Acta Brasiliensis 5(1): 1-6, 2021 *Artigo Original*
http://revistas.ufcg.edu.br/ActaBra http://dx.doi.org/10.22571/2526-4338474

In vitro evaluation of antileishmanial activity of copper (I) complexes

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Received: Outubro 21, 2020 / Accepted: Dezembro 8, 2020 / Published online: January 27, 2021

Abstract

In the research for the development of new drugs for the therapy of American tegumentary leishmaniasis, copper has been studied for its antileishmania activity. This study aims to report the activity of three copper(I) complexes on parasites of the species *Leishmania amazonensis* and *L. guyanensis*. The metal complexes were tested according to *in vitro* antileishmanial assays, against promastigote and amastigote forms of the most prevalent species in the state of Amazonas, Brazil. Cytotoxicity of the complexes was evaluated in murine macrophage-like cell line (MJ774). The results of the *in vitro* assays indicated that, among the copper complexes tested, the homoleptic phosphine complex $[Cu(thp)_4][PF_6]$(thp=tris-hydroxymethylphosphine) presented promising activity against the evolutionary forms of *L. amazonensis*, and obtained a IC_{50} of 26.45 and 24.61 µM in a period of 48 and 72 h, respectively. The results for copper complex at concentration 160 µM in amastigote forms showed a decrease in the infection index (32% of infected cells) and, in the cytotoxicity assay with MJ774, 52.43% of cell viability was observed. The results showed that the complex $[Cu(thp)_4][PF_6]$ presented significant biological activity, indicating a need for future *in vivo* studies.

Keywords: Bioassays, Leishmania, leishmaniasis, metal complexes.

Avaliação *in vitro* da atividade antileishmania de complexos de cobre (I)

Resumo

Na busca do desenvolvimento de novos fármacos para a terapia da leishmaniose tegumentar americana, o cobre tem sido estudado quanto a sua atividade antileishmania. Esse estudo tem como objetivo relatar a atividade de três complexos de cobre (I) sobre parasitas das espécies *Leishmania amazonensis* e *L. guyanensis*. Os complexos metálicos foram testados por meio de ensaios antileishmania *in vitro* contra as formas promastigota e amastigota das espécies mais prevalentes no estado do Amazonas, Brasil. A citotoxicidade dos complexos foi avaliada em linhagem de células semelhantes a macrófagos murinos (MJ774). Os resultados dos ensaios *in vitro* indicaram que, entre os complexos de cobre testados, o complexo homoléptico de fosfina [Cu(thp)4][PF6](thp=tris-hidroximetilfosfina) apresentou atividade promissora contra as formas evolutivas de *L. amazonensis*, e obtiveram IC_{50} de 26,45 e 24,61 µM em um período de 48 e 72 h, respectivamente. Os resultados para o complexo de cobre na concentração de 160 µM nas formas amastigotas reportaram diminuição no índice de infecção (32% das células infectadas) e, no ensaio de citotoxicidade com MJ774, observou-se 52,43% de viabilidade celular. Os resultados evidenciaram que o complexo $[Cu(thp)_4][PF_6]$ apresentou atividade biológica significativa, indicando a necessidade de futuros estudos *in vivo*.

Palavras-chave: Bioensaios, *Leishmania*, leishmanioses, complexos metálicos.

Introduction

American cutaneous leishmaniasis (ACL) is a disease caused by different species of the genus *Leishmania*, and is classified as cutaneous and mucocutaneous (Blanco & Nascimento-Júnior, 2017). ACL is an endemic disease in 92 countries, and in Brazil it has a high incidence in some of the poorer regions (WHO, 2020).

In 2018, the northern region of Brazil registered 7,519 cases, followed by the northeastern (3,717 cases), midwestern (2,086), southeastern (1,646) and southern (158)

regions, with the highest numbers of registered cases in the state of Pará (3,081 cases), and Amazonas with 1,684 cases (SINAN, 2019). The northern region of Brazil contributes to the highest incidence of ACL in Brazil due to the diversity of species of *Leishmania* and sandflies vectors, which is linked to the environmental conditions that favor this transmission.

Currently, meglumine antimoniate, amphotericin B and pentamidine isethionate are the standard drugs adopted for the treatment of leishmaniasis, however the combination of parenteral administration, toxicity and parasitic resistance justifies the search for new active principles (Bastos, Boechat, Hoelz, & Oliveira, 2016).

The development of metal complexes aimed at treating neglected diseases offers opportunities for pharmaceutical products because of their specific and unique physicochemical properties (Ong, Roy, Andrews, & Gasser, 2019), variety of coordination numbers, different oxidation states (Selvaganapathy & Raman, 2016) and the possibility of using a multi-target molecule approach for increasing drug efficiency (Arndt *et al*., 2017).

Arsenic complexes, mercury, bismuth, platinum, antimony, gold, iron, gadolinium, samarium, technetium, palladium and others are, or have been, used in medicine (Ong *et al*., 2019). However, copper has been the subject of many studies since it is an essential metal for most aerobic organisms. It is present as a structural and catalytic cofactor of several enzymes (Puig & Thiele, 2020) and in several studies, such as that by Gandin *et al*. (2014), it has been shown that copper(I) complexes are capable of inhibiting the growth of tumor cells *in vitro* and *in vivo*. Arndt *et al*. (2017) and Portas, Miguel, Yokoyama-Yasunaka, Uliana, and Espósito, (2012) demonstrated that different Cu(II) complexes have antileishmanial activity *in vitro* against *L. (L.) amazonensis* promastigotes.

According to Tahghighi (2014), the activity of metal complexes depends on the metal oxidation state and on the nature of the ligands. Thus, considering that the mechanism of uptake of copper cells implies a reduction from Cu(II) to Cu(I) (Ren *et al*., 2019), studies such as the one by Saeed *et al*. (2018) have focused mainly on synthesizing Cu(I) complexes as potential antileishmanial agents.

A clear example of the importance of the oxidation state of the metal is represented by Navarro *et al*. (2003a) and Navarro *et al*. (2003b) studies, in which Cu(II) and Cu(I) complexes were synthesized using the ligands ppz (dipyride[3,2-a:2',3'-c]phenazine) and dpq (dipyride[3,2-a:2',3'-h]quinoxoline). The compounds showed promising activity, but interestingly the Cu(I) $[Cu(dppz)_2]BF_4$ derivative was shown to be more active in *L. (L.) mexicana* promastigotes than the corresponding Cu(II) derivative $[Cu\ (dppz)_2(NO_3)](NO_3)$ that was tested on *L. (V.) braziliensis* promastigotes.

This study aims to report the activity of three copper(I) complexes on parasites of the species *L. amazonensis* and *L. guyanensis*.

Materials and Methods

Copper complexes

The synthesis of the copper(I) complexes $[Cu(thp)_4][PF_6]$ (thp = tris-hydroxymethylphosphine) (1), $[Cu(PTA)_4][BF_4]$ (PTA = 1,3,5-triaza-7-phosphadamantane) (2) and $[HB(pz)_3Cu(PTA)]$ ($HB(pz)_3$ = tris(pyrazolyl)borate) (3) (Figure 1) was carried out according to the procedures of Marzano *et al*. (2008), Porchia *et al*.(2009) and Gandin *et al.* (2014).

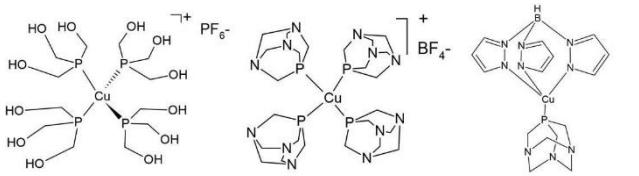

Figure 1. Chemical structures of Cu(I) complexes used in this study.

To perform the biological assays, the copper complexes were dissolved in dimethyl sulfoxide (DMSO - Vetec®, 4% v/v) and Schneider medium, filtered through a membrane with 0.22 µm pores, under sterile conditions. In the promastigote assay, concentrations ranging from 160 to 10 µM of the copper complexes were tested using serial dilution, and for the cytotoxicity and amastigote assays, a 160 µM concentration of the copper complex was evaluated. For control tests, the standard drug used was pentamidine isethionate (Pentacarinat®) (Sanofi Aventis Farmacêutica Ltda, Brazil), diluted in Schneider's insect medium (Sigma-Aldrich®), and filtered through a syringe filter (Kasvi, 0.22 µm), under sterile conditions. In the promastigote and cytotoxicity assays, ranging concentrations from 9.1 to 147 µM of the Pentacarinat® were tested by serial dilution means and in amastigotes (intracellular form) assays, 147 µM concentration of the Pentacarinat® was evaluated.

Origin and maintenance of parasites

Leishmania (Leishmania) amazonensis (MHO/BR/2006/IM5584) and *Leishmania (Viannia) guyanensis* (MHO/BR/95/M4147) were characterized, cryopreserved and maintained in the Laboratory of Leishmaniasis and Chagas' Disease, National Institute of Amazonian Research – INPA (Manaus, Amazon, Brazil). The parasites were cultured in a Novy-MacNeal-Nicolle medium (NNN). This culture was expanded in complete Schneider's insect medium (Sigma-Aldrich®), which was supplemented with 10% of inactivated fetal calf serum (iFCS) and gentamicin 40 µg mL^{-1} (Novafarma, Brazil). The J774 macrophages were kept in culture bottles with complete RPMI 1640 medium (Himedia®), supplemented with 20% of iFCS, and incubated in an oven at 37 °C.

In vitro promastigote assay

In 96-well plates promastigote forms ($2x10^6$ cells/mL) were exposed to different concentrations of copper complexes (10 to 160 µM). The control groups consisted of promastigotes treated with Pentacarinat® (9.1 to 147 µM) and promastigotes without treatment, the DMSO concentration ranged from 0.06% to 1% in the plate. The biological activity was determined by inhibiting the growth

of promastigote forms of *Leishmania* spp. and quantifying viable promastigotes in a hemocytometer using an optical microscope (Nikon Eclipse E200, Nikon, Japan) at 400x magnification, at 24, 48 and 72 hour intervals. Data are expressed as a half-maximum inhibitory concentration (IC_{50}) and parasitic viability is compared with the controls (Comandolli-Wyrepkowski *et al.*, 2017).

In vitro cytotoxicity assay

The murine macrophage-like cell line J774 (10^5 cells mL^{-1}) were incubated in 96-well plate in a complete Schneider medium at 37 °C. After 24 hours of cell adhesion. The cells were only exposed to the copper complex (1) that showed the best performance in the assay against promastigote forms, and the control groups consisted of cells treated with Pentacarinat® and cells without treatment, for a period of 24, 48 and 72 hours.

To determine cell viability, the MTT colorimetric test (Roche®) was used, after the incubation periods of 24, 48 and 72 hours. Subsequently, the optical density (OD) of each well plate was evaluated on the spectrophotometer (Bio-TEK®) at 590 nm. For morphological evaluation, a 24-well plate was used, with the same conditions for cultivation and treatment of the cells, with subsequent fixation and staining of the biological material by the Rapid Panoptic method (Laborclin®, Paraná, Brazil). The results were expressed as the percentage of viable cells (Comandolli-Wyrepkowski *et al.*, 2017).

In vitro amastigote-macrophage assay

Macrophages were incubated on a 24-well plate with 10^5 cells mL^{-1} in complete Schneider medium, incubated in an oven at 37 °C for 48 hours. Macrophages were then infected with 10^6 cells mL^{-1} for 2 hours with the *L. (L.) amazonensis* or *L. (V.) guyanensis* promastigotes. After the infection period, the wells were washed with sterile saline solution (NaCl 0.9%) for removal of the non-internalized promastigotes. The infected macrophages were incubated for 48 hours at 37 °C in the presence of copper complex (1) (160 µM) and Pentacarinat® (147 µM). After the incubation period, the wells were washed with sterile saline solution and the macrophages were then fixed and stained using the Quick Panoptic (Laborclin®). For the analysis, 100 macrophages were quantified (in triplicate) to determine infection rate considering infected and uninfected macrophages. Quantification was performed using an optical microscope at 1000x magnification (Comandolli-Wyrepkowski *et al.*, 2017).

Statistical analysis

All biological assays were performed in triplicate and the results were expressed as average ± standard deviation (SD). The data were submitted to one-away analysis of variance and *p* values below 0.05 were considered significant. Tukey's multiple comparison test was used to identify significant differences in the means among the different treatments (GraphPad Prism software, version 6.0).

Results and Discusion

In the promastigote cells sensitivity test to the three copper complexes, both species of *Leishmania* showed different behavior after cultivation in the presence of complexes. There was a significant difference between all treatments ($p < 0.001$) in the two species tested. The most promising complex against promastigote forms of *L. (L.) amazonensis* was complex (1), which presented an IC_{50} of 26.45 and 24.61 µM at 48 and 72 hours, respectively. This IC_{50} was lower than that for Pentacarinat®, however this complex did not present a similar activity against *L. (V.) guyanensis* (Table 1).

Table 1. Inibitory Concentrations (IC_{50}) of copper complexes (1, 2, 3) and Pentacarinat® calculated by linear regression, in 48 and 72 hour incubation periods with promastigotes of *Leishmania (Leishmania) amazonensis* and *Leishmania (Viannia) guyanensis*.

Copper complexes	*L. amazonensis*	
	48 h	72 h
$[Cu(thp)_4][PF_6]$ (1)	26.45 ± 0.24	24.61 ± 0.28
$[Cu(PTA)_4][BF_4]$ (2)	89.26 ± 0.79	75.03 ± 0.65
$[HB(pz)_3Cu(PTA)]$ (3)	87.17 ± 0.76	62.11 ± 0.42
Pentacarinat®	66.28 ± 0.18	27.45 ± 0.61
Copper complexes	*L. guyanensis*	
	48 h	72 h
$[Cu(thp)_4][PF_6]$ (1)	105.94 ± 0.01	177.10 ± 0.03
$[Cu(PTA)_4][BF_4]$ (2)	330.23 ± 0.05	155.49 ± 0.03
$[HB(pz)_3Cu(PTA)]$ (3)	207.43 ± 0.01	120.36 ± 0.00
Pentacarinat®	61.55 ± 0.02	58.10 ± 0.00

Considering complex (1) as the most promising, we analyzed the statistical difference between the treatments and the different concentrations of the complex (1). In *L. (L.) amazonensis* promastigotes, complex (1) showed a significant difference to the negative control in 48 hours ($p = 0.0029$) and 72 hours ($p = 0.0133$), with less parasitic viability. There was also a significant difference between the complex (1) and Pentacarinat® in the period of 48 hours ($p = 0.0049$) and 72 hours ($p = 0.0007$), with greater parasitic viability being observed in the lower concentrations of the complex (1) [Figure 2].

In promastigote forms of *L. (V.) guyanensis*, complex (1) showed a significant difference, with less minor parasitic viability when compared to the negative control in the 48 hour period ($p = 0.0429$); complex (1) also showed greater parasitic viability compared to Pentacarinat®, with significant difference in the period of 48 hours ($p = 0.0027$) and 72 hours ($p = 0.0055$) [Figure 2].

In the cytotoxicity test, the results showed that 52.45% (SD 1.29%) of the MJ774 cells were viable after 48 hours of incubation with complex (1) at concentration 160 µM. Moreover, morphological analysis of the cells exposed to complex (1) revealed that in the positive control (untreated macrophages) and Pentacarinat® assays (Figure 3A and B, respectively), the MJ774 cells did not present any morphological alterations, whereas when using treatment with complex (1) the presence of vacuoles in the cytoplasm was notable (Figure 3C).

Thus, the effectiveness of complex (1) was tested against the amastigote forms of *L. (L.) amazonensis* and *L. (V.) guyanensis* internalized in MJ774 cells. The results showed that the rate of infection of *L. (L.) amazonensis* was 32% (SD

1.4%) when macrophages were treated with a 160 µM concentration of complex (1), and 19% (SD 0.90%) when treated with Pentacarinat®.

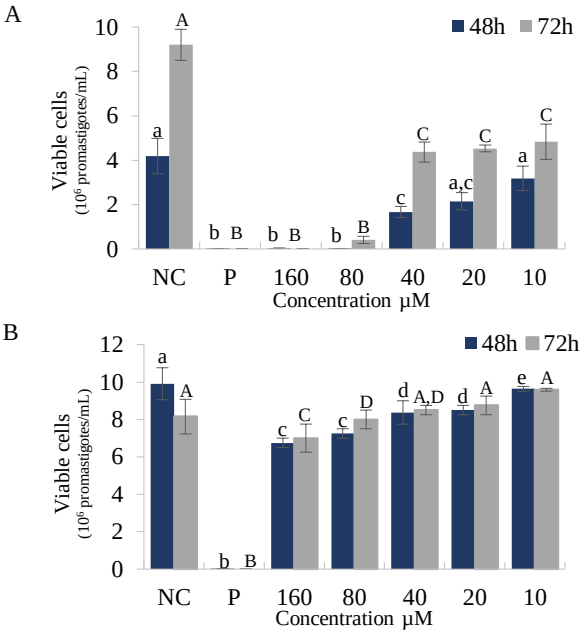

Figure 2. Antileishmanial activity of the copper complex (1) in promastigote forms of *Leishmania (Leishmania) amazonensis* (A) and *L. (Viannia) guyanensis* (B). Legend: NC: Negative control; P: Pentacarinat® (147 µM). Means followed by the same lowercase (for 48h interval) and uppercase (for 72h) letters, in each graphic, do not differ from each other by the Tukey test at 5% probability.

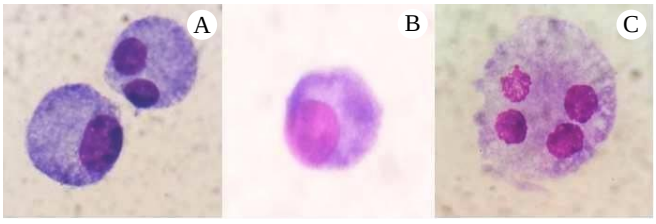

Figure 3. *In vitro* cytotoxicity morphological assay with MJ774 cells after 48 hours of incubation. A: Positive control (untreated macrophages), B: macrophages treated with Pentacarinat® C) macrophages treated with 160 µM of copper complex (1). 1000 x magnification. This figure is in color in the electronic version.

The morphological analysis of the cells exposed to the *in vitro* amastigote-macrophage assay revealed that the infected and untreated macrophages showed internalized amastigotes with the presence of small vacuoles, and the same was observed in infected macrophages and those treated with Pentacarinat®. When the infected macrophages were treated with complex (1), the presence of amastigotes interiorized in vacuoles was observed, in addition to the intense vacuolization of the macrophages (Figure 4).

When the macrophages were infected with *L. (V.) guyanensis* and treated with a 160 µM concentration of complex (1), there was a total lysis of the macrophages, and was considered a 100% infection, therefore it was not possible to perform morphological records due to cellular degradation.

In a research conducted by Portas *et al.* (2012), using copper(II) complexes, also observed antileishmanial activity against promastigote forms of *L. (L.) amazonensis*. The results showed that the acetylacetonate (acac) derivative, namely $Cu(acac)_2$, showed the best activity at 24 hours with an IC_{50} of 31 µM, but demonstrated an increase during the incubation period (IC_{50} of 94 µM at 72 hours), whereas the more lipophilic fluoroderivative $Cu(tfacac)_2$ increased its activity during the incubation period (IC_{50} of 68 and 43.0 µM at 24 and 72 hours respectively).

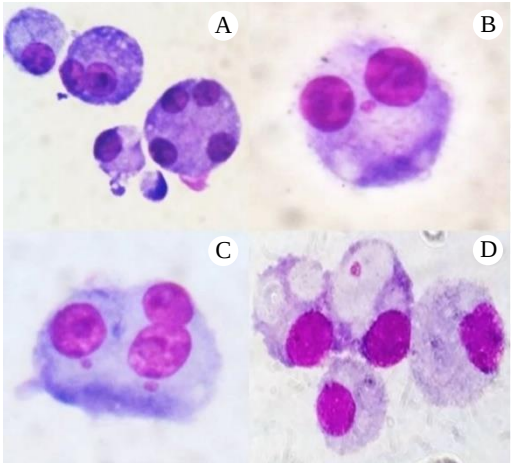

Figure 4. *In vitro* amastigote - macrophage assay with *Leishmania (Leishmania) amazonensis*. A) Positive control (non-infected and untreated macrophages, B) infected and untreated macrophages, C) infected macrophages and treated with Pentacarinat® and D) infected macrophages and treated with 160 µM of copper complex (1). 1000x magnification. This figure is in color in the electronic version.

The key factors for the efficacy of the Cu(II) complexes tested by Portas *et al*. (2012) are the high lipophilicity and the pro-oxidant activity, as lipophilic complexes are better inhibitors of trypanothione reductase (TR). In our case, we used water-soluble, or highly hydrophilic, Cu(I) compounds that presented a lower or a similar IC_{50} compared to those reported in the literature, suggesting a different mechanism of action.

In particular, complex (1) demonstrated itself to be the most promising against the promastigote forms of *L. (L.) amazonensis* when compared to the other two complexes tested, presenting an IC_{50} value lower than the values reported in previous studies (IC_{50} of 26.45 and 24.61 µM at 48 and 72 hours). Regarding the activity of the copper(I) complex against *L. (V.) guyanensis*, there was a decline in the IC_{50}, suggesting a long-term activity. However, no other study was found that used copper complexes against *L. (V.) guyanensis.*

Complex (2) is a homoleptic compound comprising the metal tetrahedrally arranged in a cationic moiety, which differs from complex (1) due to the nature of the phosphine (PTA instead of thp). This difference demonstrated a noticeable decrease in the activity against *L. (L.) amazonensis*, leading to IC_{50} values of 89.26 and 75.03 µM

at 48 and 72 hours respectively, which is about three times higher than those obtained with complex (1). As observed for complex (1), complex (2) was also even less active against *L. (V.) guyanensis*, showing significant increase in IC_{50} during the incubation period (IC_{50} of 330.23 and 155.49 µM at 48 and 72 hours, respectively).

Compound (3) is a lipophilic neutral mixed-ligand (scorpionate/phosphane) species, and its mechanism of internalization and of action might be different from those of complexes (1) and (2). In fact, complexes (1) and (2) in diluted solutions, such as those utilized for biological tests, are partially dissociated and the predominant species in the solution are the coordinatively unsaturated CuP_2^+/CuP_3^+ species. These can be internalized in the cell by an active transport mechanism using Ctr1 transporters, whereas compound (3), even at low concentrations, does not lose ligands and remains as a neutral lipophilic entity that can be internalized by a passive diffusion transport mechanism (Gandin *et al*., 2014; Tisato *et al*., 2016; Quaretti *et al*., 2018).

In fact, as it appears from the IC_{50} values, the activity of (3) is similar, or slightly better, than the activity of (2), suggesting that lipophilicity is not the key factor. However, as in the study by Portas *et al*. (2012), compound (3) showed lipophilicity and obtained a decrease in IC_{50} during the incubation period, suggesting long-term activity.

In the literature, the activity of a cationic drug, such as paromomicyn, can be related to its ability to bind to the negatively-charged leishmanial glycoprotein−polysaccharide layer covering the cell membrane (Jhingran, Chawla, Saxena, Barrett, & Madhubala, 2009). In our case, both complexes (1) and (2) are positively charged, so the charge is not a key factor for explaining the remarkable activity of (1). Among the three complexes tested, (1) is the most active compound against both evolutionary forms of the life cycle of *L. (L.) amazonensis*.

Further studies are needed to elucidate the mechanism(s) of action of the compounds tested in this study, in particular their ability to inhibit TR, since this is an enzyme which is exclusive to organisms that belong to the Trypanosomatidae family, this made it a potential chemotherapeutic target. In addition, it was found that antimony compounds have the ability to inhibit TR, which may be one of the mechanisms of action for the current treatment of leishmaniasis (Tunes *et al*., 2020). Studies, such as the one by Tunes *et al*. (2020), have sought to investigate the ability of gold complexes to inhibit TR and it was found that gold complexes were active against *Leishmania* spp. (IC_{50} of 0.5 to 5.5 µM) and all gold(I) complexes were potent inhibitors of TR, which demonstrates that although antileishmanial activity may involve several mechanisms, one strategy is to ascertain the potential for inhibiting TR.

In relation to cytotoxicity, Singh *et al*. (2017) performed biological cytotoxicity assays on macrophages of mammalian hosts and the results revealed that copper salisylaldoxime (30 µM and 45 µM) is almost non-toxic to host cells. In our study, we observed intense vacuolization in macrophages treated with complex (1). This fact may be associated with the mechanisms described by Tisato *et al*. (2016), who suggested that complex (1) does not induce cell apoptosis or DNA fragmentation, but it does induce cell paraptosis through the stress of the endoplasmic reticulum in cancer cells, causing their death.

In experimental studies with intracellular forms, Britta *et al*. (2012) evaluated the activity of a copper complex against *L. (L.) amazonensis* and the infectivity rate was 62.3% to 14.4%. The results presented for Britta *et al*. (2012) are similar to those of this study since the infectivity rate was 32%.

A relevant factor in assays with amastigote forms was the intense vacuolization of macrophages exposed to complex (1), which maybe associated with an increase in infection. Young and Kima (2019) recently showed that *L. (L.) amazonensis* infections are more successful when autophagy increases in host cells, in addition to an increase in parasitic load in BALB/c macrophages.

Conclusion

The phosphine copper(I) complex $[Cu(thp)_4][PF_6]$ (thp = tris-hydroxymethylphosphine) (1) presented promising activity against both evolutionary forms in the life cycle of *L. (L.) amazonensis* and showed moderate toxicity in murine macrophage line J774 in the concentration tested in this study.

Acknowledgments

The authors would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq, Fundação de Amparo à Pesquisa do Estado do Amazonas - FAPEAM, and FP7-PEOPLE-2011-IRSES- 295262: VAIKUTUS for their financial support.

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