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Study of in vitro biological activity of thiazoles on *Leishmania (Leishmania) infantum*

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ABSTRACT

Objectives: In the prospection of possible agents against neglected diseases, thiazole compounds are presented as promising candidates and are known to have activity against trypanosomatid parasites. Thus, this work aimed to evaluate the effects of thiazole compounds on *Leishmania infantum*, etiological agent of visceral leishmaniasis.
Methods: Thiazole compounds, being 5 thiazoacetylpyridines (TAPs-01; 04; 05; 06; 09) and 5 thiazopyridines (TPs-01; 04; 05; 06; 09) were tested regarding its leishmanicidal activity on both promastigote and amastigote forms of *L. infantum*. Its cytotoxicity was tested using peritoneal macrophages of BALB/c mice. Ultrastructural analyzes were performed to identify possible intracellular targets of the most effective compound on promastigote forms. In order to observe routes that can clarify the possible mechanism of action of the compounds on the intracellular amastigote forms, the Nitrite dosage was performed.
Results: All compounds inhibited the growth of promastigote and presented low cytotoxicity, being more selective to the parasite than to mammalian cells. All compounds tested were able to decrease macrophage infection. There was a significant decrease in the survival rate of the amastigote when compared to the untreated cells, with TAP-04 presenting the best index. TAP-04 compound induced ultrastructural changes that are relted to cell death by apoptosis. None of the macrophage groups infected with *L. infantum* and subsequently treated showed increased nitrite release.
Conclusions: The low toxicity to mammalian cells and the leishmanicidal activity observed demonstrate that the synthesis of drugs based in thiosemicarbazone nucleus, thiazole and pyridine derivatives are promising to the treatment of VL.

Keywords: Ultrastructure; cytotoxicity; leishmaniasis

1. Introduction

Visceral leishmaniasis (VL) is an important parasitic zoonosis caused by the protozoan *Leishmania infantum* [1, 2]. It is known that dogs are the main reservoirs of the parasite in the urban environment in Brazil [3]. The transmission of VL among mammals is essentially through vectors [4], although there is a possibility of direct dog-dog transmission, such as transplacental vertical transmission in female dogs [5], as well as sexual transmission [6].

Despite of the existence of several canine leishmaniasis (CanL) treatment protocols [7], the combination of allopurinol with either meglumine antimonate or miltefosine is currently considered the treatment of choice. These drugs trigger a number of side effects, are difficult to administer and can lead to parasite resistance to the treatment [8]. These substances may also further potentiate kidney damage [7]. Thus, alternative drugs, such as aminosidine (paromomycin) has been shown to have

leishmanicidal activity. However, these drugs are expensive and are also reported to be toxic to mammals [9]. It is clear that new alternatives for the treatment against CanL and by extension also against VL in humans, are necessary through the use of safer and effective drugs.

Among other chemical groups that have been studied for anti-protozoal activities, thiosemicarbazones [10] are promising. In addition, the cyclization of the thiosemicarbazone in thiazole and the condensation of the pyridine ring with this new molecule have shown anti-Trypanosoma activity [11]. The thiazole is an important class of heterocyclic compounds that exhibit a broad biological activity spectrum, such as antitumor [12], antibacterial [13], anti-inflammatory [14] and leishmanicidal activities [15]. It has been demonstrated that cysteine-protease enzymes of *L. infantum* are identified as targets of thiazolic compounds [16, 11]. These enzymes play an important role in Leishmania *sp.* virulence, in its viability maintenance, in its morphology, and in the host mononuclear phagocytic system (SFM) immune response [17].

Thus, the goal of this work was to evaluate the *in vitro* biological activity of different thiazoles compounds in *L. infantum*.

2. Material and methods

2.1. Thiazolic derivatives

Five thiazol derivatives of the thiazopyridines series (TP) and five of the thiazolacepyridines series (TAP) were used. They are N- (2-methyl-pyridine) -N '- (phenylthiazol-2-yl) -hydrazine (TP-01); N- (2-methyl-pyridine) -N '- (p-bromophenylthiazol-2-yl) -hydrazine (TP-04); N- (2-methyl-pyridine) -N '- (P-fluorophenylthiazol-2-yl) -hydrazine (TP-05); N- (2-methyl-pyridine) -N '- (p-anisal thiazol-2-yl) -hydrazine (TP-06); N- (2-methyl-pyridine) -N '- (3,4-dichlorophenylthiazol-2-yl) -hydrazine (TP-09); N- (1-methyl-2-methyl-pyridine) -N '- (phenylthiazol-2-yl) -hydrazine (TAP-01); N- (1-methyl-2-methyl-pyridine) -N '- (p-bromophenylthiazol-2-yl) -hydrazine (TAP-04); N- (1-methyl-2-methyl-pyridine) -N '- (P-fluorophenylthiazol-2-yl) -hydrazine (TAP-05); N- (1-methyl-2-methylpyridine) -N '- (p -anisaltiazol-2-yl) -hydrazine (TAP-06) and N- (1-methyl-2-methylpyridine) -N' - (3,4-dichlorophenylthiazol-2-yl) -hydrazine (TAP-09)]. The synthesis of these compounds was developed by using high purity chemical reagents described by Cardoso et al [11] (Fig. 1).

2.2. Parasites

Promastigote forms of *L. infantum* (Strain BH 46) were maintained at 26°C in Schneider's medium (Sigma) supplemented with 10% fetal bovine serum (FBS) and used in the exponential growth phase. Amastigote forms were obtained from the inoculation of infective promastigote forms in cultures of peritoneal macrophages of BALB/c mice.

2.3. Analysis of cytotoxicity in peritoneal macrophages of BALB/c mice

Peritoneal macrophages of BALB/c mice were treated with/without different concentrations (2.78, 5.56, 11.12, 22.25, 44.5 μM) of the compounds to evaluate its cytotoxicity in mammalian cells using the CellTiter-Glo® Luminescent Cell Viability Assay method. Cells were incubated in 96-well plates (10^6 cells/mL). After a 48h-treatment with the compounds, it was possible to determine the cytotoxic concentration for 50% of the peritoneal macrophages (CC_{50}). Its cytotoxicity and its activity against promastigote parasites were compared to each other to determine the Selectivity Index (SeI/pro= CC_{50} of macrophages divided by IC_{50} of promastigote forms).

2.4. Effect of the compounds on promastigate parasites

Promastigote forms of *L. infantum* were incubated in Schneider's medium (Sigma) supplemented with 10% FBS at a concentration of $1x10^6$ cells/mL. After diluting the cells, they were incubated in the presence of different concentrations (2.78-44.5 μM) of the compounds for 48h to determine the concentration that inhibited 50% growth of the parasites (IC_{50}/pro). Cell growth was monitored by CellTiter-Glo® Luminescent Cell Viability Assay. Cells incubated with only Schneider's medium were used as cell control. Each test was done in triplicate and in 3 independent experiments.

2.5. Effect of the compounds on amastigote forms

Peritoneal macrophages of BALB/c mice were incubated with RPMI-1640 medium (Sigma-Aldrich, Co., St. Louis, MO, USA) supplemented with 10% FBS in 24-well plates containing coverslip (10^6 cells/mL). Macrophages were incubated at 37ºC and 5% CO_2 for 2 hours for adhesion and then infected with promastigote parasites using a 1:15 parasite/cell ratio at 37ºC for 14h. Parasites that didn't adhere on the coverslips were washed out. Then, TAP-01, TAP-04 and TAP-06 (compounds selected by previous tests) were added in the cell culture in different concentrations (0, 0.25, 0.5 and 1 μg/mL) for 24h. The coverslips were stained with Giemsa (Sigma-Aldrich, Co., St. Louis, MO, USA). The concentration capable of inhibiting 50% of macrophage infection by amastigote forms (IC_{50}/ama) was estimated from direct counting of intracellular amastigote/100 infected cells by optical microscopy. Survival index was determined by multiplying the number of amastigote by the number of infected macrophages.

2.6. Ultrastructural analysis

In order to identify the drug's possible intracellular target and also deleterious effects of the compounds on the parasite membrane, ultrastructural analyzes using transmission electron microscopy (MET) and scanning electron microscopy (MEV) were performed. To perform MET, promastigotes forms of *L. infantum* with/without TAP-04 (1/2 IC_{50} and IC_{50} incubation for 48h) were fixed for 60 min in a solution containing 2.5% glutaraldehyde in sodium cacodylate buffer 0,1M (pH 7.2) and post-fixed for one hour in a solution containing 1% osmium tetroxide (OsO 4), 0.8% potassium ferricyanide, 5 mM CaCl 2 in 0.1M cacodylate buffer. After this step, the samples were dehydrated in increasing concentrations of acetone, infiltrated and included in epoxy resin (Fluka Analytical). Ultrafine cuts of approximately 70 nm thickness were obtained in ultramicrotome (Leica EMUC6). Then, 5% uranyl acetate and lead citrate were added for analysis on the Zeiss EM109B transmission microscope. For MEV, promastigote forms of *L. infantum* treated with TAP-04 were fixed as described above and placed to adhere to coverslips previously mentioned with poly-lysine. After 20 minutes, the coverslips were washed with PBS to remove unbound and post-fixed cells as described for MET. The cells were then washed in 0.1 M sodium cacodylate buffer, dehydrated in increasing ethanol series and subjected to critical-point drying at Critical Point dryer HCP-2 (Hitachi), metallized with 20 nm gold on the JFC metallizer -1100 (Jeol) and viewed through the scanning electron microscope JEOL T-200.

2.7. Nitric oxide production

To analyze the effect of the compounds using nitrite dosage, 100µL of the culture's supernatant from infected macrophages with and without treatment with the compounds for 24 hours were incubated with 100µL of Griess reagent (1% sulfanilamide and 0.1 % N - (1-naphthyl) -ethylenediamine dihydrochloride / 2.5% H 3 PO 4) at room temperature for 10 minutes. The absorbance was measured with a 540nm filter in the Benchmark Plus ELISA reader (Bio-Rad Laboratories, Philadelphia, PA, USA). The nitrite concentration was determined using a standard curve of sodium nitrite.

2.8. Data analysis

Linear regression analyzes were performed in SPSS 8. (IBM Co., New York, USA) for Windows and significance analyzes, considering $p < 0.05$ as significant values, were performed using the ANOVA test using the program GraphPad Prism 5. (GraphPad, Calif., USA) for Windows.

3. Results

3.1. Cytotoxicity of compounds on peritoneal macrophages of balb/c

When tested against peritoneal macrophages, some compounds showed cytotoxic effect at concentrations of CC_{50} close to the IC_{50}/pro values determined for *L. infantum*. However, calculation of SeI /pro demonstrated that all compounds were more selective for the promastigote parasite than for the mammalian cells. Among the compounds that presented the best selectivity index, we highlight TAP-01 (SeI = 12.73), TAP-04 (SeI = 18.94) and TAP-06 (SeI = 7.79) (Table 1).

3.2. Effect of compounds on promastigotes forms of *L. infantum*

All thiazolic compounds were able to inhibit *L. infantum* promastigote growth. The IC_{50} values ranged from 0.42 to 22.55 μM (Table 1). The most effective compounds, ie those with the lowest IC_{50} concentrations, were TAP-05 (0.42 ± 0.03 μM), TAP-09 (2.73 ± 0.61 μM) and TAP-01 (3.57 ± 0.95 μM).

3.3. Effect of compounds on amastigotes forms of *L. infantum*

The amastigote parasites are responsible for the clinical manifestation of the disease. In this way, the compounds TAP-01, TAP-04 and TAP-06 with highest SeI values for *L. infantum* promastigote were chosen for the following tests. All compounds were more effective against amastigotes than for promastigotes. TAP-06 and TAP-04 were about seven times more effective for intracellular forms of the parasite than for promastigote forms, whereas for TAP-01 this difference was approximately 3 times. The IC_{50}/ama values regarding TAP-01, TAP-04 and TAP-06 were 0.99, 0.43 and 0.59 μM, respectively. The differences in the activity of the compounds on amastigotes were directly related to the selectivity index of these parasites corresponding to the values of 46, 10, 137.37 and 59.05 for TAP-01, TAP-04 and TAP-06, respectively. Thus, according to results TAP-04 had the best selectivity index for *L. infantum* amastigote (Table 1). For the three concentrations tested (0.25, 0.5 and 1 μg / mL) there was a significant ($p < 0.05$) decrease in the survival index of the parasites inside the macrophage when compared to the untreated cells. TAP-04 was the compound that presented the highest rate of the amastigote viability inhibition inside the macrophage. At the concentration of 1μg/mL the macrophage infection was almost 100% abolished (Fig. 2).

3.4. Ultrastructural analysis

Considering that TAP-04 was the one that presented the best activity against both forms of the parasite and was also the least toxic, we performed new experiments to investigate possible intracellular targets of the compound as well as its effects on the parasites' cellular membrane using MET and MEV. As observed by MET, promastigotes without addition of TAP-04 showed regular morphology, with the nucleus well preserved, occupying the most central part of the cytoplasm and chromatin

associated with the inner nuclear membrane. A single mitochondria containing a kinetoplast, region where mitochondrial DNA (k-DNA) is concentrated, was found close to the flagellar pocket where the flagellum emerges (Fig. 3a).

After adding 1/2x IC_{50} of TAP-04, promastigote started to present swollen mitochondria, besides intense disorganization of the mitochondrial ridges and in the kinetoplast region, cellular disorganization with displacement of the nucleus to the periphery of the cell and an increased profile of the endoplasmic reticulum. In addition to these alterations, disorganization of Golgi complex cisternae (Fig. 3b, c, d) was observed. These changes were more dramatic in cells treated with IC_{50}/pro. It was also possible to observe a change in the acidocalcisome volume and in the electron-dense deposits present in this organelle, loss of mitochondrial matrix material and cytoplasm compatible with possible damage to the plasma membrane. (Fig. 3e, f).

MEV experiments without addition of TAP-04 showed normal promastigote shape, presenting an elongated cell body and an intact membrane (Figure 4a, b). Cells treated with 1/2x IC_5/pro (Fig. 4c, d) showed changes in the shape of the cell body which started to assume a rounded morphology and loss of the flagellum. In the most drastically affected cells it was possible to observe loss of cell volume with intense cell membrane wrinkling. However, it was not possible to observe the presence of cellular debris or apparent damages in the cell membrane, suggesting a cell death by apoptosis.

3.5. Effect of compounds on the production of NO

Considering the effect of the selected compounds (TAP-01, TAP-04 and TAP-06) on amastigotes, we ran the NO experiment to see if these effects could be related to an activation of the microbicidal capacity of the macrophages through the production of nitric oxide (NO). Nitrite dosage was developed using the supernatant of infected cultures with and without addition of the drugs. The data indicated that none of the groups of macrophages infected with *L. infantum* and subsequently treated with TAP-01, TAP-04 and TAP-06 showed increased NO production when compared to the untreated infected cells. In cells treated with TAP-04 there was a significant decrease in the highest concentrations tested (Fig. 5).

4. Discussion

Several studies have demonstrated that thiazole-derived compounds may exhibit an important activity against trypanosomatid parasites [18, 19, 20, 21]. To evaluate the leishmanicidal potential of thiazole derivatives, we performed a screening of the biological activity of ten compounds on *L. infantum* promastigote parasites. All compounds inhibited promastigote growth of forms. However, treatment with TAP-01, TAP-04 and TAP-06 were considered more effective because they presented lower values of IC_{50}/pro and higher survival index when compared to the other compounds, with TAP-04 being the compound that presented highest SeI /ama

For the TP series, comparing the compound without modification (TP-01, <2.78 μM) to the substituted ones, it is possible to observe only TP-06 (OMe) showed

leishmanicidal activity. Compounds TP-04 (Br), TP-05 (F) and TP-09 (Cl) were less potent than TP-01. For the TAP series, all compounds presented relevant leishmanicidal activity, with TAP-05 (F, 0.42 μM) and TAP-09 (Cl, 0.99 μM) being the ones with the best results. In fact, TAP-05 was up to 8 times more potent than the base compound without substituent (TAP-01, 2.73μM). These results are very similar to those found in trypomastigote and epimastigote of *Trypanosoma cruzi* [11]. These authors showed that the TAP series were an excellent inhibitor of cruzaine activity, being greater than the TP series. Both results demonstrate the importance of methyl for antichagasic and leishmanicidal activity. In fact, the presence of methyl in the chemical structure usually improves the liposolubility, thus improving the drug bioavailability with increasing of the compounds biological activity [11, 20].

Similarly to the results observed by thiazolic compounds' experiments, it was possible to observe potent activity against naphthotiazoles *L. braziliensis* promastigotes; also, the compounds tested by them reduced the survival rate of amastigote forms in mammalian macrophages [22]. In addition, studies by Nava-Zuazo et al. [15] also showed that compounds containing the thiazolic ring in their structure inhibited *L. amazonensis* promastigote. These results demonstrated that thiazole derivatives have promising leishmanicidal activity. It is important to emphasize that it's the first time that the compounds used in all experiments developed by us were tested for *in vitro* biological activity on *L. infantum*.

The important requirement to develop new medicine routes with leishmanicidal action is that they must not be toxic to mammalian cells [23]. Therefore, not only the effect of the compounds on parasite growth was considered but also the cytotoxic potential of these on mammalian cells was evaluated. The low toxicity of some thiazole derivatives to mammalian cells when compared to reference drugs, such as Glucantime (727.63 μM) [24], had a direct reflection on SeI /pro values, which were always more selective for parasites than for mammalian cells. Even the drug with the highest CC_{50} value among the 10 ones tested so far, TP-05 (109.67 μM), was almost 7x less cytotoxic than Glucantime. Cytotoxicity in peritoneal macrophages treated with thiazopyridine derivatives can be attributed to the presence of the pyridine ring, since it has been demonstrated that the thiazole group is non-toxic and it improves the cellular viability of thiosemicarbazones through the functionalization of thiocarbonyl to thiazole [11].

TAP-04 was selected for ultrastructural analysis through MET after demonstrating the highest index selectivity to the parasite when compared to the host cell. After 48h-treatment, it was possible to observe different morphological features in the parasites in a dose-dependent relation, such as complete disorganization of mitochondrial ridges. In fact, mitochondria is a main target of several drugs [25, 26] because this organelle is crucial for the survival of several cell types, especially for trypanosomatids, which present only a single mitochondria. Modifications on the trypanosomatid mitochondria structure caused by drugs are commonly observed, showing that this organelle is chemotherapeutic sensitive [27]. In addition, there are modifications in the endoplasmic reticulum profile, vesiculation and fragmentation of the Golgi complex and appearance of structures typical of the autophagic process.

Bilbao-Ramos et al [28] developed ultrastructural experiments on *L. infantum* promastigote with another class of thiazole derivatives. However, they observed similar leishmanicidal effect, such as damage to the nucleus, mitochondria and kinetoplast. Finally, electron microscopy studies demonstrated that the parasites treated with TAP-04 clearly showed ultrastructural modifications compatible with programmed cell death [29], such as, cell rounding, chromatin condensation and mitochondrial swelling.

Taking into account that the thiazole derivatives showed good leishmanicidal activity against *L. infantum* promastigotes and low toxicity in host cells, we expected that intracellular amastigote might be also sensitive to treatment with the chosen drugs. In this case, the compounds might have two mechanism of action: a direct effect on the host cells making them more efficient in combating infection [30] or an effect on therapeutic targets important to the survival of amastigotes in the macrophage. In addition, the compounds selected for evaluation in amastigotes present cysteine-protease inhibition activity [11]. This enzyme plays an important role on the parasite viability, such as in its morphology maintenance [31, 17]. In fact, cysteine-protease is found in large quantities in lysosomes, which are particularly abundant in *Leishmania* amastigotes [16].

The current progress within drug development is possible because it was established a few criteria that classify the compound efficiency [32]. These criteria include biological, physicochemical and pharmacokinetic aspects, as well as safety and toxicological aspects of drug discovery for several neglected diseases, including leishmaniasis [32, 33]. Among these criteria, there are also the definition of chemical structure; the synthetic route establishment; and the amastigote selectivity index value that has to be higher than 20 [32]. In fact, TAP-01, TAP-04 and TAP-06 fulfill those last three criteria [11], highlighting TAP-04 for presenting the highest selectivity index (SeI /ama=137.37), which was approximately 7 times higher than the minimum established for a promising drug [32].

In the vertebrate host, the early stages of the trypanosomatid parasites' infection are capable of triggering inflammation mediators from infected cells, such as Th1 or Th2 cytokines, as well as reactive oxygen species (ROS) and nitrogen species (eg NO) [34]. Data from the present study showed a significant reduction of peritoneal exudate (PEC) cells infected by *L. infantum* after treatment with thiazole derivatives. However, analysis of nitric oxide production by these cells demonstrated that there was no significant increase in NO in infected macrophages and in macrophages treated with the compounds. Several authors describe that the release of cytokines from the Th2 profile triggered by the activation of inflammatory regulation factors are the defense mechanism against *Leishmania* sp. [35, 36]. Bcl-2 family members are known as apoptosis regulators in mammalian cells and therefore mediators of parasite infection response. They may be related to the elimination of parasites by the activation of inflammatory cytokines [37]. Thus, the activation of such regulatory mediators could modulate the inflammatory process that normally occurs during Leishmania infection and thus inhibit the exacerbated production of nitric oxide in these infected

macrophages [38, 39]. However, further studies are necessary to understand the mechanism of action of these compounds in the intracellular *L. infantum* amastigote.

5. Conclusions

These results demonstrated that thiazole compounds of the series thiazopyridines (TP) and thiazoacetylpyridines (TAP) have promising leishmanicidal activity. In addition, it is important to emphasize its high selective activity against *L. infantum* with low toxicity against host cells.

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Ethical approval
The present study was carried out in accordance with the ethical principles adopted by Brazilian law 11.794/2008 and approved by the Ethical Committee for Animal Research of the Instituto Aggeu Magalhães/Fundação Oswaldo Cruz (N° 039/12).

Competing interests
None declared.

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References

[1] J.J. Shaw, Taxonomy of the genus Leishmania: present and future trends and their implications, Mem Inst Oswaldo Cruz. 89 (1994) 471–478. doi:S0074-02761994000300033 [pii].

[2] C.H.N. Costa, Characterization and speculations on the urbanization of visceral leishmaniasis in Brazil, Cad. Saude Publica. 24 (2008) 2959–2963. doi:10.1590/S0102-311X2008001200027.

[3] S.A. Diniz, F.L. Silva, A.C. Carvalho Neta, R. Bueno, R.M.S.N.C. Guerra, A.L. Abreu-Silva, R.L. Santos, Animal reservoirs for visceral leishmaniasis in densely populated urban areas., J. Infect. Dev. Ctries. 2 (2008) 24–33. doi:10.3855/jidc.318.

[4] V.V.G. de Oliveira, L.C. Alves, V.A. da Silva Junior, Transmission routes of visceral leishmaniasis in mammals, Ciência Rural. 45 (2015) 1622–1628. doi:10.1590/0103-8478cr20141368.

[5] T.J. Naucke, S. Lorentz, First report of venereal and vertical transmission of canine leishmaniosis from naturally infected dogs in Germany, Parasites and Vectors. 5 (2012). doi:10.1186/1756-3305-5-67.

[6] F.L. Silva, R.G. Oliveira, T.M.A. Silva, M.N. Xavier, E.F. Nascimento, R.L. Santos, Venereal transmission of canine visceral leishmaniasis, Vet. Parasitol. 160 (2009) 55–59. doi:10.1016/j.vetpar.2008.10.079.

[7] R.M. Reguera, M. Morán, Y. Pérez-Pertejo, C. García-Estrada, R. Balaña-Fouce, Current status on prevention and treatment of canine leishmaniasis, Vet. Parasitol. 227 (2016) 98–114. doi:10.1016/j.vetpar.2016.07.011.

[8] D. Yasur-Landau, C.L. Jaffe, L. David, G. Baneth, Allopurinol Resistance in

Leishmania infantum from Dogs with Disease Relapse, PLoS Negl. Trop. Dis. 10 (2016). doi:10.1371/journal.pntd.0004341.

[9] D. Kasabalis, M.K. Chatzis, K. Apostolidis, P.G. Xenoulis, A. Buono, T. Petanides, L.S. Leontides, Z.S. Polizopoulou, J.M. Steiner, J.S. Suchodolski, M.N. Saridomichelakis**.** Evaluation of nephrotoxicity and ototoxicity of aminosidine (paromomycin)-allopurinol combination in dogs with leishmaniosis due to Leishmania infantum: A randomized, blinded, controlled study. Exp. Parasitol. 206 (2019) 167-76. doi: 10.1016/j.exppara.2019.107768.

[10] S. Singh, N. Bharti, F. Naqvi, A. Azam, Synthesis, characterization and in vitro antiamoebic activity of 5-nitrothiophene-2-carboxaldehyde thiosemicarbazones and their Palladium (II) and Ruthenium (II) complexes, Eur. J. Med. Chem. 39 (2004) 459–465. doi:10.1016/j.ejmech.2004.02.003.

[11] M.V.D.O. Cardoso, L.R.P. De Siqueira, E.B. Da Silva, L.B. Costa, M.Z. Hernandes, M.M. Rabello, R.S. Ferreira, L.F. Da Cruz, D.R. Magalhães Moreira, V.R.A. Pereira, M.C.A.B. De Castro, P. V. Bernhardt, A.C.L. Leite, 2-Pyridyl thiazoles as novel anti-Trypanosoma cruzi agents: Structural design, synthesis and pharmacological evaluation, Eur. J. Med. Chem. 86 (2014) 48–59. doi:10.1016/j.ejmech.2014.08.012.

[12] M. Popsavin, S. Spaić, M. Svirčev, V. Kojić, G. Bogdanović, V. Popsavin, Synthesis and in vitro antitumour screening of 2-(β-d-xylofuranosyl) thiazole-4-carboxamide and two novel tiazofurin analogues with substituted tetrahydrofurodioxol moiety as a sugar mimic, Bioorganic Med. Chem. Lett. 22 (2012) 6700–6704. doi:10.1016/j.bmcl.2012.08.093.

[13] X. Lu, X. Liu, B. Wan, S.G. Franzblau, L. Chen, C. Zhou, Q. You, Synthesis and

evaluation of anti-tubercular and antibacterial activities of new 4-(2,6-dichlorobenzyloxy)phenyl thiazole, oxazole and imidazole derivatives. Part 2, Eur. J. Med. Chem. 49 (2012) 164–171. doi:10.1016/j.ejmech.2012.01.007.

[14] S. Shafi, M. Mahboob Alam, N. Mulakayala, C. Mulakayala, G. Vanaja, A.M. Kalle, R. Pallu, M.S. Alam, Synthesis of novel 2-mercapto benzothiazole and 1,2,3-triazole based bis-heterocycles: Their anti-inflammatory and anti-nociceptive activities, Eur. J. Med. Chem. 49 (2012) 324–333. doi:10.1016/j.ejmech.2012.01.032.

[15] C. Nava-Zuazo, F. Chávez-Silva, R. Moo-Puc, M.J. Chan-Bacab, B.O. Ortega-Morales, H. Moreno-Díaz, D. Díaz-Coutiño, E. Hernández-Núñez, G. Navarrete-Vázquez, 2-Acylamino-5-nitro-1,3-thiazoles: Preparation and in vitro bioevaluation against four neglected protozoan parasites, Bioorganic Med. Chem. 22 (2014) 1626–1633. doi:10.1016/j.bmc.2014.01.029.

[16] T. Ueda-Nakamura, M. Attias, W. De Souza, Megasome biogenesis in Leishmania amazonensis: A morphometric and cytochemical study, Parasitol. Res. 87 (2001) 89–97. doi:10.1007/s004360000319.

[17] J.C. Mottram, G.H. Coombs, J. Alexander, Cysteine peptidases as virulence factors of Leishmania, Curr. Opin. Microbiol. 7 (2004) 375–381. doi:10.1016/j.mib.2004.06.010.

[18] D.A. Patrick, T. Wenzler, S. Yang, P.T. Weiser, M.Z. Wang, R. Brun, R.R. Tidwell, Synthesis of novel amide and urea derivatives of thiazol-2-ethylamines and their activity against Trypanosoma brucei rhodesiense, Bioorganic Med. Chem. 24 (2016) 2451–2465. doi:10.1016/j.bmc.2016.04.006.

[19] D.R.M. Moreira, A.C. Lima Leite, M.V.O. Cardoso, R.M. Srivastava, M.Z.

Hernandes, M.M. Rabello, L.F. Da Cruz, R.S. Ferreira, C.A. De Simone, C.S. Meira, E.T. Guimaraes, A.C. Da Silva, T.A.R. Dos Santos, V.R.A. Pereira, M.B. Pereira Soares, Structural design, synthesis and structure-activity relationships of thiazolidinones with enhanced anti-Trypanosoma cruzi activity, ChemMedChem. 9 (2014) 177–188. doi:10.1002/cmdc.201300354.

[20] N. Zelisko, D. Atamanyuk, O. Vasylenko, P. Grellier, R. Lesyk, Synthesis and antitrypanosomal activity of new 6,6,7-trisubstituted thiopyrano[2,3-d][1,3]thiazoles, Bioorganic Med. Chem. Lett. 22 (2012) 7071–7074. doi:10.1016/j.bmcl.2012.09.091.

[21] M.J. Chan-Bacab , E. Hernández-Núñez , G. Navarrete-Vázquez . Nitazoxanide, tizoxanide and a new analogue [4-nitro-N-(5-nitro-1,3-thiazol-2-yl)benzamide; NTB] inhibit the growth of kinetoplastid parasites (Trypanosoma cruzi and Leishmania mexicana) in vitro. J Antimicrob Chemother. 63(6) (2009) 1292-3. doi: 10.1093/jac/dkp117.

[22] J.S. de Toledo, P.E.S. Junior, V. Manfrim, C.F. Pinzan, A.S. de Araujo, A.K. Cruz, F.S. Emery, Synthesis, cytotoxicity and in vitro antileishmanial activity of naphthothiazoles, Chem. Biol. Drug Des. 81 (2013) 749–756. doi:10.1111/cbdd.12123.

[23] P. Bhargava, R. Singh, Developments in diagnosis and antileishmanial drugs, Interdiscip. Perspect. Infect. Dis. 2012 (2012). doi:10.1155/2012/626838.

[24] S. Paladi Cde, I.A. Pimentel, S. Katz, R.L. Cunha, W.A. Judice, A.C. Caires, C.L. Barbieri, In vitro and in vivo activity of a palladacycle complex on Leishmania (Leishmania) amazonensis, PLoS Negl Trop Dis. 6 (2012) e1626. doi:10.1371/journal.pntd.0001626.

[25] J.L. Affranchino, C.F. Ibañez, A.O. Luquetti, A. Rassi, M.B. Reyes, R.A. Macina, L. Åslund, U. Pettersson, A.C.C. Frasch, Identification of a Trypanosoma cruzi antigen that is shed during the acute phase of Chagas' disease, Mol. Biochem. Parasitol. 34 (1989) 221–228. doi:10.1016/0166-6851(89)90050-9.

[26] J.C.F. Rodrigues, W. de Souza, Ultrastructural alterations in organelles of parasitic protozoa induced by different classes of metabolic inhibitors., Curr. Pharm. Des. 14 (2008) 925–938. doi:10.2174/138161208784041033.

[27] R.M. Santa-Rita, A. Henriques-Pons, H.S. Barbosa, S.L. de Castro, Effect of the lysophospholipid analogues edelfosine, ilmofosine and miltefosine against Leishmania amazonensis, J. Antimicrob. Chemother. 54 (2004) 704–710. doi:10.1093/jac/dkh380.

[28] P. Bilbao-Ramos, C. Galiana-Roselló, M.A. Dea-Ayuela, M. González-Alvarez, C. Vega, M. Rolón, J. Pérez-Serrano, F. Bolás-Fernández, M.E. González-Rosende, Nuclease activity and ultrastructural effects of new sulfonamides with anti-leishmanial and trypanocidal activities, Parasitol. Int. 61 (2012) 604–613. doi:10.1016/j.parint.2012.05.015.

[29] A. Jiménez-Ruiz, J.F. Alzate, E.T. MacLeod, C.G.K. Lüder, N. Fasel, H. Hurd, Apoptotic markers in protozoan parasites, Parasites and Vectors. 3 (2010). doi:10.1186/1756-3305-3-104.

[30] J. MacMicking, Q.W. Xie, C. Nathan, Nitric oxide and macrophage function., Annu. Rev. Immunol. 15 (1997) 323–50. doi:10.1146/annurev.immunol.15.1.323.

[31] P.M. Selzer, S. Pingel, I. Hsieh, B. Ugele, V.J. Chan, J.C. Engel, M. Bogyo, D.G. Russell, J.A. Sakanari, J.H. McKerrow, Cysteine protease inhibitors as

chemotherapy: Lessons from a parasite target, Proc. Natl. Acad. Sci. 96 (1999) 11015–11022. doi:10.1073/pnas.96.20.11015.

[32] S. Nwaka, B. Ramirez, R. Brun, L. Maes, F. Douglas, R. Ridley, Advancing drug innovation for neglected diseases - Criteria for lead progression, PLoS Negl. Trop. Dis. 3 (2009). doi:10.1371/journal.pntd.0000440.

[33] R. Pink, A. Hudson, M.A. Mouriès, M. Bendig, Opportunities and challenges in antiparasitic drug discovery, Nat. Rev. Drug Discov. 4 (2005) 727–740. doi:10.1038/nrd1824.

[34] M.K. Ibrahim, J.L. Barnes, G.M. Anstead, F. Jimenez, B.L. Travi, A.G. Peniche, E.Y. Osorio, S.S. Ahuja, P.C. Melby, The Malnutrition-Related Increase in Early Visceralization of Leishmania donovani Is Associated with a Reduced Number of Lymph Node Phagocytes and Altered Conduit System Flow, PLoS Negl. Trop. Dis. 7 (2013). doi:10.1371/journal.pntd.0002329.

[35] V. Rodrigues, A. Cordeiro-Da-Silva, M. Laforge, R. Silvestre, J. Estaquier, Regulation of immunity during visceral Leishmania infection, Parasites and Vectors. 9 (2016). doi:10.1186/s13071-016-1412-x.

[36] I. Vouldoukis, P.-A. Bécherel, V. Riveros-Moreno, M. Arock, O. Da Silva, P. Debré, D. Mazier, M.D. Mossalayi, Interleukin-10 and interleukin-4 inhibit intracellular killing of Leishmania infantum andLeishmania major by human macrophages by decreasing nitric oxide generation, Eur. J. Immunol. 27 (1997) 860–865. doi:10.1002/eji.1830270409.

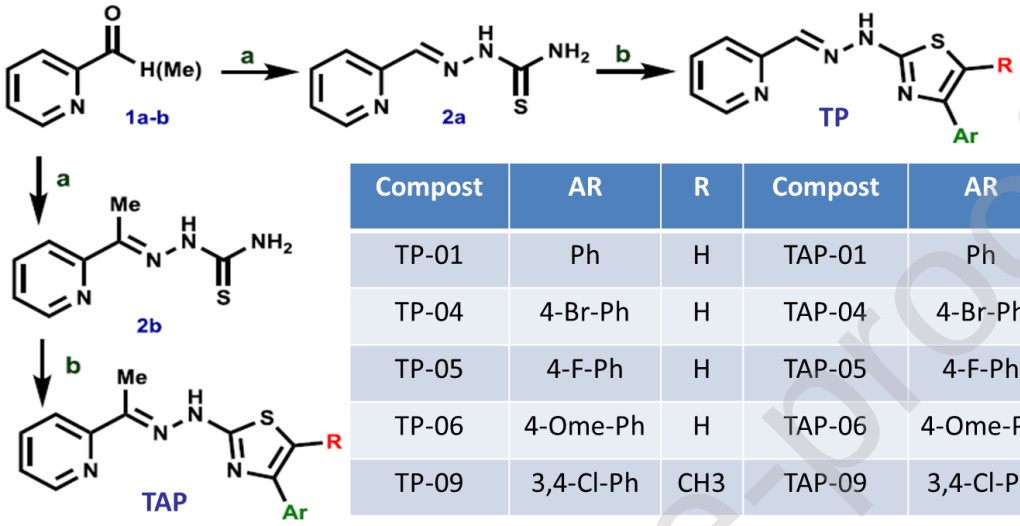
[37] R.K. Pandey, S. Mehrotra, S. Sharma, R.S. Gudde, S. Sundar, C. Shaha, Leishmania donovani-induced increase in macrophage Bcl-2 favors parasite survival, Front. Immunol. 7 (2016). doi:10.3389/fimmu.2016.00456.

[38] A. Geiger, G. Bossard, D. Sereno, J. Pissarra, J.L. Lemesre, P. Vincendeau, P. Holzmuller, Escaping deleterious immune response in their hosts: Lessons from trypanosomatids, Front. Immunol. 7 (2016). doi:10.3389/fimmu.2016.00212.

[39] G. Forget, D.J. Gregory, L.A. Whitcombe, M. Olivier, Role of host protein tyrosine phosphatase SHP-1 in Leishmania donovani-induced inhibition of nitric oxide production, Infect. Immun. 74 (2006) 6272–6279. doi:10.1128/IAI.00853-05.

Figure legends

Fig. 1. Synthetic procedures for thiosemicarbazones (2a-b) and 2- (pyridin-2-yl) 1,3-thiazole (TPs and TAPs). Reagents and conditions: (a) thiosemicarbazide, 2-propanol, acetic acid (3 drops), ultrasonic irradiation, r.t., 120 min; (B) Substituted 2-bromoacetophenones, 2-propanol, CaCO 3, ultrasonic irradiation, r.t., 60 min. Adapted from Cardoso et al [11].



Compost	AR	R	Compost	AR	R
TP-01	Ph	H	TAP-01	Ph	H
TP-04	4-Br-Ph	H	TAP-04	4-Br-Ph	H
TP-05	4-F-Ph	H	TAP-05	4-F-Ph	H
TP-06	4-Ome-Ph	H	TAP-06	4-Ome-Ph	H
TP-09	3,4-Cl-Ph	CH3	TAP-09	3,4-Cl-Ph	CH3

Fig. 2. Survival Index of *Leishmania infantum* within macrophages after 24 hours of treatment with TAP-01, TAP-04 and TAP-06. Note: Asterisks correspond to significant values ($p < 0.05$)

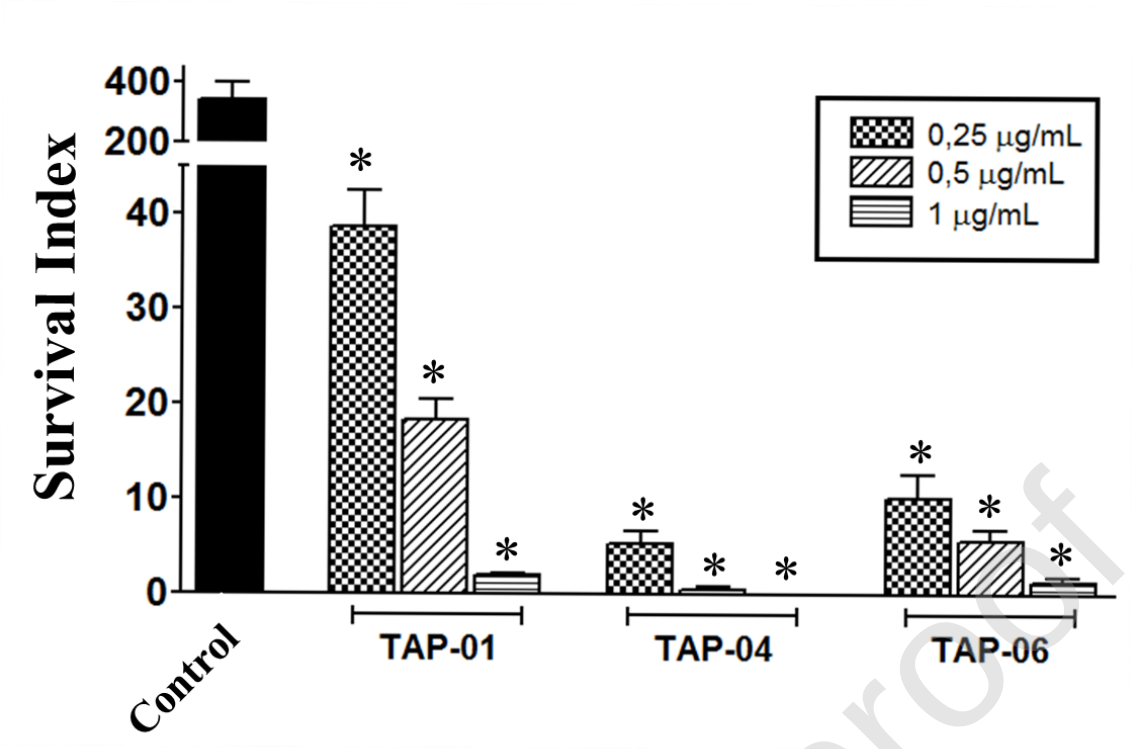


Fig. 3. Transmission Electron Microscopy (MET) of *Leishmania infantum* promastigote parasites after treatment with TAP-04. A: promastigote without addition of TAP-04 demonstrating preserved morphology of mitochondria (M), nucleus (N), kinetoplast (C), flagellar purse (BF), flagellum (F) and glycosome (*). B, C, D: Promastigote treated with 0.5x IC_{50}. Note disorganization of the mitochondrial ridges and kinetoplast, cellular disorganization with displacement of the nucleus to the periphery of the cell and increase of the endoplasmic reticulum (ER) profiles and formation of atypical vesicles in cisterns of the Golgi complex. E, F: Promastigote treated with IC_{50} showing condensation of the chromatin and greater disorganization of the mitochondrial ridges.

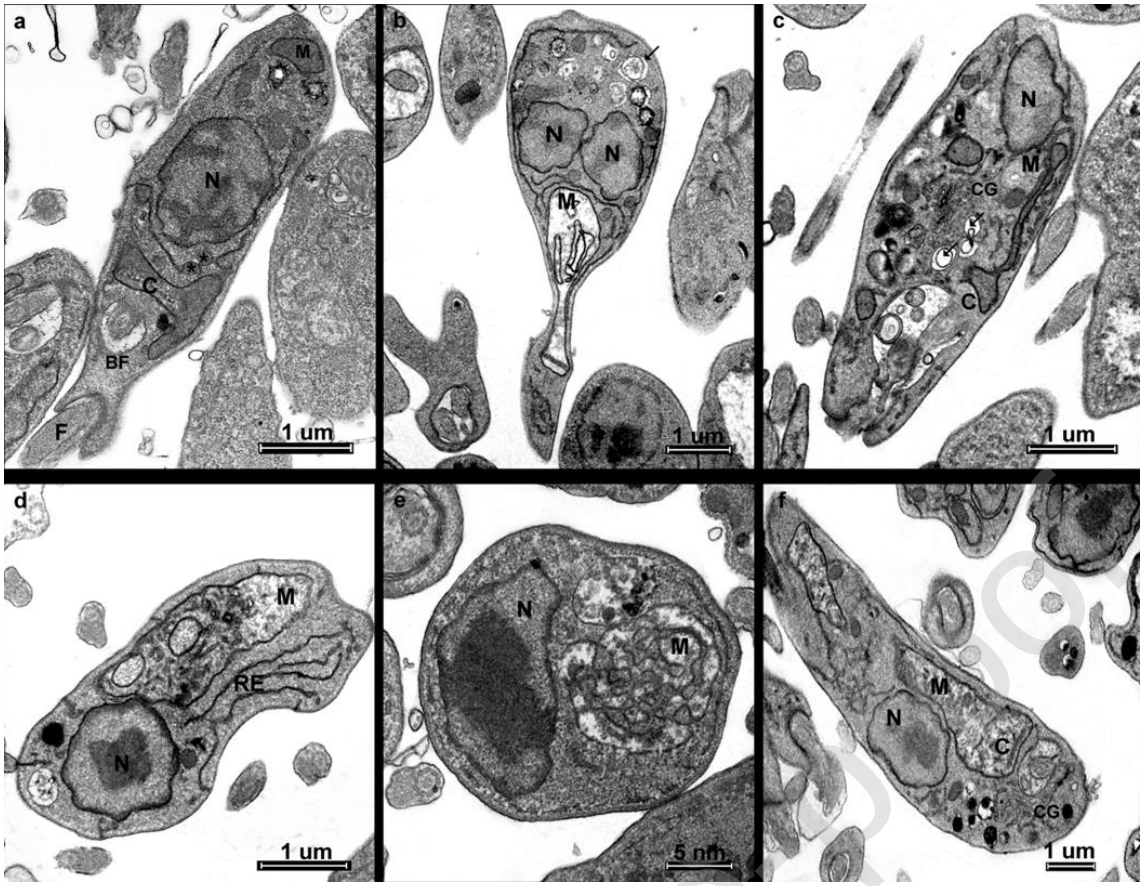


Fig. 4. Scanning Electron Microscopy (MEV) of *Leishmania infantum* promastigote parasites after treatment with TAP-04. A, B: Promastigote without addition of TAP-04 presenting an elongated cell body and an integral membrane. C, D: Promastigote treated with 0.5x IC_{50} showing changes in morphology, rounded forms and loss of flagellum. E, F: Parasite treated with IC_{50} showing loss of membrane integrity with exposure of the cytoplasmic material demonstrated with the appearance of electron-sparing regions in the cytoplasm region.

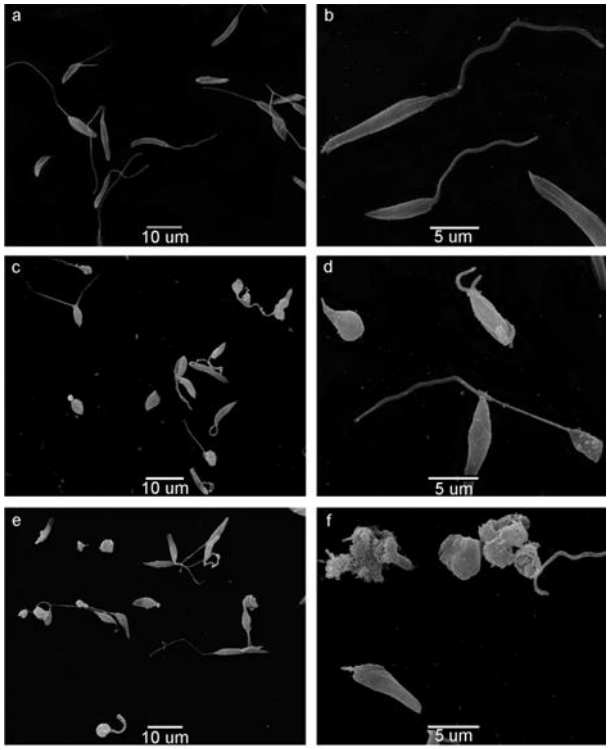


Fig. 5. Nitric oxide dosage in the supernatant of the infected cultures cells with and without addition of TAP-01, TAP-04 and TAP-06.

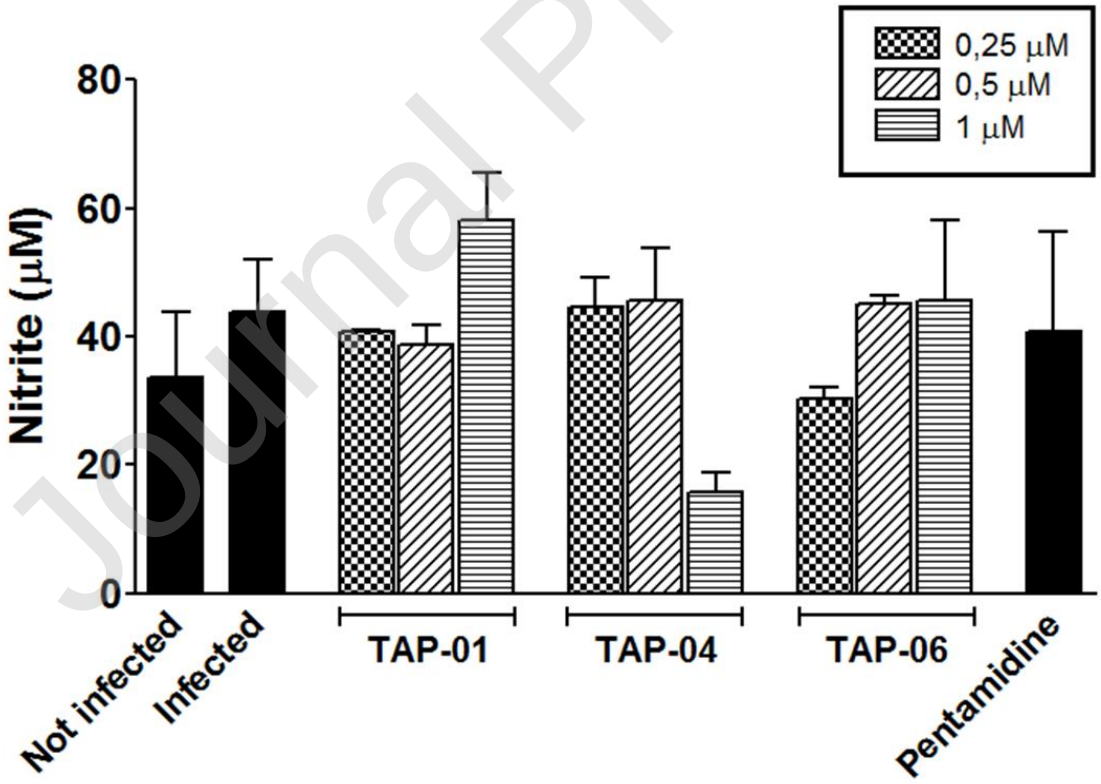


Table 1
Effect of the thiazole derivatives on the promastigotes growth and amastigotes growth of *Leishmania infantum* and on peritoneal macrophages of BALB/c mice.

	Promastigotes **IC_{50}**	Macrophages **CC_{50}**	Promastigotes **SeI**	amastigotes **IC_{50}**	Amastigotes **SeI**
	µM			**µM**	
TAP-01	3.57 ± 0.95	45.46 ± 11.66	12.73	0.99 ± 0.03	46.10
TAP-04	3.12 ± 0.86	59.09 ± 15.19	18.94	0.43 ± 0.11	137.37
TAP-05	0.42 ± 0.03	2.95 ± 0.29	7.07	-	-
TAP-06	4.44 ± 1.27	34.62 ± 13.21	7.79	0.59 ± 0.09	59.05
TAP-09	2.73 ± 0.61	4.31 ± 0.86	1.57	-	-
TP-01	<2.78	11.71 ± 1.21	*	-	-
TP-04	7.01 ± 3.72	41.51 ± 1.70	5.92	-	-
TP-05	22.55 ± 0.97	109.67 ± 39.15	4.86	-	-
TP-06	<2.78	1.35 ± 0.19	*	-	-
TP-09	5.52 ± 0.29	4.68 ± 1.87	0.84	-	-

* it was not possible to determine.