ejpmr, 2017,4(7), 799-812

SJIF Impact Factor 4.161

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

THIOSEMICARBAZONES AND THIAZOLIDINONES, SAR-STUDIES AND ANTI-EXTRA AND INTRACELLULAR PARASITIC ELIMINATION OF *LEISHMANIA AMAZONENSIS*

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Article Received on 16/05/2017 **Article Revised on 06/06/2017** **Article Accepted on 29/06/2017**

ABSTRACT

Leishmaniasis has a high prevalence in under-developed and developing countries and can be lethal if untreated. Nonetheless, the available treatments, including pentavalent antimonials or second-line drugs, can cause a wide variety of drawbacks, or the drug activity can stop. Considering the prominent role of the thiosemicarbazones and thiazolidinones as therapeutic agent candidates due to their versatility in production and activity, these compounds were tested against *Leishmania amazonensis.* To verify their capacity to eliminate extra and intracellular parasites as well as the structure-activity relationship using an *in silico* approach. Initially, proliferative promastigotes were incubated at different times and concentrations. Thereafter, infected macrophages with proliferative intracellular amastigotes were subjected to the same conditions. The antiparasitic analyses showed the elimination of both extra and intracellular forms with 24 hours of incubation. The morphological/ultrastructural studies revealed both parasite forms with progressive internal organelles disorganization including promastigotes with rounded shape, dense nucleus and loss of flagellum whereas amastigotes were at different stage without plasma membrane rupture. No effect was observed against host cells with a selective index higher than 10 times when analysing the amastigotes effects. The SAR studies indicate that lower values of Energy of HOMO and number of hydrogen bond acceptors (nOHNH-HBD) and higher values of Energy of LUMO, hydrogen bond donors (nON-HBA), Dipole, Area, Volume, and PSA lead to the safer compounds represented by thiazolidinone group. These data pointed these compounds as good antiparasite prototypes for further studies as they affected both *L. amazonensis* forms.

KEYWORDS: amastigotes; *Leishmania amazonensis;* promastigotes; SAR-studies; Thiazolidinones; Thiosemicarbazones.

1 INTRODUCTION

Trypanosomatidae protozoans from the Leishmania genus are ethiological agents of Leishmaniasis, a neglected disease.[1] The life cycle of this causing agent involves the female Phlebotomine sandflies that transmit the promastigotes during feeding from vertebrates.[2] They invade the host mononuclear system blood cells and settle into phagosome where they differentiate into amastigotes. The intravacuollar amastigotes modulate the host cell microbicide system mainly in the phagosome-lysosome pathway to survive and replicate,[3] leading to the host cell rupture when they gain access to the blood stream. Thus they are phagocytised by new cells, establishing the vertebrate host infection. During intracellular development, amastigotes modulate the host cell microbicide system, mainly the phagosome-lysosome pathway, to survive and replicate.[3] These host-cell parasite interactions are responsible for different clinical disease manifestations in the three main forms: cutaneous, mucocutaneous and visceral Leishmaniasis.[3–8] L. amazonensis is the species directly involved in the high incidence of Leishmaniasis in Brazil [9,10] and other countries.

The most commonly used drugs against *Leishmania spp.* are pentavalent antimonials, such as Pentostan® and Glucantime®. However, they present problems (*eg.* long-term and painful treatment) with a broad range of

toxic effects. In addition, parasite resistance also limits the available treatment options.[11,12] Due to the emergence of parasite resistance, associated treatments are currently in use (*eg.* Paramomycin®, Pentamidine® and Amphotericin B®). However, they also present high toxicity and new drugs such as Miltefosine is the current drug for visceral leishmaniasis, but it is very expensive.[13– 15]

Thiosemicarbazones (TSCs) and Thiazolidinones (TZDs) are known as potential chemical leading groups for producing new therapeutic agent prototypes due to: *i*) diverse biological profile; *ii*) easy, low-cost and rapid production; and *iii*) high stability.[16–18] TSCs have been studied for several decades as they allow different substitution patterns. The pharmacological properties of TSCs and TZDs in complex with metalloproteins have been explored since 1946 [19] and the potential for coordination has been responsible for avoiding drug resistance. [16] Earlier studies of our group suggest that these same classes of compounds are able to arrest the parasite's life cycle, allowing the host cell to eliminate intracellular forms of *T. cruzi and T. gondii* (which present different forms of parasitism).[20-30] This feature is of interest for drug design area specially when involving diseases caused by resistant strains such as Leishmaniasis.

In this work we evaluated a series of TSCs and their compounds TZDs and evaluated their anti-leishmanial activity, representing a new parasitism model not studied with these compounds (Figure 1). These compounds were tested against extracellular and intracellular *L. amazonensis* forms and have their mechanism against the parasite components analyzed by an ultrastructural approach. Finally, we performed a brief structure-activity relationship study of these molecules using a molecular modelling approach.

2- MATERIALS AND METHODS

2.1. Cell cultures maintenance

Leishmania amazonensis promastigotes (LV79 strain) were maintained in Warren's medium (90% Brain heart broth, SIGMA ALDRICH, St. Louis, Missouri, EUA) containing 10% heat-inactivated foetal bovine serum (FBS, SIGMA ALDRICH) and enriched with 0.01% folic acid and 0.4% hemin at 28°C. An aliquot (1 mL) was transferred to a new medium every four days to maintain the exponential growth curve.

Macrophages were obtained from normal Suisse mice through peritoneal washing using Hank's solution at 4°C and cultivated in 24-well plates containing glass cover slips at a growth rate of 5 x 10^5. After 1 hour of culture, the Hank's solution was replaced by DMEM 1152 (SIGMA ALDRICH) medium supplemented with 10% of FBS, and the macrophages were maintained in an atmosphere at 37°C with 5% of CO2.

All procedures for the use of the animals followed the Ethical Guidelines for Animal Experimentation of the North Fluminense State University - Darcy Ribeiro, protocol number 104.

2..2 Host cell-parasite interaction and incubations

Promastigotes were centrifuged for 10 minutes at 300 *g*. The pellet was suspended in 1 mL of phosphate buffer solution (PBS), pH 7.2 at 4°C and counted using a Neubauer chamber. The promastigotes were incubated with the compounds at 10^6 parasites/mL (exponential growth) for 1–6 days. All compounds were diluted in DMSO at 1.5% v/v, which was not toxic for the parasites (data not shown), and Warren's medium at 0.1, 1 and 10 mM. Hydroxyurea (HU, SIGMA ALDRICH) and Pentamidine (SIGMA ALDRICH) were used as reference drugs. An aliquot of parasites was obtained each 24 h for quantification and monitoring of toxicology using a Neubauer chamber.

After 24 h of culturing macrophages, 24 randomly fields were counted with around 200 cells each one (3 fields of 8 wells) and calculated the average. The promastigotes were estimated as describe above and incubated with macrophages at a rate of (10:1 parasites/host cell), and after 1 hour, the medium was replaced to remove the extracellular parasites. The culture was maintained in an atmosphere at 37°C with 5% of CO2 for 24 h. For drugs assays, the compounds were diluted as described above and added at 0.1, 1, 5 and 10 mM for an additional 24 h. HU and Pentamidine were used as reference drugs. All experiments were performed in triplicates.

The HU is used in these assays as reference drug because it is a cellular cycle controller, what it is suspected that these drugs are, and the used drugs have as the central structure a carbon binds with three nitrogenous (the same structure of HU).

2.3. Quantification and structural analyses

For quantification, a promastigote sample was collected each day of treatment through 6 days, fixed with formaldehyde (SIGMA ALDRICH) solution (formaldehyde 4% + PBS), and counted using a Neubauer chamber.

We harvested a 0.5 mL sample of the control and treated promastigotes and fixed the sample with formaldehyde solution (formaldehyde 4% + PBS) for morphological analyses. The promastigotes were heated to adhere to the slips, stained with Giemsa's (SIGMA ALDRICH) solution (methylene blue 10% + ultrapure water 90%) for 15 minutes and washed in ultrapure water. The slips were heated again to dry, and we covered the cells with a coverslip, which was adhered to the slip through Canadian balsam.

The slips were observed in a ZEISS AXIOPLAN microscope, and morphological analyses were obtained. Infected macrophage cultures treated or not with

compounds were washed in PBS solution and fixed in Bouin's solution (picric acid 67%, formaldehyde 22% and acetic acid 11%) for 5 minutes. The cells were washed three times with PBS to remove the fixative solution and stained with Giemsa's solution for 6 hours at room temperature. The samples were then washed with ultrapure water to remove the staining solution. The slips were dehydrated with an acetone-xylene solution and fixed as histological slips for visualization and quantification using a Zeiss AXIOPLAN microscope.

The macrophages were scored under 40 x magnification using three different view fields for each slide (each one with around 200 cells), as a total of 8 slides per treatment. Four parameters were analysed: number of (1) uninfected macrophages, (2) infected macrophages, (3) unaltered amastigotes and (4) vacuoles containing morphologically altered parasites. Thereafter, LD 50 values were calculated.

2.4. Ultrastructural analyses

The promastigotes and infected macrophages were incubated with compound **10** at 1 mM for 12 and 24 h. Both were fixed in a solution containing glutaraldehyde (SIGMA ALDRICH) 2%, formaldehyde 4%, sodium cacodylate buffer 0.1 M, calcium chloride 5 mM and sucrose 5% for 1 h at room temperature, and they were post-fixed for 1 h in a solution containing osmium tetroxide (SIGMA ALDRICH) 2% and potassium ferrocyanide (SIGMA ALDRICH) 0.8%. The cells were rinsed with cacodylate buffer 0.1 M, dehydrated with acetone and embedded in PolyBed® 812 (POLYSCIENCE INC, Valley Road, Warrington, Philadelphia, EUA). Thin sections obtained using an ultramicrotome were stained with uranyl acetate for 20 min and lead citrate for 5 minutes and were then observed under a Zeiss 900 Transmission Microscope.

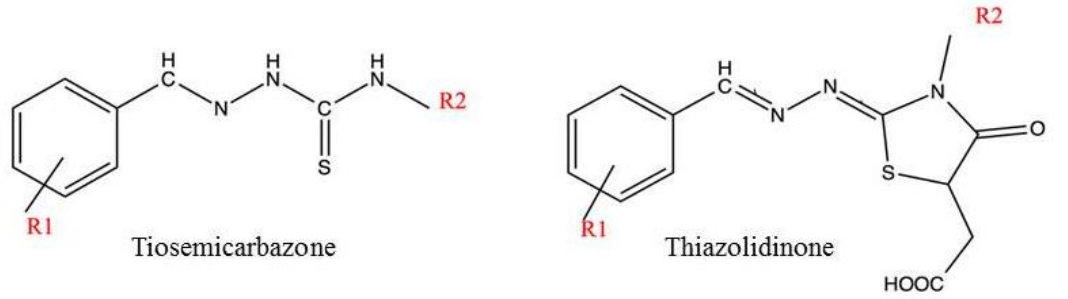
2.5. Structure-activity relationship studies

TSC and their 4-TZD derivate chemical structures were constructed using the Spartan'10 program (Wavefunction©, Inc.) and then subjected to conformational analyses based on Monte Carlo using an MMFF force field (Merck Molecular Force Field). The *in silico* study of 4-TZD compounds were carried out in the ionized state simulating the intracellular pH range condition. The geometry optimization assays for the more stable conformer were performed using the Recife Model 1 (RM1) semi-empirical method. The stereoelectronic properties (energy of Highest Occupied Molecular Orbital -HOMO and Lowest Unoccupied Molecular Orbital - LUMO, density maps, orbital coefficients distribution, molecular dipole moment and molecular electrostatic potential maps - MEP) were characterized for each compound using Hartree-Fock *ab initio* calculation and the 6-311G** basis set.

In order to evaluate the lipophilicity of the compounds we calculated LogP and LogD values (octano-water partition coefficient) with ChemAxon Calculator (Copyright © 1998-2016 ChemAxon Ltd.). We also evaluated *in silico* toxicity with *DataWarrior* program (www.openmolecules.org).

2.6. Chemical synthesis

The molecules were synthesised and firstly published in Tenório *et al.*[20] Briefly, TSC (**1-8**) and TZD (**9-16**) compounds (Figure 1) were synthesised through a reaction between the thiosemicarbazide and respective benzaldehydes substituted in an acidic medium under reflux with satisfactory yields. These compounds were then cyclized with excess maleic anhydride in dry toluene and *N*, *N*-dimethylformamide to yield the respective 4-TZD compounds. All compounds are stable in both the solid and solution states. Spectroscopic data for these compounds were consistent with their structures. HU was obtained from Sigma.



Compounds	1	9	2	10	3	11	4	12	5	13	6	14	7	15	8	16
R1	p-NO_2		m-NO_2		p-NO_2		o-NO_2		m-NO_2		p-NO_2		o-NO_2		m-NO_2	
R2	H		CH_3		CH_3		CH_2CH_3		CH_2CH_3		CH_2CH_3		C_6H_5		C_6H_5	

Figure 1: The representative scheme of the 16 compounds from Thiosemicarbazone (1-8) and Thiazolidinone (9-16) classes including 2D structure, radicals (R1/R2) and nomenclature.

3. RESULTS AND DISCUSSION

3.1. TSC and TZD antiparasitic profile against *L. amazonensis* extracellular form (promastigotes)

This work explored the effects of the compounds from TSC and TZD series against *L. amazonensis* extracellular form (promastigotes) at 0.1, 1 and 10 mM during 6 days (Figure 2 and 3) since metacyclic promastigotes are highly infective and to verify whether the drugs are able to eliminate the parasites. After the first day of treatment with TSC, the number of parasites significantly decreased for all compounds whereas the control culture remained at exponential growth (Figure 2A).

The compounds effects were dose and time dependent with the best profile detected for **5** and **8**, which eliminated all parasites after 2 days of incubation at 10 mM, similar to Hydroxyurea, the control drug. Nonetheless, the Pentamidine showed an IC 50 value of 0.46 M (data not shown). The other compounds showed variable parasite number decrease whereas **1** allowed the parasite exponential growth at the end of the treatment (Figure 2A). We also aligned the TSCs data at 1 mM concentration on 1, 3 and 6 days based on parasite survival to allow a better evaluation (Figure 2B), which reinforced that **5, 6** and **8** exhibited higher antiparasitic effects than **1**, **4** and **7** (Figure 2B).

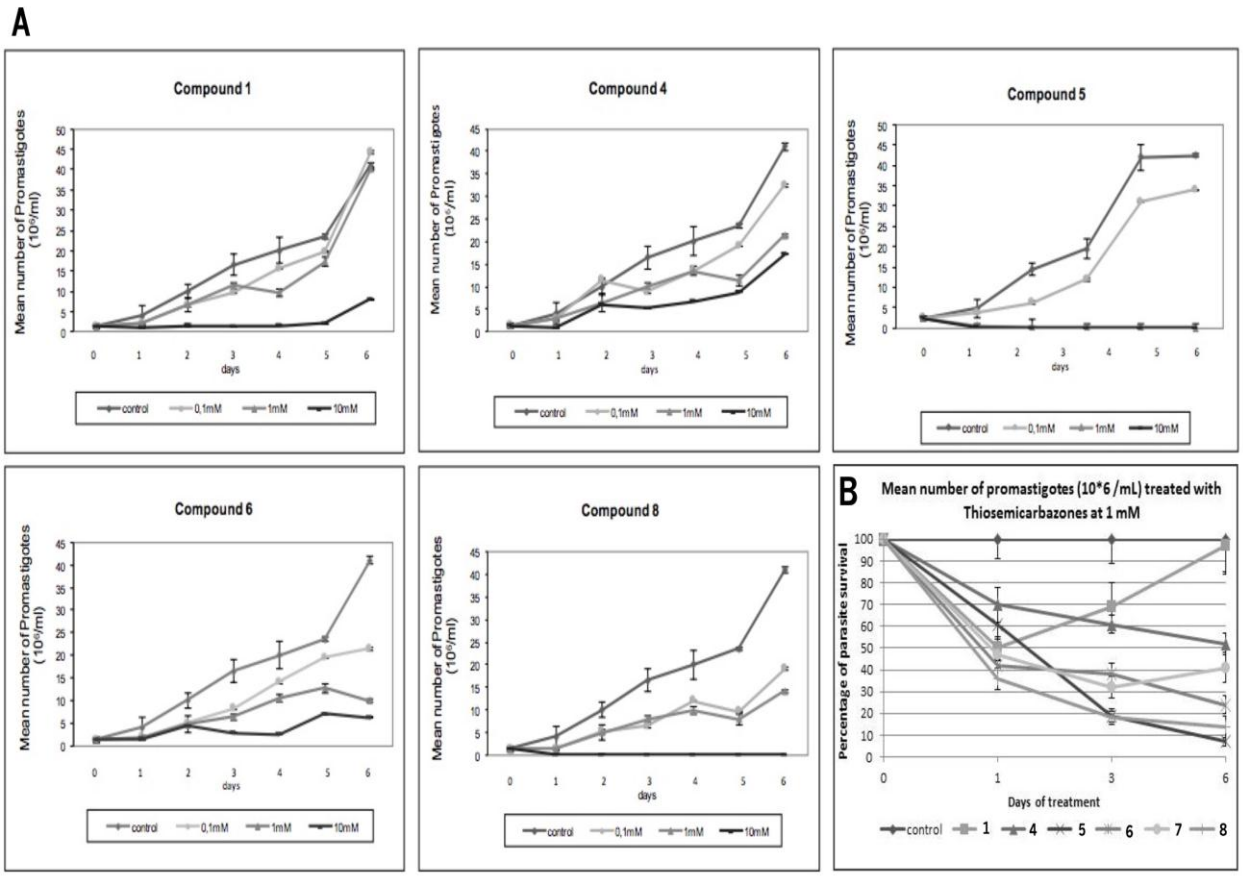


Figure 2: Effects of Thiosemicarbazones on *Leishmania amazonensis* extracellular form (promastigotes) after 1-6 days of incubation. A) Remaining promastigotes (Mean Number) at 0.1, 1 and 10 mM, and B) Parasite survival (%) at 1 mM.

Promisingly, the cultures did not grow after TZD treatments at 1 and 10 mM whereas some compounds eliminated all promastigotes after 1, 2 or 3 days of incubation (**12, 9** and **16** respectively) in contrast to TSC data. This pointed TZD as more effective than TSC (Figure 3A). The comparison of TZD compounds, revealed **14** as capable of eliminating all parasites after 3 days of treatment, while the remaining compounds exhibited antiparasitic effects that varied from 55 to 70% (Figure 3B).

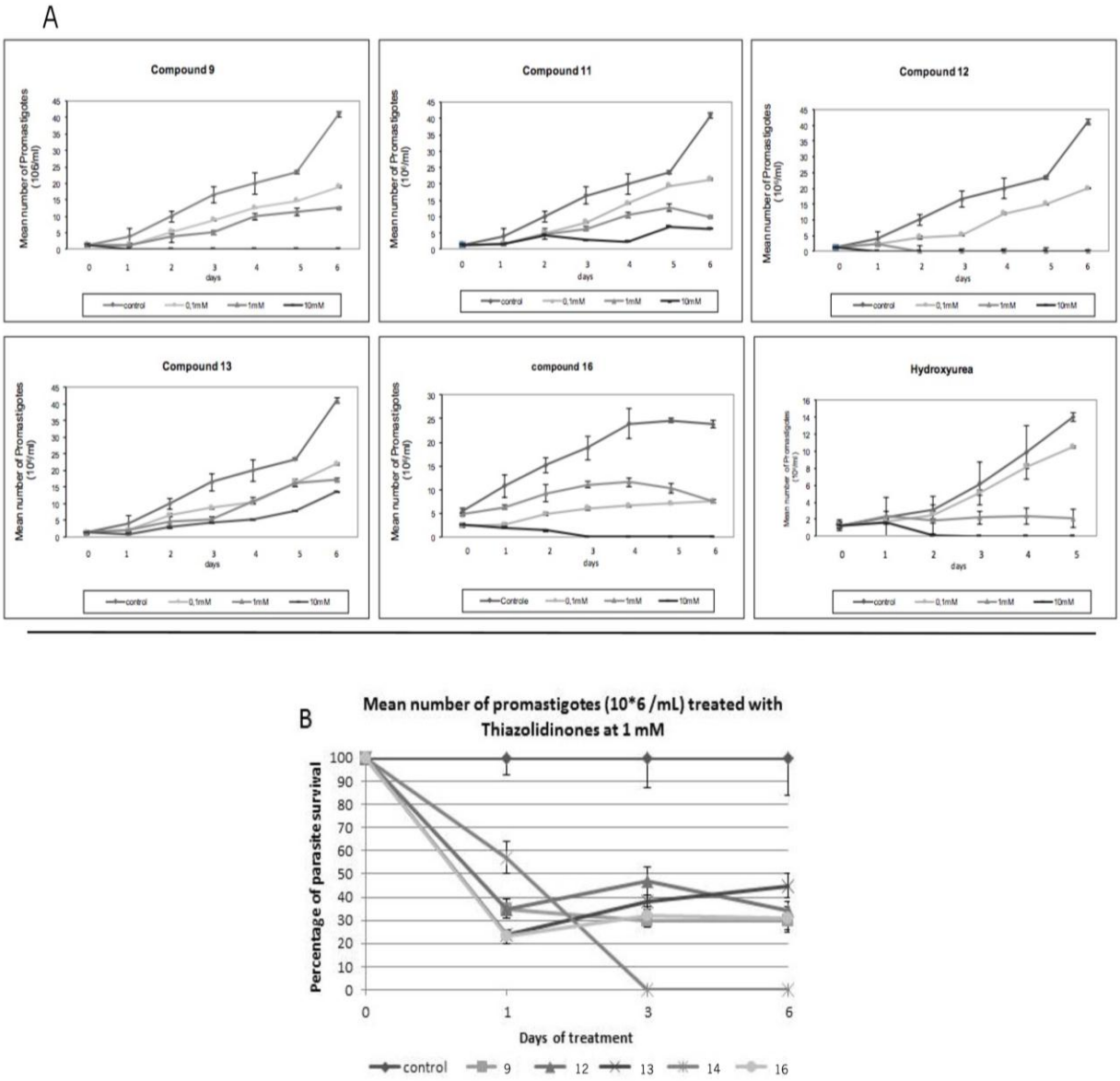


Figure 3: Effects of Thiazolidinones on *Leishmania amazonensis* extracellular form (promastigotes) after 1- 6 days of incubation. A) Remaining promastigotes (Mean Number) at 0.1, 1 and 10 mM, and B) Parasite survival (%) at 1 mM.

Recently, the literature described several synthetic and natural compounds with leishmanicidal activity but mostly only against promastigotes. Among the molecules described are ferrocenyl N-heterocyclic analogues against the *L. infantum,*[31] tetrazole compounds and their pyrazole-4-carbonitrile precursors against *Leishmania* spp, [32] alkyl-substituted benzophenones,[33] lipophilic extracts from Hypericum species[34] and recombinant and natural defensins from *Vigna unguiculata* seeds against *L. amazonensis.* [35,36] Although these studies presented interesting results, they lack the evaluations against the intracellular amastigote form that should complement the results.

3.2. TSC and TZD synthesis and evaluation against *L. amazonensis* intracellular form (amastigotes)

The 16 compounds from Thiosemicarbazone (TSC 1-8) and Thiazolidinone (TZD 9-16) classes were tested against amastigotes, the most infective and important *L. amazonensis* form (Figure 4).

The screening analysis using *L. amazonensis* amastigotes confirmed the antiparasitic profile of TSC and TZD compounds at 1 mM (Figure 4). All compounds were able to decrease the macrophages infection as well as the number of parasites within whereas the untreated controls showed proliferative amastigotes and typical number and morphological characteristics as expected after 24h incubation (Figure 4).

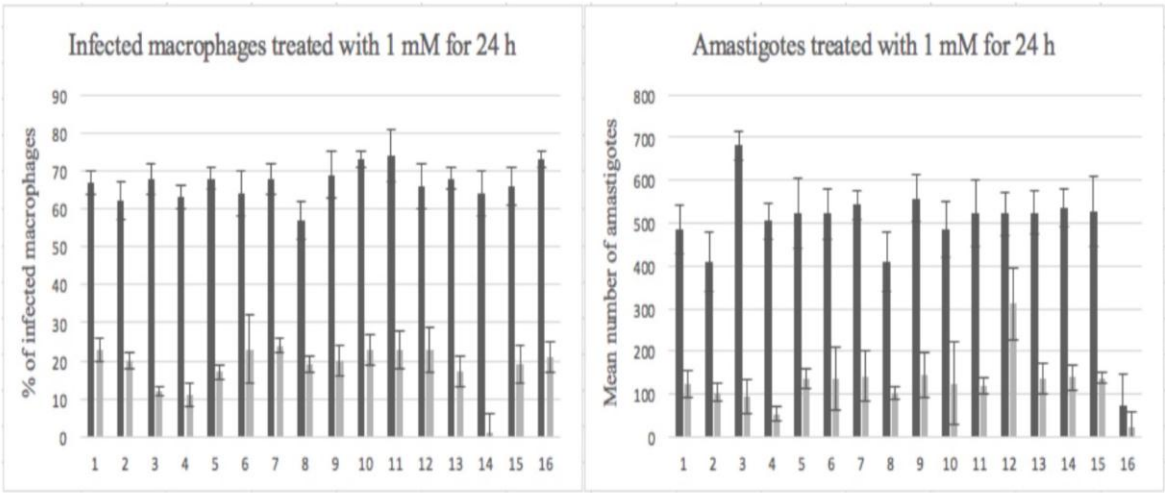


Figure 4: Comparison of the effects of Thiosemicarbazones (1-8) and Thiazolidinones (9-16) on macrophages infected with intracellular parasites (amastigotes) after incubation at 1 mM, 24h, including A) level of infection (%) and B) number of parasites within.

Due to limitations to evaluate a large number of compounds in macrophage culture, we picked up the compounds 1, 4, 6, 7, 9, 10, 12 and 14 at different concentrations (0.1, 1, 5 and 10 mM) to further studies, which revealed a dose-dependent effect (Table 1). A decrease on infected cells was detected ranging from 66% (1) to 91% (6) at 1 mM whereas at 5 mM, 75-96 % of decrease was observed (9 and 4, respectively). Interestingly, a reduction was detected on number of amastigotes ranged from 0-30 % at 0.1 mM despite its low decrease of infection > 10% (1 and 14, respectively) (Table 1). The number of amastigotes was also affected from 74 (1 and 9) to 97 % (4) at 1 mM and 86-100 % (9 and 4, respectively) at 5 mM. Importantly, these compounds eliminated all amastigotes at 10 mM, except for compound 9. The CC50 (cytotoxic concentration to kill 50% of the cells) was in the range of 0.2-0.4 mM in both group of compounds (Table 1), while the Pentamidine showed a IC50 = 118 M for amastigotes.

Table 1: Comparison of the effects of different concentrations (0.1, 1, 5 and 10 mM) of active Thiosemicarbazones and Thiazolidinones on macrophages infected with intracellular parasites (amastigotes) after incubation for 24h.

Compound		Infected cells (%)				Number of Intracellular parasites (Mean±SD)					CC_{50} (mM)
TSC/TZD(mM)	Control	0.1	1	5	10	Control	0.1	1	5	10	
1 (TSC)	67±3	75±5	23±3	13±3	0	486±58	519±86	126±33	46±14	0	0.3
4 (TSC)	67±3	65±1	8±2	3±2	0	1196±128	1032±72	39±9	4±3	0	0.2
6 (TSC)	70±2	65±6	6±1	6±2	0	861±119	799±115	46±8	10±7	0	0.2
7 (TSC)	65±2	67±2	9±3	12±6	0	812±53	687±156	27±9	11±5	0	0.2
9 (TZD)	69±6	62±5	20±4	17±2	13±4	558±116	542±111	147±52	77±19	51±19	0.4
12 (TZD)	67±2	71±2	10±2	6±2	0±0	1224±124	1216±75	47±12	8±4	0±0	0.2
14 (TZD)	84±3	78±2	11±2	11±4	0±0	1480±207	1041±129	85±25	23±9	0±0	0.2

Since some infected macrophages treated with Thiosemicarbazones and Thiazolidinones showed disorganized intravacuolar amastigotes, we also evaluated the number of vacuoles containing morphologically altered parasites after 24h incubation (Table 2).

Table 2: Comparison of number of vacuoles containing morphological altered parasites after 24 h of incubation with active compounds at different concentrations.

Compound	Vacuoles with altered parasites (Mean+ SD)			
TSC/TZD	0.1 mM	1 mM	5 mM	10 mM
1 (TSC)	0	36±5	28±5	12±2
4 (TSC)	10±2	35±4	6±2	6±1
6 (TSC)	11±4	46±8	11±2	7±1
7 (TSC)	9±3	36±11	11±5	6±2
9 (TZD)	0	31±2	28±4	18±3
12 (TZD)	9±2	45±7	10±2	7±1
14 (TZD)	20±4	60±8	13±3	9±1

The number of vacuoles containing morphologically disorganized amastigotes increased from 0.1 to 1 mM whereas decreased at higher concentrations of 1 and 10 mM of the compounds, probably because these last concentrations exhibited quick and drastic lethal effect (Table 2).

3.3. *In vitro* and *in silico* toxicity profile of TZC and TSC

In order to analyze the toxicity profile of TSC and TZD, we determined the CC50 against macrophages, also comparing these data with that found against amastigotes and calculating their selective index (Figure 5). Interestingly, when comparing the effects against infected macrophages and amastigotes, most compounds presented a selective index greater than 10-fold (Figure 5).

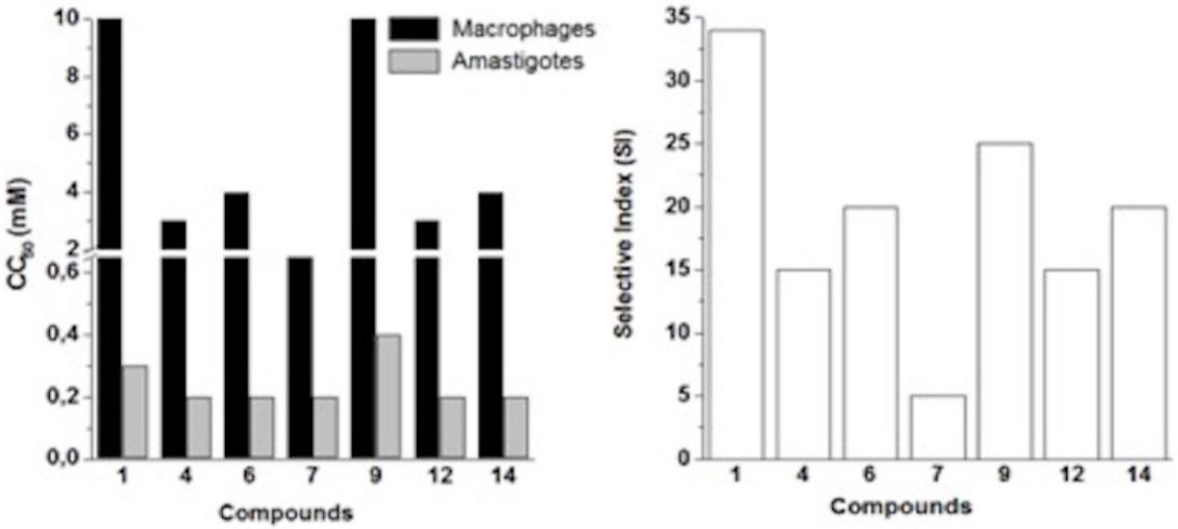


Figure 5: Comparison of Thiosemicarbazones and Thiazolidinones cytotoxic effects (CC50) against host cell and amastigotes after 24h (left), and Selective Index (right). Compounds 1 and 9 showed CC50>10 mM.

In this work we also evaluated the in silico toxicity profile of these compounds using molecular modelling tools. According to our theoretical data, TZD presented low risk of toxic effects generating no mutagenic, tumorigenic, reproductive and irritant theoretical alerts, similar to Pentamidine and Miltefosine. In contrast, TSC presented high risks of mutagenic (compounds 2-8), tumorigenic (compounds 4-6), and reproductive (compounds 2-8) effects similar to Hydroxyurea. None of the compounds presented irritant effects but Hydroxyurea, which presented high risks of toxicity for all categories evaluated.

3.4. Morphological studies of the effects of a Thiazolidinone against *L. amazonensis* extra and intra cellular forms

Herein the morphological aspects of the mechanism of these molecules was also explored by analyzing both intra and extracellular forms of *L. amazonensis* treated with one of the safest compound TZD **(10)** and comparing with the untreated controls (Figure 6). Different from the untreated parasite that showed conserved flagellar extensions, nuclei and kinetoplasts (Figure 6 A), the morphological analyses of promastigotes treated with the selected TZD during 12 h showed a rounded shape, dense nucleus and loss of flagellum (Figure 6 B). Drastic morphological alterations were detected as time progressed and the compound concentrations increased (data not shown). The cultures treated with TZD also presented a decrease in intracellular amastigotes as showed in Figure 6. The remaining parasites showed morphological changes (Figure 6 D) whereas the host cell did not show any alterations (Figure 6 C-D) in agreement to the previous analysis of the citotoxity (CC50) and selective index values.

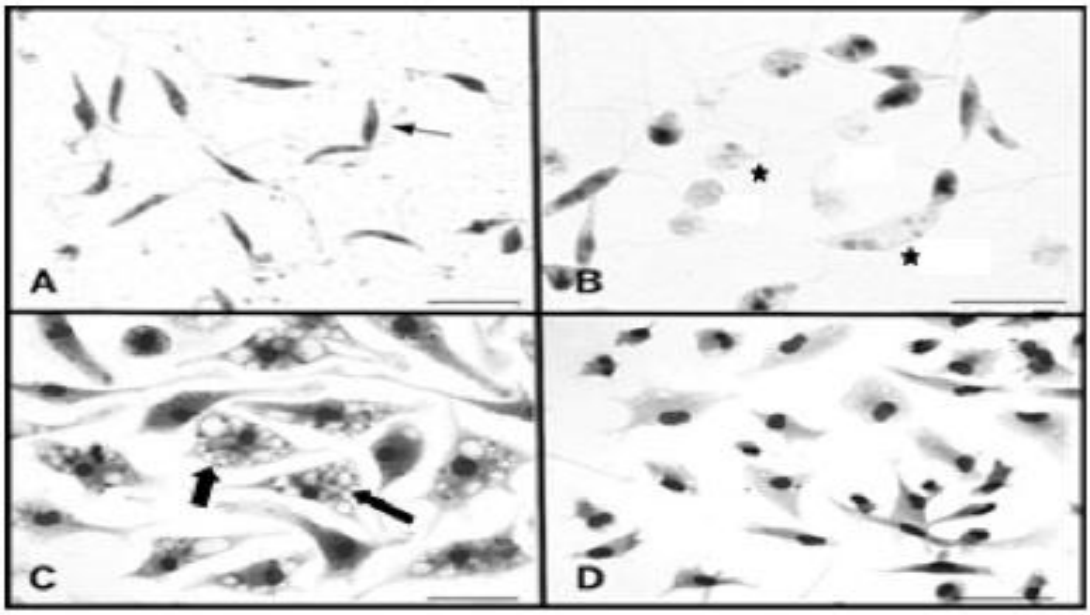


Figure 6: Morphological analysis of *Leishmania amazonensis* extracellular and intracellular forms treated with a TZD. (A) Untreated promastigotes. (B) treated promastigotes with TDZ at 1 mM, 12 h.

(C) Untreated infected macrophages and (D) infected macrophages treated with TDZ at 1 mM, 24h. Black arrowhead: control promastigote; stars: promastigotes in assynchronic destruction; Black arrow: amastigotes. Scale Bars: 20 µm.

3.5. Ultrastructural studies of the effects of a Thiazolidinone against *L. amazonensis* extra and intra cellular forms

The ultrastructural analysis of the promastigotes performed in this work showed the untreated parasites with normal features (Figure 7 A). However, after treating with TZD at 1 mM for 12 h as described in the material and methods section, the samples showed internal structure alterations, including membranes, organelles, and cytoplasm extraction, without external membrane rupture (Figure 7 B). Comparing the macrophages and amastigotes under the same treatment conditions, we observed vacuoles containing parasites in different stages of destruction and, again, internal structures in advanced stages of disorganization (Figure 7 C). After 24h, the parasites were fully destroyed, and no recognizable structures were observed whereas no alterations were observed in the host cell.

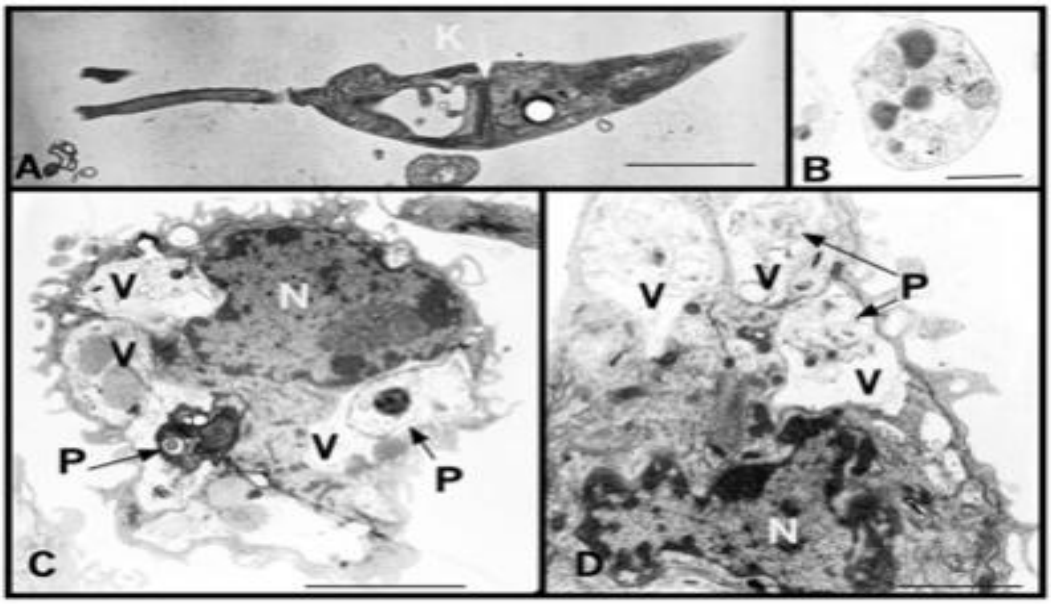


Figure 7: Ultrastructural analysis of promastigotes and macrophages containing amastigotes under treatment with a TDZ. (A) Untreated promastigotes, (B) destroyed promastigote after the treatments with TDZ at 1 mM, 12 h. (C) Infected macrophages treated with TDZ at 1 mM, 12 h and (D) 24h. K: Kinetoplast; N: Host cell nuclei; V: vacuole containing parasite and P: parasite. Scale Bars: A and B: 2 µm; C and D: 10 µm.

Our research group has been testing TSC and TZD compounds and already detected activity against *Toxoplasma gondii* and *Trypanosoma cruzi* comparable with Hydroxyurea, the control drug [20-30]. These previous studies confirmed that TSC and TZD arrested *T. gondii* proliferation cycle and intracellular pathways, causing lysosome fusion and acidification of the parasitophorous vacuole in Vero cells whereas caused *T. cruzi* acidification and autophagic vacuole formation on Vero cells.[32] These results suggest that these classes of drugs act through the inhibition of the parasite replication cycle and so, the parasites lost the ability to subvert the host cell microbicide responses, being eliminated by them. The control of the parasite's cell cycle is low explored even being very promising. The usual drugs utilize other different pathway to eliminate the parasites.

The pentavalent antimonial mechanism of action is unclear but some authors suggest DNA fragmentation as the current targets [37] as well as the inhibition of trypanothione reductase, an enzyme that protects the parasites against host reactive nitrogen and oxygen species.[38] For Pentamidine, the leishmanicidal activity is due to the decrease in mitochondrial membrane potential and inhibition of mitochondrial topoisomerase II.[39] Amphotericin B inhibits sterol production and causes pore formation in the promastigotes cell membrane, which leads to parasite death through osmotic shock.[40] The Miltefosine mechanism of action remains unclear; however, the apoptosis pathway was observed for promastigote and amastigote forms of *Leishmania donovani* with the decrease in lipid content and increase in phosphatidylethanolamine in promastigote membranes. Amphotericin B inhibits sterol production and causes pore formation in the promastigotes cell membrane, which leads to parasite death through osmotic shock.[40] The Miltefosine mechanism of action remains unclear; however, the apoptosis pathway was observed for promastigote and amastigote forms of *Leishmania donovani* with the decrease in lipid content and increase in phosphatidylethanolamine in promastigote membranes.

This suggests inhibition of phosphatdylethanolamine-N-methyltransferase, which yields a lower parasite proliferation rate.[41-42] The literature also described the evaluation of leishmanicidal potential of some TSC and TZD series. Some of these reports indicated cell proliferation as the target of these chemical classes (*eg.* the ribonucleotide reductase and DNA synthesis).[43] According to the authors, this effect is due to their ability to form a complex with metal cations; acting as chelators, targeting metalo-enzymes that are present in protozoans (*eg.* cysteine proteases).[44] Others TSC have been reported as affecting the mitochondrial function and structure, also including the lipid metabolism, in *L. amazonensis* promastigotes.[45]

3.6. Structure-activity relationship study of Thiosemicarbazones and Thiazolidinone

In order to observe the structural features that may be involved in the antiparasitic and toxicity profiles of TSC and TZD series, we evaluated their stereoelectronic properties by using an *in silico* approach (Table 3 and 4). According to our results, some differences were detected in the parameters calculated for TSC and TZD, which seems to be more related to the toxicity profile (Table 3) than to the antiparasitic activity level detected (Figures 2-3). TZD showed lower values for Energy of HOMO and the number of hydrogen bond acceptors (nOHNH-HBD) and higher values for Energy of LUMO, hydrogen bond donors (nON-HBA), Dipole, Area, Volume, and PSA than TSC (Table 4) directly or indirectly analogous to its lower toxicity profile.

Table 3: Comparison of the theoretical risks of Thiosemicarbazones and Thiazolidinones calculated in the Osiris program including Mutagenic, tumorigenic, reproductive and irritant side effects.

Compound	Mutagenic	Tumorigenic	Reproductive	Irritant
1 (TSC)	-	-	-	-
2 (TSC)	+	-	+	-
3 (TSC)	+	-	+	-
4 (TSC)	+	+	+	-
5 (TSC)	+	+	+	-
6 (TSC)	+	+	+	-
7 (TSC)	+	-	+	-
8 (TSC)	+	-	+	-
9 (TZD)	-	-	-	-
10 (TZD)	-	-	-	-

11 (TZD)	-	-	-	-
12 (TZD)	-	-	-	-
13 (TZD)	-	-	-	-
14 (TZD)	-	-	-	-
15 (TZD)	-	-	-	-
16 (TZD)	-	-	-	-
Hydroxyurea	+	+	+	+
Pentamidine	-	-	-	-
Miltefosine	-	-	-	-

In the TSC group, the dipole moment varied from 4.70 (4) to 7.43 Debye (7) whereas the area and volume were higher for molecules 7 and 8 but lower for molecule 1 (Table 4). In the TZD group, dipole moment values varied from 2.28 (12) to 8.23 Debye (9 and 14) whereas 15 and 16 exhibited the greatest area and volume values and the lowest polar surface area in contrast to 9 exhibited the greatest polar surface area, and the number of hydrogen bond acceptors (nOHNH-HBD). The n-octanol-water coefficient partition (log P) that refers to lipophilicity, revealed it higher with the addition of the propyl and butyl chains with the nitro group at the same position (1 < 2,3 < 4-6 < 7,8) (Table 4).

Table 4: *In silico* theoretical analysis of Thiosemicarbazone and Thiazolidinone. The stereoelectronic parameters were calculated including: HOMO energy (EHOMO), LUMO energy (ELUMO), Dipole moment (Dipole), Area, Volume, Polar surface area (PSA), hydrogen-bond acceptor (nON-HBA), hydrogen-bond donor (nOHNH-HBD), LogP and Druglikeness.

Molecule	EHOMO (eV)	ELUMO (eV)	Dipole (debye)	Area (A^2)	Volume (V^3)	PSA (A^2)	nOHNH-HBD	nON-HBA	LogP (1-9)/ LogD (9-16)	Druglikeness
1	-8.82	0.58	5.22	231.60	199.83	82.086	2	7	1.61	-3.02
2	-8.78	0.63	5.33	252.64	220.25	68.704	2	7	1.83	-2.47
3	-8.81	0.53	7.16	235.01	220.35	68.916	2	7	1.83	-2.47
4	-8.64	0.86	4.70	271.73	238.71	67.276	2	7	2.19	-2.68
5	-8.73	0.63	4.96	273.45	239.06	68.336	2	7	2.19	-2.68
6	-8.76	0.50	7.11	273.56	239.07	68.419	2	7	2.19	-2.68
7	-8.39	0.83	7.43	313.02	285.23	67.933	2	7	3.85	-3.03
8	-8.48	0.73	5.74	313.87	285.68	67.831	2	7	3.85	-3.03
9	-6.42	3.53	8.23	292.42	273.01	107.341	0	9	1.34	0.96
10	-6.08	3.73	7.58	307.49	291.53	93.381	0	9	1.57	1.68
11	-6.36	3.49	8.11	321.24	292.67	94.833	0	9	1.57	1.68
12	-6.34	3.76	2.28	323.01	310.78	90.390	0	9	1.92	2.09
13	-5.95	3.78	7.01	322.04	309.65	92.024	0	9	1.92	2.09
14	-6.36	3.52	8.23	331.11	311.43	94.352	0	9	2.73	-1.98
15	-6.03	3.73	3.02	362.53	356.75	89.710	0	9	3.29	0.84
16	-6.05	3.69	6.30	358.15	354.94	89.582	0	9	2.23	0.27

Druglikeness is a theoretical value calculated based on fragments database of traded drugs and unoccupied molecular orbital concentrated in nitrobenzene group (Figure 8). Differently, the analysis of the TZD HOMO orbital distribution maps showed that the highest occupied molecular orbital was concentrated in the hydrazinecarbothioamide and benzene ring regions. For the LUMO orbital distribution, the lowest unoccupied molecular orbital was most concentrated in the nitrobenzene rings, except for the molecules 13 and 16, which were similar to TSC group (Figure 8). A significant difference was observed in the dipole moment due to NO2 group position, which interferes with the charge distribution. The Electrostatic potential map and HOMO/LUMO distribution coefficient and density maps point to different reactivity profile and ability to interact with the target in the parasites of these molecules (Figure 8).

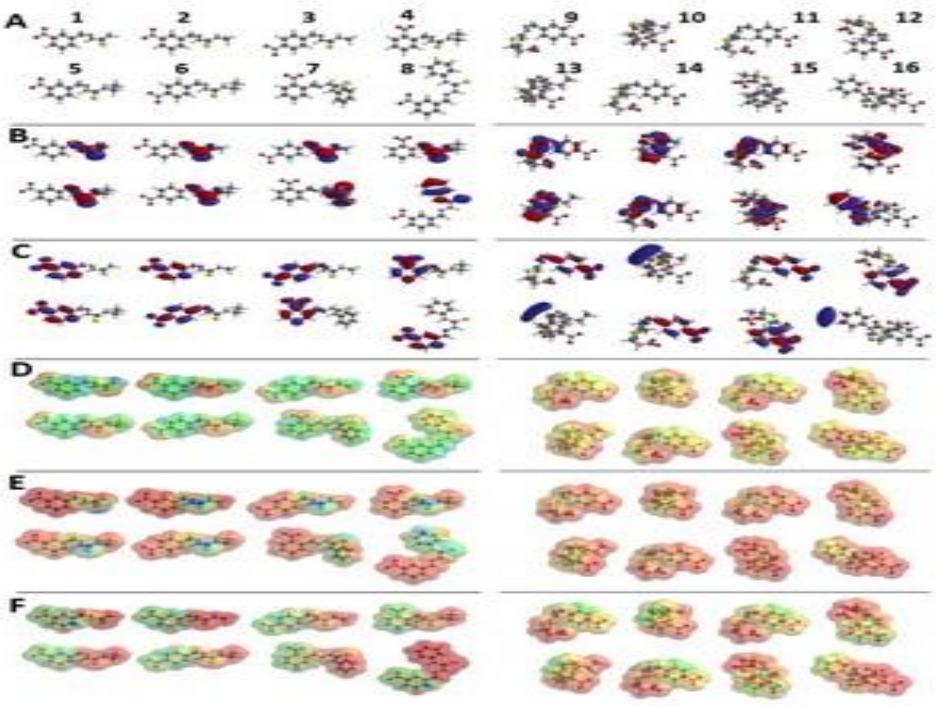


Figure 8: In silico evaluation of Thiosemicarbazones and Thiazolidinones stereoelectronic profile. A) 3D structure, B) HOMO distribution coefficient, C) LUMO distribution coefficient, D) Electrostatic Potential Map, E) HOMO density map, and F) LUMO density map.

Since the current limitation for the Leishmaniasis treatments is the toxicity profile, the design of new antiparasites should take the safety feature into consideration when planning new prototypes. Thus, according to our *in silico* results, it is necessary to maintain the TDZ parameters with lower values of the energy, density and distribution of HOMO and number of hydrogen bond acceptors (nOHNH-HBD) and higher values for energy of LUMO, hydrogen bond donors (nON-HBA), Dipole, Area, Volume, and PSA.

Other studies showed the N-alkyl indole derivatives and the non-polar bicyclic compounds, both thiosemicarbazone derivatives compounds tested against intracellular amastigotes of *Leishmania dovani* specie. The toxicity of these compounds was also tested in mammalians cell lines MRC-5 – human fetal fibroblast, and THP-1 – acute monocyclic leukaemia cells revealing them as toxic. Interestingly, these authors suggested that apolar molecules could be advantageous to the antileishmanial activity. In agreement to the literature that reported aromatic moieties with higher activities,[46] our study found the compound 9, which presented the highest polar surface area (107.341A2) and dipole moment (8.23 debye), without ability to fully eliminate all amastigotes at 10 mM. In naphthalene thiosemicarbazone derivatives, the substitutions methoxy, ethoxy, butoxy, methyl or a fluor favour the antileishmanial activity, with toxicity in most cases.[47] These data are also according to our *in silico* data that showed TSC series with mutagenic, tumorigenic and reproductive risks alerts. Pervez and co-workers described leishmanicidal effects of different N4-substituted 5-nitroisatin-3-thiosemicarbazones compounds against *Leishmania major*. Almost all the compounds showed leishmanicidal activity as well as our compounds. Depending on the position (*ortho*, *meta*, *para*), the activity was detected and reported, without clarity about the preferential position as it depends on the group added to it.[47] In our compounds we could also notice that the NO2 position interferes in the activity profile, being related to the dipole moment distribution. A recent study of Leite et al, analysed thiazolidine-2,4-dione derivatives and suggest that they can inhibit Pteridine Reductase 1 of Leishmania major. In vitro assays, molecular dockings and molecular dynamics shows that these inhibitors are probably non-competitive inhibitors of LmPTR1.[48]

Our *in silico* studies showed different chemical features for TSC and TZD (*eg.* 3D structure and conformation, and stereoelectronic distribution), which could lead to targeting different *Leishmania* enzymes or receptors. Our results showed HOMO distribution concentrated in hydrazinecarbothioamide, which could favour a nucleophilic attack in enzymes such as ribonucleotide reductase, where interactionof HOMO region with iron atoms are favoured, as well as cysteine protease, in which a covalent bond may be formed with the catalytic cysteine. Most importantly, as the cost of discovering new antiparasites should be kept as low as possible and the investments should be rational, these structural features apparently lead to toxic (TSC) and non-Toxic (TZD) profiles of these chemical groups, which is are

essential parameters to be used to select TZD compounds to carry on with further *in vitro* and *in vivo* assays.

4. CONCLUSION

Overall this work reports the series of Thiosemicarbazones (TSC) and Thiazolidinones (TZD) that presented antiparasitic activity against both intra and extra cellular forms of *Leishmania amazonensis*. These effects compromised the cellular structure of these parasites as detected by ultrastructural evaluations. The low replication rate might facilitate the parasite elimination by the host cell microbicide mechanisms while no toxic effect is observed on macrophages. The *in silico* toxicity studies pointed TZD as the safest compounds to further analysis, which showed a specific strcutural and stereoelectronic profile in the SAR studies. The structural differences and *in silico* toxicity profile of these compounds may suggest different targets for these two groups of molecules. These data pointed these compounds, specially TZD as good candidates for further studies due to the good toxicity features and ability to affect extracellular and intracellular parasite development.

ACKNOWLEDGMENT FOR FINANCIAL SUPPORTS: Fundação de Amparo à Pesquisa do Rio de Janeiro (FAPERJ), Coordenação de Aperfeiçoamento de Pessoas de Nível Superior (CAPES) and Conselho Nacional de Pesquisa (CNPq).

List of abreviations: Thiosemicarbazones (TSC); Thiazolidinones (TZD); Hydroxyurea (HU); Polar surface area (PSA); hydrogen-bond acceptor (nON-HBA); hydrogen-bond donor (nOHNH-HBD); energy of Highest Occupied Molecular Orbital (HOMO) and Lowest Unoccupied Molecular Orbital (LUMO).

Conflicts of Interest: The authors declare no conflict of interest.

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