramacrophage amastigotes. Complex **4** promoted a progressive reduction on the promastigotes size and a remarkable increase on the granularity/complexity as judged by flow cytometry. Transmission electron microscopy (TEM) analysis revealed extensive mitochondrial and plasma membrane alterations, although plasma membrane integrity remained. The mitochondrial changes observed by TEM were accompanied by a decrease in the activity of mitochondrial dehydrogenases with increased production of reactive oxygen species. Interestingly, promastigotes also showed changes in lipid metabolism. Besides the very low toxicity to macrophages, complex **4** had a great effect on intramacrophage amastigotes, displaying an IC_{50} of 3.91 μM. Collectively, the data presented here reinforce the potential of aminopyridyl compounds complexed to zinc against *L. amazonensis*. Thus, our work serves as a basis for *in vivo* assays to be designed or even the synthesis of more selective/effective compounds with lower cost.

1. Introduction

Leishmaniasis is a vector-borne protozoonosis endemic in poverty-stricken countries within Southeast Asia, East Africa, and Latin America. On a global scale, there are an estimated 14 million people directly affected by one (or more than one) of the three forms of the disease (visceral, cutaneous and mucocutaneous). Morbidity from leishmaniasis is the third among parasitic diseases and the second most common cause of mortality [1]. As with any neglected disease, pharmaceutical options for fighting leishmaniasis run into a number of problems. Therefore, new therapeutic alternatives, with high efficacy, simple to administer, low toxicity and low cost need to be developed [2].

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Metallodrugs are an interesting therapeutic approach in the treatment of tropical diseases, especially those ones with broad spectrum of activity [3]. In this line, our group previously detailed the synthesis and evaluation of the anti-parasitic activity of novel aminopyridyl metal complexes against *Trypanosoma cruzi* and *Leishmania amazonensis* [4]. Particularly, our results revealed the potent and metal-dependent activity for the aminopyridyl compounds, in which Zn^{2+} complexes presented excellent activity against *L. amazonensis* promastigotes. Among them, bis(*N*-[4-(hydroxyphenyl)methyl]-2-pyridinemethamine)zinc perchlorate monohydrate **4** (complex **4**) (Fig. S1) presented a 50% inhibitory concentration (IC_{50}) value of 1.3 μM. With the concentration of 5 μM, complex **4** reduced the promastigotes' viability in 83.2%, while the maximum concentration employed (10 μM) eradicated the viability of all parasites in culture. Besides that, complex **4** showed excellent selectivity index (90.9) and very low toxicity to RAW macrophages (CC_{50} of 118.2 μM), besides no deleterious effect to *Galleria mellonella* larvae model (no mortality and any melanization of their cuticle at 100 μM) [4].

The results detailed above are excellent indicators that complex **4** may be a promising anti-leishmania candidate and, therefore, further assays should be conducted. In this sense, the present work verified possible physiological targets of the metallic agent in the promastigotes of *L. amazonensis*. Moreover, the effect of complex **4** on the intramacrophage amastigotes was evaluated.

2. Material and methods

2.1. Treatment of L. amazonensis promastigotes with complex 4

For the following assays (items 2.2–2.7), promastigotes (10^5/ml) of *L. amazonensis* Josefa strain (LTCC WDCM-731) were maintained according to described in [4] in the absence or presence of the ½-fold (0.65 μM), 2-fold (2.6 μM) and IC_{50} (1.3 μM) doses of complex **4** (Fig. S1). Afterwards, promastigotes were washed in phosphate-buffered saline (PBS) and the viability was monitored by motility and lack of Trypan blue staining. Untreated cells and promastigotes maintained in the presence of dimethylsulfoxide (DMSO), the drug vehicle, were used in all experiments [4].

2.2. Morphometrics

Promastigotes were fixed in 0.4% paraformaldehyde for 30 min at 25 °C. Each experimental population was acquired in a flow cytometer (FACSCalibur, USA) and mapped ($n = 10.000$) by using a two-parameter histogram of forward-angle light scatter (FSC) *versus* side scatter (SSC) in order to measure two morphometric parameters: size and granularity.

2.3. Transmission electron microscopy (TEM)

Promastigotes were fixed with 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer at 25 °C for 40 min and post-fixed with a solution of 1% OsO_4, 0.8% potassium ferricyanide, and 2.5 mM $CaCl_2$ in the same buffer for 30 min at 25 °C. The samples were dehydrated in an ascending acetone series and embedded in PolyBed 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate; these sections were examined under a Jeol 1200 EX transmission electron microscope (Tokyo, Japan) at Centro Nacional de Biologia Estrutural e Bioimagem (CENABIO) [5].

2.4. Plasma membrane integrity

Promastigotes were incubated with propidium iodide (PI) (1 mg/ml) for 5 min in PBS. Then, parasites were washed in PBS and immediately analyzed in a flow cytometer (FACSCalibur, USA) for log red fluorescence. Parasites permeabilized with 4% paraformaldehyde were used for positive staining [5].

2.5. Neutral lipids accumulation

Promastigotes were incubated with 5 mg/ml Nile red in PBS for 30 min. Then, parasites were washed with PBS and immediately analyzed in a flow cytometer (FACSCalibur, USA) for log red fluorescence [5].

2.6. Mitochondrial function

Parasites were adjusted for the concentration of 10^6 cells/ml, washed with PBS and the formation of formazan was measured by incubating the wells for 3 h in the dark at 28 °C with methylthiazolyldiphenyl-tetrazolium bromide (MTT) (5 mg/ml). Then, the plates were centrifuged at 500 ×*g* for 10 min, the supernatant removed, the pellet with the formazan crystals were dissolved in DMSO and the absorbance measured in a microplate reader (Molecular Devices, USA) at 570 nm [5].

2.7. Production of reactive oxygen species (ROS)

Promastigotes were incubated with the cell permeable probe dichlorofluorescein (H_2DCFDA) (40 μg/ml in PBS) for 30 min at 25 °C. After incubation, the cells were washed in PBS and immediately analyzed in a flow cytometer (FACSCalibur, USA) for log green fluorescence. Parasites treated with hydrogen peroxide (H_2O_2) at 1 mM were used as positive controls of ROS production [5].

2.8. Effects of complex 4 on intramacrophage amastigotes

RAW 264.7 murine macrophages (ATCC TIB-71) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS at 37 °C and 5% CO_2. First of all, MTT assay was performed in order to ensure safety dosages of complex **4** to macrophages, as described in [4]. Macrophages (10^5/ml) were placed to adhere for 4 h in glass coverslips allocated in a 24-well plate containing DMEM (10% FBS). Then, macrophages were pre-stimulated with lipopolysaccharide for a period of 3 h. The cells were washed with sterile PBS and the plates completed with medium (2% SFB). The macrophages were then infected with promastigotes in the proportion of 10:1 (parasites/macrophage) for 1 h. The free parasites were removed by washes with sterile PBS and new medium (2% SFB) was added. The systems were treated for 24 h with 1.56 μM, 3.12 μM and 6.25 μM of the complex **4** and then fixed in Bouin and stained with Giemsa. The association index was obtained by multiplying the percentage of infected macrophages by the number of amastigotes per infected cell. The IC_{50} for amastigotes was determined by linear regression analysis.

3. Results and discussion

The design of metallodrugs appears as an interesting strategy in view of the necessity of low-cost, alternative and more efficient treatments for leishmaniasis. The incorporation of metal ions on several compounds can enhance their effectiveness while decreasing the cytotoxicity effects [3,6,7]. The metal ion zinc is extensively used alone or complexed to organic and inorganic ligands due to its well know antimicrobial activity [8–10]. For instance, zinc sulfate is administered though oral and intralesional routes as an alternative option in the treatment of cutaneous leishmaniais [11]. In this line, our group previously revealed the potent and metal-dependent activity of the novel aminopyridyl Zn^{2+} complex **4** against *L. amazonensis* promastigotes [4]. The complex **4** reduced drastically the viability of the parasites, presenting an IC_{50} value of 1.3 μM. The remaining aminopyridyl analogues synthetized (without Zn^{2+} or substituted by Cu^{2+}) had little or no effect on the parasites, reinforcing the premise that the conjugation with the Zn^{2+} potentiated the efficacy [4].

In the present work, flow cytometry analyses revealed that the treatment of promastigotes with complex **4** induced a progressive

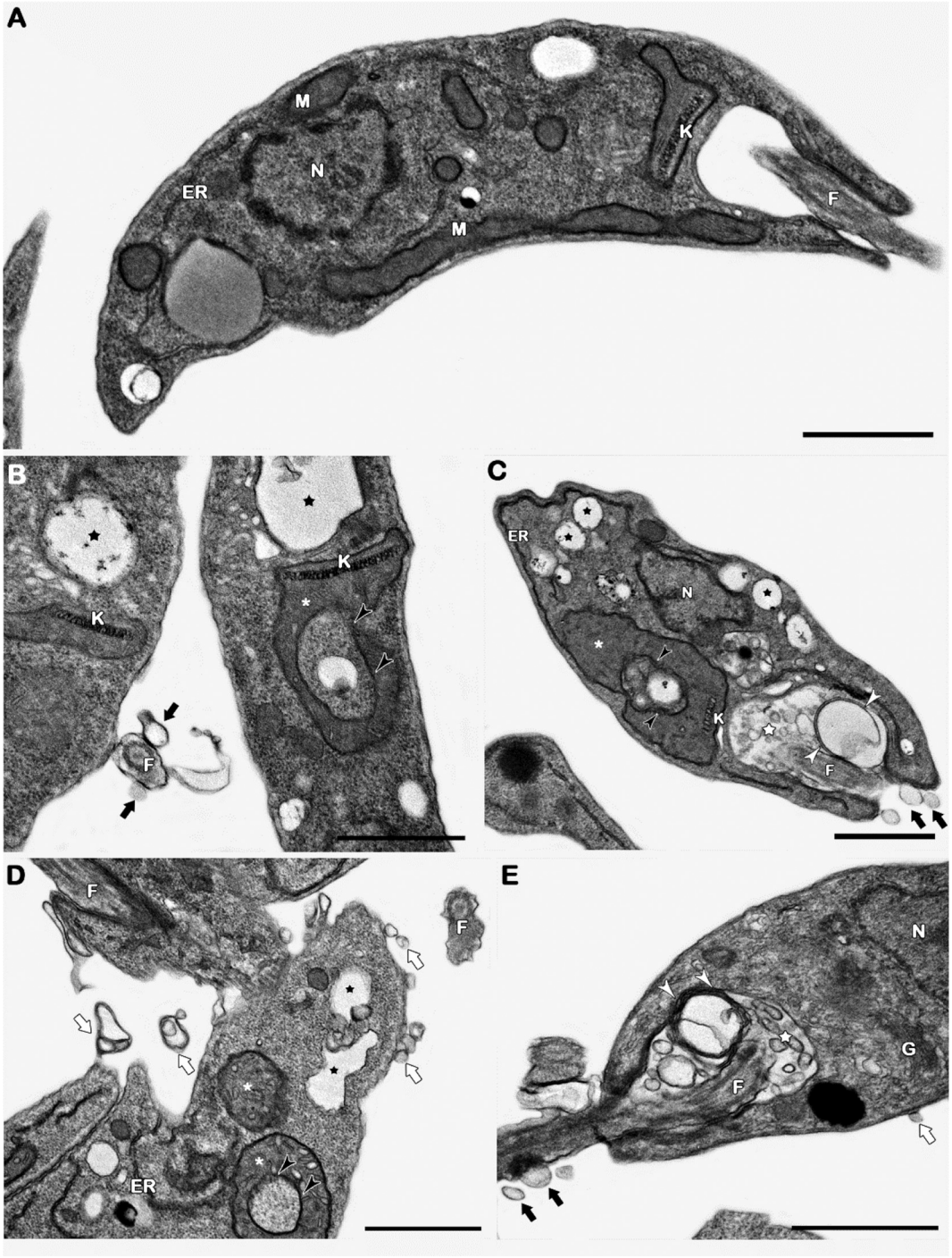

Fig. 1. Effects of complex 4 on the ultrastructure of *L. amazonensis* promastigotes. (**A**) Control parasites presented normal morphology of organelles. (**B-E**) Promastigotes treated with the IC_{50} dosage (1.3 μM) showed mitochondrial swelling (*white asterisks*), with concentric membranar structures inside the organelle (*black arrowheads*). Additionally, the treatment with compound 4 promoted the intense formation of extracellular vesicles inside the flagellar pocket (*white stars*), being the presence of concentric membranar structures also observed (*white arrowheads*), as well as prominent cytoplasmic vacuolization (*black stars*) and the appearance of membranar shedding (*white arrows*) and/or small vesicles (**black arrows**) distributed throughout the parasite's plasma membrane. Nucleus (N), kinetoplast (K), mitochondrion (M), endoplasmic reticulum (ER), flagellum (F) and Golgi (G). Bars = 0.5 μm. The images are a representative set of three independent experiments.

reduction on the promastigotes size (Forward scatter-FSC), with the IC_{50} dosage promoting a reduction of 34.7% (Fig. S2). Conversely, the treatment induced a remarkable increase (18.2% at IC_{50}) on side scatter (SSC), altering other important morphometric parameter. SSC signal represents the light refracted or reflected at the interface between the laser and intracellular structures, providing additional information about the internal complexity/granularity of a cell (Fig. S2).

Then, we proceeded a TEM analysis of the promastigotes to visualize the ultrastructural alterations promoted by the treatment with the IC_{50} dosage of complex **4**. The mitochondrion of treated promastigotes showed a dilated morphology (Fig. 1B–D), and alterations in the plasma membrane were also extensively detected throughout the parasite's body. Membranar shedding and the presence of small vesicles were observed in all extension of the plasma membrane, including inside the flagellar pocket (Fig. 1B–E). Additionally, cytoplasmic vacuolization (Fig. 1B–D) as well as concentric membranar structures were found

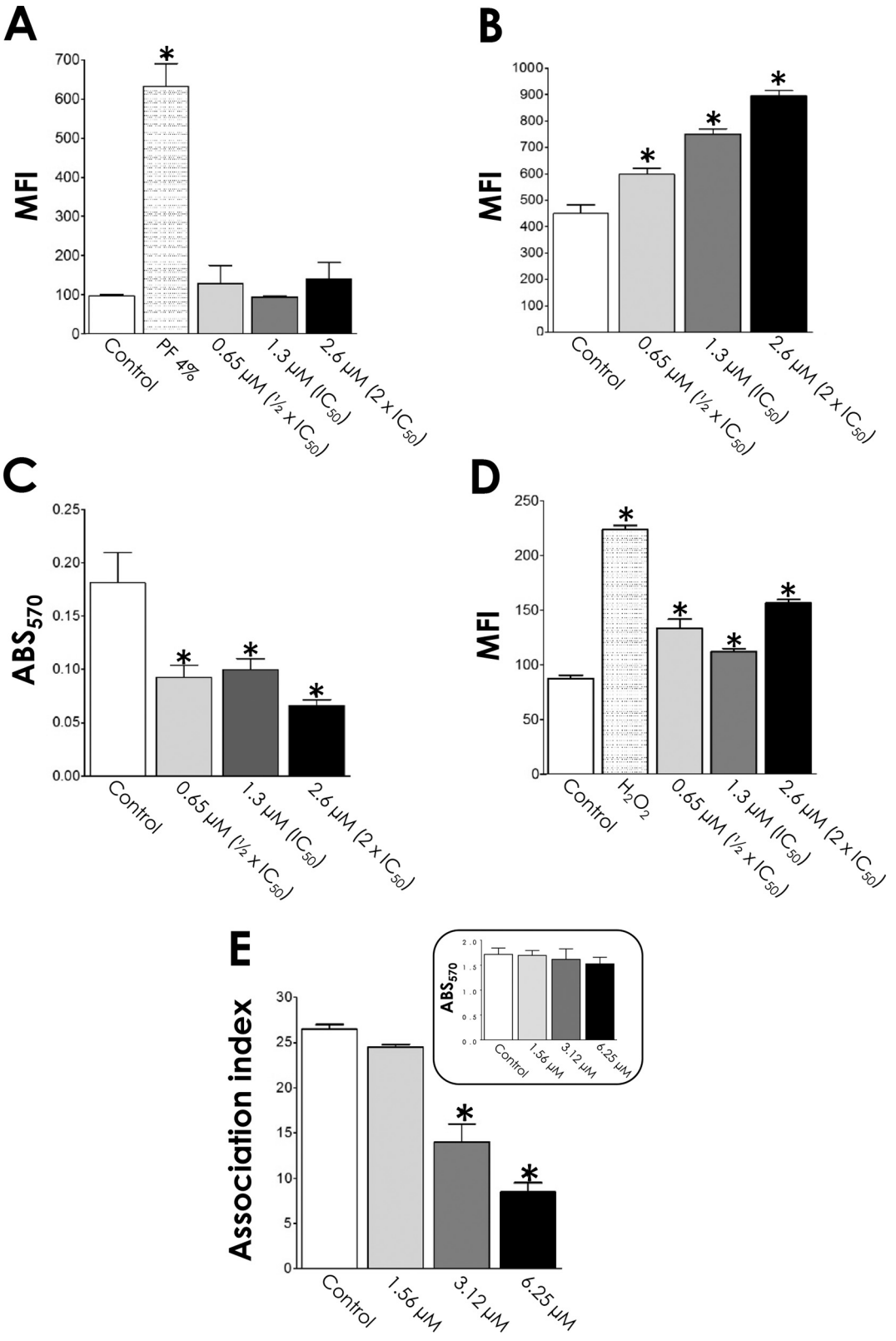

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Fig. 2. Effects of complex 4 on *L. amazonensis* promastigotes physiology and on the susceptibility of intramacrophages amastigotes. In all experimental conditions in A, B, C and D, the assays were carried out with parasites untreated (control) and treated with different concentrations (½ × LD_{50}, LD_{50} and 2 × LD_{50}) of compound 4 for 72 h. (A) Flow cytometry analysis of the incorporation of PI in control and treated promastigotes. The results express the mean of the fluorescence intensity (MFI) of each population. Parasites fixed with 4% paraformaldehyde (PF) were used as non-viable cells (PI-positive staining). (B) The incorporation of Nile red was quantified by flow cytometry in the absence (control) or in the presence of complex **4** at the indicated dosages. The results are expressed as MFI. (C) The mitochondrial dehydrogenase activities were determined spectrophotometrically (ABS, absorbance) by MTT assay. (D) Production of ROS was analyzed by flow cytometry in the control and treated cells incubated with the green fluorescent probe H_2DCFDA. Cells treated with 1 mM H_2O_2 were used as a positive control to the intracellular generation of ROS. The results are expressed as MFI. (E) RAW macrophages were pre-stimulated with lipopolysaccharide (LPS) for a period of 3 h. Then, the macrophages were infected with promastigotes at a ratio 10:1 (parasite/cell) for 1 h at 37 °C. After this period, systems were washed to remove non-internalized parasites and then treated or not (control) with complex 4 at different concentrations for 24 h. The concentrations employed did not display toxic effects to RAW cells, as demonstrated by MTT assay in the inset. Data shown are the mean ± standard deviation (SD) of three independent experiments performed in triplicate. The symbols represent significant statistical differences compared to the respective negative controls. Data were analyzed statistically ($P < 0.01$) by means of one-way analysis of variance (ANOVA) using GraphPad Prism software 6.0 (GraphPad Software Inc., La Jolla, CA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inside the mitochondrion and flagellar pocket (Fig. 1B–E). Some of these ultrastructural changes could be suggestive of programmed cell death (PCD) that includes phenotypically distinct processes [12]. In this context, some of the ultrastructural alterations, such as the formation of concentric membrane structures are suggestive of autophagy. The mitochondrial swelling and vacuolization are common in apoptosis and necrosis, but the presence of membrane structures within the mitochondrion may indicate degradation by autophagy, a tentative of the parasite to survive in the stress conditions promoted by the drug [12]. In addition, one of the major changes observed in the treatment was the alterations in the plasma membrane, where the membrane shedding suggests apoptosis [12].

Given the alterations in parasite membranes described herein, it is also plausible to propose the components of the plasma membrane as a possible target of complex **4**. Indeed, some works have reported that zinc complexes recognize anionic cell surfaces through interaction of the Zn^{2+} with the phospholipids in the plasma membrane [13]. However, PI staining did not confirm membrane injuries since this fluorescent DNA intercalating agent did not permeate the parasite cells (Fig. 2A), suggesting that the plasma membrane is intact and, therefore, giving no evidence of classical necrosis [12]. Therefore, besides being suggestive of apoptosis, the membrane changes that were visualized by TEM, in particular the presence of membranar shedding and the intense presence of vesicles inside the flagellar pocket, can also be an attempt of the parasite to externalizing the compound as a form of defense [14,15].

In order to evaluate the effects of complex **4** on the lipid metabolism, additional analysis was also performed by incubating promastigotes with Nile red. The results showed that in all the concentrations used, the compound induced a huge dose-dependent increase in the accumulation of neutral lipids (Fig. 2B). It is known that disorders on mitochondrial functions and excessive ROS production can be associated to imbalance in lipid metabolism [16]. Indeed, TEM analyzes showed ultrastructural alterations on the mitochondrion of *Leishmania* parasites. These morphological changes were accompanied by metabolic changes in the organelle. By measuring the activity of mitochondrial NAD(*P*)H-dependent dehydrogenases, we could observe that there was a significant and proportional reduction (around 50%) with all tested concentrations of complex **4** (Fig. 2C). After that, we also decided to determine the effects of complex **4** in the generation of oxidative stress using the probe H_2DCFDA. In this context, the compound induced a significant generation of ROS in all tested concentrations (Fig. 2D). Several studies have demonstrated that zinc complexes are interesting for treating leishmaniasis, especially for their capacity to generate extensive ROS [8–10]. Dysfunction of electron transport chain can result in excessive release of ROS from the mitochondrion. The deviation of electrons from the mitochondrial complex II in trypanosomatids is the primary source of endogenous ROS [17].

We also investigated the effects of complex **4** on the infection inside macrophages, since *Leishmania* amastigotes have an important role in the persistence and spread of infection to other body sites [1]. Previously, it was demonstrated that the compound **4** showed low toxicity to RAW macrophages [4], with a CC_{50} of 118.2 μM. For the post-treatment assay, we choose the highest concentrations that maintained at least 90% of viable macrophages. This low toxicity was also ensured by an additional MTT assay (Fig. 2E, inset). Thereafter, our results evidenced that the two highest tested concentrations of complex **4** (3.12 μM and 6.25 μM) were able to significantly reduce the number of intracellular amastigotes in a clear time- and dose-dependent manner. The highest concentration used showed a 67.9% reduction in the number of amastigote forms in relation to the control (Fig. 2E) and the IC_{50} calculated for these forms was 3.91 μM. With the IC_{50} value, the selectivity index for amastigotes forms was calculated, resulting in the ratio of 30.23, which means, in theory, that the compound is at least 30 times more toxic to the parasite than to a host cell.

The IC_{50} value for amastigotes found herein was 3 times higher than that found previously for promastigotes [4]. These results may be due to the differences in the susceptibility profiles related to the distinct mechanisms of uptake/extrusion and cellular targets between amastigote and promastigote forms [18]. Another factor to consider is that complex **4** was in direct contact with promastigotes whereas for amastigotes, it first had to permeate through the membrane of the RAW macrophages in order to reach parasites within acidic phagolysosomal vesicles [19]. Therefore, amastigotes are subjected to lower concentrations of complex **4**, in addition to the possibility of its degradation by macrophage enzymes.

4. Conclusion

Altogether, our results indicate that complex **4** affects the physiology of promastigotes of *L. amazonensis*, presenting low toxicity to the host cells and a good effect against intramacrophage amastigotes. Complex **4** has the advantage to be easily synthesized in few steps with low cost; thus, it can be considered as an interesting candidate for further optimization to use against *Leishmania* spp.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2021.102376.

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