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Effects of nitro-heterocyclic derivatives against *Leishmania (Leishmania) infantum* promastigotes and intracellular amastigotes

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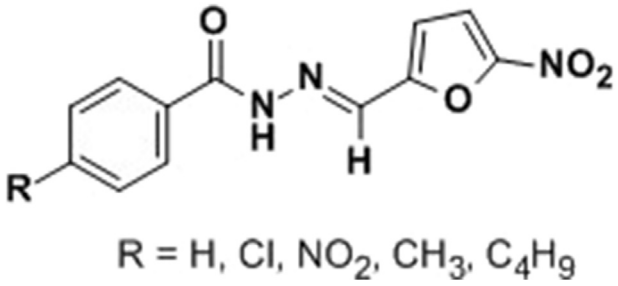
GRAPHICAL ABSTRACT



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ABSTRACT

Leishmaniasis is an overlooked tropical disease affecting approximately 1 million people in several countries. Clinical manifestation depends on the interaction between *Leishmania* and the host's immune response. Currently available treatment options for leishmaniasis are limited and induce severe side effects. In this research, we tested nitro-heterocyclic compounds (BSF series) as a new alternative against *Leishmania*. Its activity was measured in *Leishmania (Leishmania) infantum* promastigotes and intracellular amastigotes using MTT colorimetric assay. Additionally, we assessed the phosphatidylserine exposure by promastigotes, measured by flow cytometry, as well as nitric oxide production, measured by Griess' method. The nitro-heterocyclic compounds (BSF series) showed activity against *L. (L.) infantum* promastigotes, inducting the phosphatidylserine exposition by promastigotes, decreasing intracellular amastigotes and increasing oxide nitric production. The selectivity index was more prominent to *Leishmania* than to macrophages. Compared to amphotericin b, our compounds presented higher IC_{50}, however the selectivity index was more specific to parasite than to amphotericin b. In conclusion, these nitro-heterocyclic compounds showed to be promising as an anti-*Leishmania* drug, in *in vitro* studies.

1. Introduction

Leishmaniasis is a disease caused by a protozoan from *Leishmania* genus which affects over one million people worldwide,

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causing disability-adjusted life years and death, mainly in the tropical region, and is present in 98 countries from five continents (WHO). Leishmaniasis is considered one of the most overlooked tropical diseases and has highest priority by the World Health Organization (WHO). Clinical manifestation is characterized by visceral and tegumentary involvement (Lindoso and Lindoso, 2009). Although tropical diseases account for 11.4% of the global disease burden, only 21 (1.3%) out of 1556 drugs registered between 1975 and 2004 were developed specifically for these diseases (DNDi, 2012). The therapeutic arsenal for leishmaniasis is very limited (Copeland and Aronson, 2015). In Brazil, there are only three drugs available (pentavalent antimonial, amphotericin B and pentamidine), and these present several drawbacks to patients related to the route of application (parenteral via) and induction of severe side effects, such as renal injury, pancreatitis and cardiac injury (Kaur et al., 2015; Gontijo and Melo, 2004). This scenario highlights the need for new therapies that might be effective against *Leishmania*, with fewer side effects. Nitro-heterocyclic compounds could be good candidates, since some studies have shown that these compounds present action against protozoan, such as *Trypanossoma cruzy* (Palace-Berl et al., 2013). These compounds have a nitro-heterocyclic moiety which can induce oxidative stress, either by producing radicals toxic to the parasite, which is involved in cellular mechanism for detoxification of free radicals, or by inducing the occurrence of oxidative stress (Hall et al., 2011; Aguirre et al., 2005; Oliveira et al., 2008; Blumenstiel et al., 1999). Due to these features, it may be an interesting alternative to anti-*Leishmania* studies, since its physical and chemical properties showed anti-Chagas action (Krauth-Siegel et al., 2005; Krauth-Siegel and Inhoff, 2003; Coura e Castro, 2002; Urbina, 2001; Blumenstiel et al., 1999; Viodé et al., 1999; Krauth-Siegel and Schöneck, 1995; Jockers-Scheruibel et al., 1989; Henderson et al., 1988). This study explores anti-*Leishmania* activity of a series of nitro-heterocyclic compounds against *Leishmania (Leishmania) infantum* promastigotes and intracellular amastigotes, as well as the cytotoxicity against THP-1 cells. Additionally, we assessed the nitric oxide produced by infected macrophages upon nitro-heterocyclic derivate and the occurrence of promastigotes apoptosis induced by these compounds.

2. Materials and methods

2.1. Nitro-heterocyclic compounds

The five compounds, *4-R-substituted-N'-[(5-nitrofuran-2-yl) methylene]benzhydrazide* (R = H, Cl, NO_2, CH_3, C_4H_9), were obtained through the molecular modification of nifuroxazide, *4-Hydroxy-N'-[(5-nitrofuran-2-yl)methylene]benzohydrazide* (Paula et al., 2009). These compounds were previously synthesized by Tavares and coworkers (Tavares et al., 1996; Paula et al., 2009; Palace-Berl et al., 2013). Compounds structures are elucidated in the graphical abstract. A stock solution of each drug was prepared in DMSO p.a. in glass vials. The dilutions of the drugs were subsequently carried out in RPMI without phenol red in order to obtain the drug at the desired testing concentration.

2.2. Leishmania parasite culture and drugs screening test

L(L) infatum (MHOM/BR/1972/LD46) promastigotes were cultured in 199 medium Hanks (Gibco, USA), supplemented with L-glutamine (Sigma Chemical, USA), 10% fetal bovine serum (FBS) (Sigma Chemical, USA) and 100UI/ml and 100 μg/mL streptomicyn (Sigma Chemical, USA) at 27 °C. The parasites (2×10^6/mL) in growth stationary phase were transferred to 96 wells flat bottom microplates (Sigma–Aldrich, USA) in serial dilutions (range 400 μM–0.1953 μM) of each nitro-heterocyclic compound and amphotericin B for 48 h at 26 °C. The assays were carried out in sextuplicate. The activity was measured by MTT method. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) was diluted (5 mg/ml) in sterile phosphate buffered saline solution (PBS). 20 μL of MTT solution were added to each well and incubated for 4 h at 26 °C. The reaction was stopped with 10% sodium dodecyl sulfate (SDS) and incubated for 18 h at 26 °C. The optical density (OD) was determined in a spectrophotometer Multiskan® card reader MCC/340 (Brazil) in 570 nm filter (Morais, 2014). The drug concentration corresponding to growth inhibition of 50% of the parasites was expressed as the inhibitory concentration (IC_{50}) in μM.

2.3. Cell culture and differentiation

THP1 cells (human monocytes) were maintained in RPMI medium without phenol red supplemented with 20% FBS at 36 °C in 5% CO_2. Macrophages differentiation was induced by PMA (final PMA concentration25 ng/mL) (Sigma Chemical, USA) for 48 h at 36 °C in 5% CO_2. Differentiated cells were incubated in RPMI with 20% FBS for 48 h at 36 °C in 5% CO_2, in 24 wells flat bottom microplates (Jain et al., 2012).

2.4. In vitro macrophage infection

Parasites in growth stationary phase were added to wells containing macrophages at a ratio of 1:10 (cell/parasites) in fresh medium for 4 h at 36 °C in 5% CO_2. After that, the infected cells were washed to remove extracellular parasites and the compounds were added (range 400μM-0.39 μM) and incubated for 48 h at 36 °C in 5% CO_2. The medium was removed and the cells were washed with RPMI. Infected cells were fixed using methanol and stained with Giemsa (Sigma Chemical, USA). The percentage of infected macrophages in each assay was determined microscopically at X 1000 magnification.

2.5. Cytotoxicity assay for THP 1 cells

After adhesion in 96-well plates, THP-1 cells were incubated

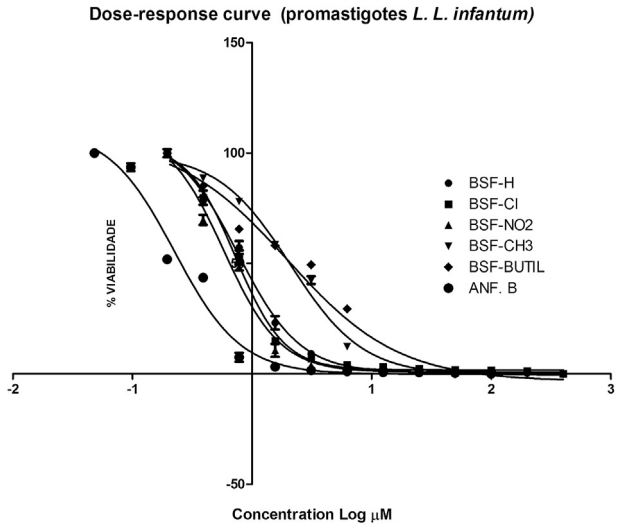

Fig. 1. Effective concentration 50% against Leishmania (L.) infantum promastigotes. Sigmoid dose–response curves analyzed by Graph Pad Prism software. The concentration is expressed in logarithm (μM).

Table 1
Activity of nitro heterocyclic compounds and amphotericin B against L. (L.) infantum promastigotes.

	BSF-H	BSF-CL	BSF-NO_2	BSF-CH_3	BSF-BUTIL	ANF.B
EC_{50} (μM)	0.76	0.72	0.58	2.0	1.97	0.22
95%IC	0.68 ± 0.85	0.66 ± 0.79	0.49 ± 0.68	1.8 ± 2.3	1.3 ± 2.8	0.18 ± 0.27

EC_{50}: 50% effective concentration determined after 48 h of incubation.

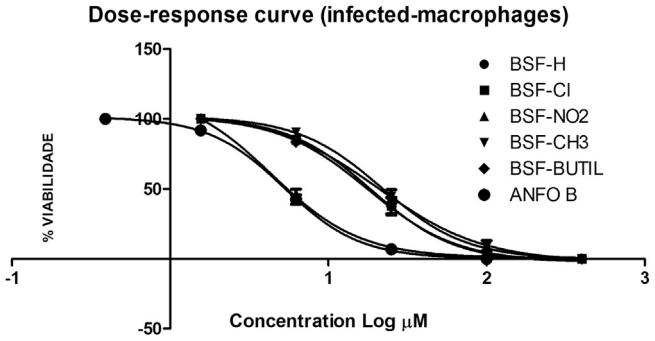

Fig. 2. Effective concentration 50% against Leishmania (L.) infantum amastigotes. Sigmoid dose–response curves analyzed by Graph Pad Prism software. The concentration is expressed in logarithm (μM).

with the nitro-heterocyclic compounds for 48 h at 37 °C in a CO_2 incubator (5%) in a concentration range of 400 μM–0.097 μM. After a 48-h incubation period, the cytotoxicity (CC_{50}) was estimated by MTT assay as described above.

2.6. Selectivity index calculation

The selectivity index (SI) was measured by IC_{50} to CC_{50} ratio.

2.7. Phosphatidylserine exposition by L.L. infantum promastigotes using flow cytometry

Phosphatidylserine externalization was detected by Annexin V-FITC method (BD, USA) according to the manufacturer's instructions. Briefly, 10^6 *Leishmania (L.) infantum* promastigotes were incubated with the nitro-heterocyclic compounds in the range from 400 μM to 0.1953 μM, for 48 h at 26 °C. Parasites were washed in sterile PBS and immediately resuspended in binding buffer (1X). Five μL of fluorochrome-conjugated Annexin V were added to each 100 μL of the cell suspension and incubated for 15 min at room temperature protected from light. After incubation, the cells were washed in binding buffer again and resuspended in 200 μL of binding buffer. Five μL of propidium iodide staining solution (Becton–Dickinson, USA) were added and the samples were analyzed in Fortessa SRL flow cytometer (Becton–Dickinson, USA). The positive control for phosphatidylserine detection was induced by camptothencin. Cells were gated on forward and side scatter signals to eliminate debris from analysis. All fluorescence parameters were recorded with logarithmic amplification. The analysis was performed on 10,000 gated events, stored on FACS DIVA and data was analyzed using the FlowJo software program (Treestar, USA). Three independent experiments were performed.

Table 2
Evaluation of cell viability macrophages THP-1 cells without infection incubated with BSF series compounds.

	BSF-H	BSF-Cl	BSF-NO_2	BSF-CH_3	BSF-Butil	ANF. B
CC_{50} (μM)	10.5	0.06	10.9	1.9	NP	0.08
95%CI	9.02 ± 12.3	0.005 ± 3	9.2 ± 13	1.7 ± 2.16	NP	0.03 ± 2.6
IS	13.84	0.079	18.79	0.91	NP	0.36

CC_{50}: cytotoxicity concentration, CI- confidence interval, IS- index selective = IC_{50}/CC_{50}*L(L) infantum*promastigotes.

Table 3
Cytotoxicity of the compounds in infected macrophage THP-1 cells.

	BSF-H	BSF-Cl	BSF-NO_2	BSF-CH_3	BSF-Butil	ANF. B
CC50 (μM)	10.5	0.06	10.9	1.9	Nd	0.08
95%CI	9.02 ± 12.3	0.005 ± 3	9.2 ± 13	1.7 ± 2.16	Nd	0.03 ± 2.6

CC_{50}: cytotoxicity concentration, CI- confidence interval-ND-no determinad.

Table 4
Macrophages assessment that remained with intracellular Leishmania (L.) infantum amastigotes after exposure of the nitro heterocyclic series.

Macrophages infected %												
Concentration μM	BSF-H		BSF-Cl		BSF-NO2		BSF-CH3		BSF-BUTIL		ANF. B	
	% Ø	% Ø infectious	% Ø	% Ø infectious	% Ø	% Ø infectious	% Ø	% Ø infectious	%Ø	% Ø infectious	%Ø	%Ø infectious
400	56.8	43.2	79.4	20.6	60.8	39.2	48.8	51.2	37.3	62.7		
100	54.7	45.3	69.2	23	55.6	44.9	42.2	57.8	63.7	36.3	82.7	17.3
25	45.8	54.2	58.8	41.2	35	65	40.6	59.4	51.7	48.3		
6.25	59.7	40.3	46.6	53.4	32.9	67.1	42.9	57.1	18.6	77.7		
1.56	45.3	54.7	52.2	47.9	32.4	67.6	33	67	49.1	50.9		
0.39											63.1	36.9

Ø: Macrophage without infection; Ø infected: macrophage infected with *L. (L) infantum.*

2.8. Measurement of nitric oxide

Nitric oxide (NO) production was assessed in the supernatants of infected macrophages. 100 μL of culture supernatant were placed in 96-well plates added with 100 μL of Griess Reagent (SIGMA) and incubated for 30 min protected from light at room temperature.

Nitrite concentration was measured using the standard curve (Ribeiro, 2014). The results were expressed as millimolar concentrations.

2.9. Statistical analysis

The data represents the mean and standard deviation of triplicate samples from at least three independent experiments. IC_{50}, EC_{50} and 95% CI values were calculated using sigmoidal dose-response curves of Graph Pad Prism 5.0 software (GraphPad Software, USA).

3. Results

3.1. Dose-response curve and determination of IC_{50} against L.L.infantum promastigotes

The compounds were biologically active against the parasite in a dose-dependent fashion (Fig. 1). The IC_{50} values (CI 95%) in *L.infantum* promastigotes after a 48-h exposure to the BSF series compounds (Table 1), showed higher efficacy in lower doses for the following compounds: BSF-H (0.76 μM), BSF-Cl (0.72 μM), and BSF-NO_2 (0.58 μM) when compared to BSF-CH_3 (2.08 μM) and BSF-Butyl (1.97 μM) compounds. Amphotericin B showed an IC of 0.22 μM.

3.2. Dose-response curve and CC_{50} of THP1cells and infected THP1 cells

Cellular viability of THP-1 cells incubated for 48 h in the BSF series compounds, which was measured by MTT, is shown in a dose-response curve of BSF series compounds (Fig. 2). BSF-Cl and BSF-CH_3 series showed similar response to amphotericin B. When analyzing the selectivity index, it was observed that BSF-H and BSF-NO_2 compounds were 13.84- and 18.79-fold more cytotoxic than amphotericin B, respectively (Table 2). However BSF-CL series showed lower cytotoxicity (Table 3).

3.3. Infectivity index in infected THP1 macrophages

In order to check the infectivity index regarding the series actions in different concentrations, macrophages were infected with *L. Leishmania infantum* promastigotes in the ratio 1:10 and treated with five BSF series compounds for 48 h in a range from 400 μM to 1.56 μM. Untreated macrophages were used as control. Table 4 shows BSF- H and BSF-Cl, highlighted on 400 μM, 100 μM and 6.25 μM, above 50% of non-infected macrophages, which indicates that the compounds were effective. However, amphotericin b had higher values than the five compounds.

3.4. Phosphatidylserine assessment by flow cytometry analysis

Taking into account the exhibition of phosphatidylserine to external cell surfaces, which is indicative of cellular membrane injury allowing the PI to diffuse inside, and marker of apoptosis, we observed that the compounds BSF-H, BSF-Butil on 400 μM, and BSF CL on 100 μM induced apoptosis in *L. infantum* promastigotes on 63%, 61% and 50%, respectively (Fig. 3 and Table 5).

3.5. Nitric oxide production assessment

The compounds induced higher production of nitric oxide as measured by Griess' method, when compared with LPS. Additionally, infected THP1 under amphotericin B produced higher concentrations as well (Fig. 4).

4. Discussion

To search for new drugs, it is necessary to know the structure and metabolic processes of the etiologic agent, such as *L. L. infantum*. The Trypanosomatidae family (family to which the genus *Leishmania* belongs) has distinct characteristics for the study of new therapeutic targets. Studies comparing mammalian hosts and trypanosomes' metabolism have demonstrated the existence of specific enzyme targets to study the action of novel compounds with anti-*Leishmania* activity. Among these targets, one can quote cruzipain, trypanothione reductase, reductase fumerate, glyceraldehyde-3-phosphate dehydrogenase, esqueleno epoxidase and parasitic trans-sialidase (Krauth-Siegel et al., 2005; Siegel Krauth and Inhoff, 2003; Coura and Castro, 2002). New anti-Leishmania drugs are needed due to few drugs available and increased resistance in some regions. Nitro-heterocyclic compounds - based drugs alternating the radical in ring heterocyclic could be a good alternative. Palace-Berrl et al. (2013) studied the influence of 5-nitrofurfuriliden derivate on the anti-*Trypanosoma cruzi* activity of these compounds. The mechanism of action by which the compounds act on the parasite is not well understood. However, it is known that the nitro group present in BSF series has parasitic action when it binds to furan rings, tiophenics or imidazole in carbon, acting on nitro group bioreduction (Arguello et al., 2006). These promising results encouraged the testing of *L. infantum* promastigotes, since they belong to the same family. Initially, the compounds series in our study showed a dose-dependent behavior, and these results were confirmed when analyzed on cytotoxicity assay. Two series of *L.L.infantum* promastigotes were highlighted, BSF-NO_2 and BSF-Cl, which presented better activity/structure value, although amphotericin B was one of the most active anti-leishmanial drugs (Kaur et al., 2015; Lucero et al., 2015; Sundar et al., 2015; Vakil et al., 2015; Yesilova et al., 2015). During cytotoxicity assessment in THP1 macrophages with the compounds, it was observed that BSF-H and BSF-NO_2 compounds showed a much higher selectivity index compared to amphotericin B, and these compounds were 13- and 18-fold more active against parasites than THP1 cells. These cytotoxicity results are favorable to the compounds, and lead us to believe that since these compounds present activity on intracellular amastigotes with less toxicity in the host cells (THP-1 macrophage), it strengthens the point that these are promising compounds for the treatment of leishmaniasis, which may have fewer adverse effects than the reference drug. Interestingly, when infected macrophages were exposed to compounds series, the non-constant percentage of intracellular amastigotes was above 50%, meanly BSF-Cl. The values next to amphotericin B probably indicate amastigotes death or more potentiality of macrophages activity.

In this study, the five BSF series compounds showed some anti-Leishmania activity, with different EC_{50}. This fact is probably due to certain substituents, as in the case of Cl and NO_2 had lower EC_{50}, present better activity/structure value. In promastigotes, BSF-NO_2 (EC_{50}: 0.58 μM), BSF-M (EC_{50}: 0.76 μM) and BSF-Cl (EC_{50}: 0.58 μM) compounds were more active, as determined by MTT method. When comparing the EC_{50} of our compounds with amphotericin B (EC_{50}: 0,022 μM), it is noted that the latter has lower EC_{50}, ad is more effective when compared to the number of compounds used. This point is crucial, since amphotericin B has been shown as one of

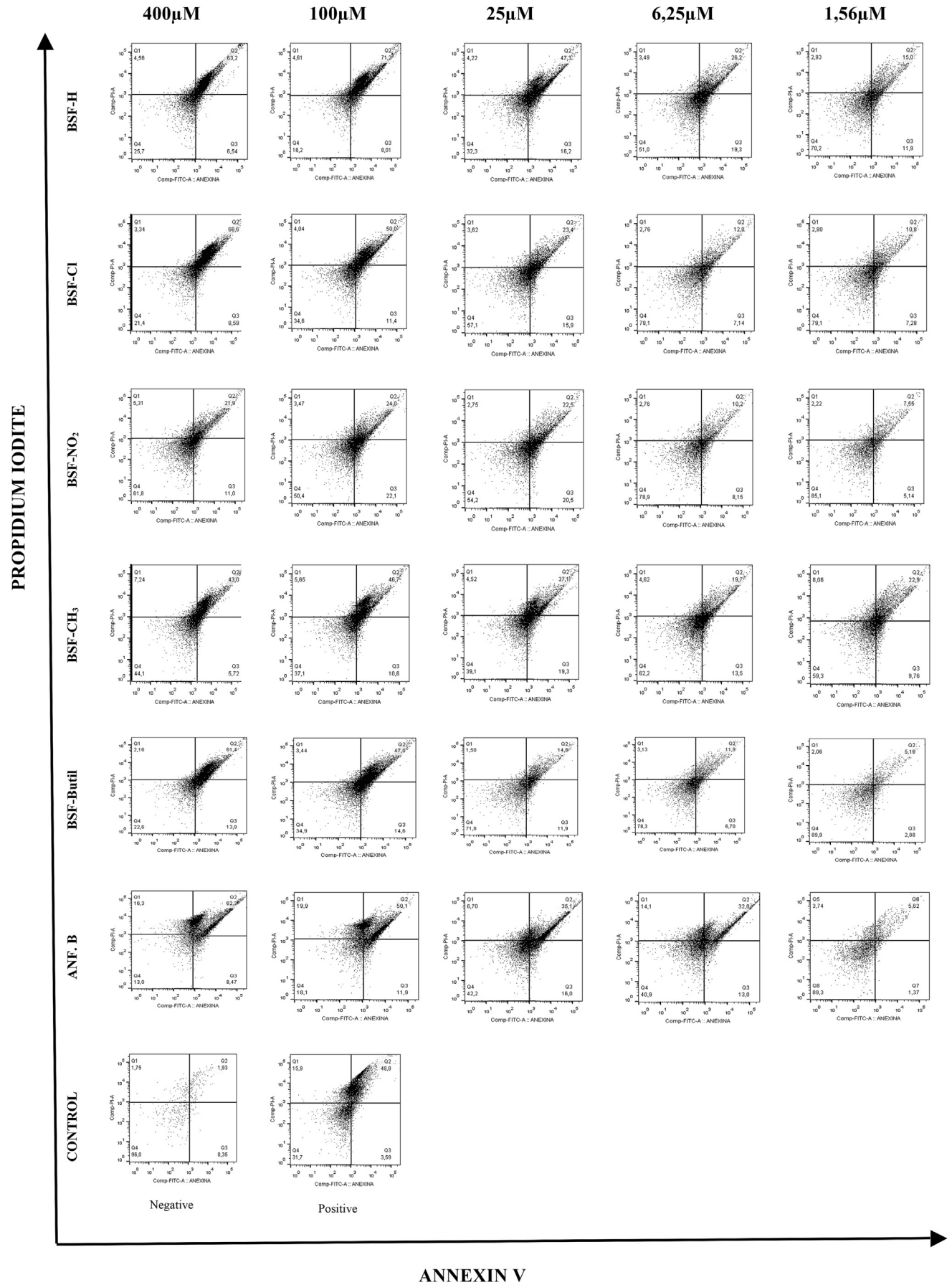
400µM
100µM
25µM
6,25µM
1,56µM
BSF-H
BSF-Cl
BSF-NO2
BSF-CH3
BSF-Butil
ANF. B
CONTROL
PROPIDIUM IODITE
ANNEXIN V
Negative
Positive

Table 5
Percentage of the average fluorescence intensity of Annexin V-FITC on L.(L)infantum promatigotes submitted for different concentrations of compounds derived from the nitro-heterocyclic BSF series and amphotericin B.

	PI+	AV+	AV + PI(+) +
[400 μM]			
CN	1.75	0.35	1.93
CP	15.9	3.59	48.8
Anf. B	NP	NP	NP
BSF-H	4.56	6.54	63.2
BSF-Cl	3.34	8.59	6.66
BSF-NO_2	5.31	11.0	21.9
BSF-CH_3	7.24	5.72	43.0
BSF-Butil	21.6	13.9	61.4
[100 μM]			
CN	1.75	0.35	1.93
CP	15.9	3.59	48.8
Anf. B	19.9	11.9	50.1
BSF-H	4.61	8.1	71.2
BSF-Cl	4,04	11.4	50
BSF-NO_2	3,37	22.1	24.0
BSF-CH_3	5,85	10.6	46.7
BSF-Butil	3,44	14.6	47
[25 μM]			
CN	1.75	0.35	1.93
CP	15.9	3.59	48.8
Anf. B	16.3	8.47	62.3
BSF-H	4.22	16.2	47.3
BSF-Cl	3.62	15.9	23.4
BSF-NO_2	2.75	20.5	22.5
BSF-CH_3	4.52	19.3	37.1
BSF-Butil	1.50	11.9	14.8
[6.25 μM]			
CN	1.75	0.35	1.93
CP	15.9	3.59	48.8
Anf. B	6.70	16.0	35.1
BSF-H	3.49	19.3	26.2
BSF-Cl	2.76	7.14	12
BSF-NO_2	2.76	8.15	10.2
BSF-CH_3	4.62	13.5	19.7
BSF-Butil	3.13	6.70	11.9
[1.56 μM]			
CN	1.75	0.35	1.93
CP	15.9	3.59	48.8
Anf. B	14.1	13	32
BSF-H	2.93	11.9	15
BSF-Cl	2.80	7.28	10.8
BSF-NO_2	2.22	5.14	7.55
BSF-CH_3	8.06	9.76	22.9
BSF-Butil	2.06	2.88	5.16
[0.39 μM]			
CN	1.75	0.35	1.93
CP	15.9	3.59	48.8
Anf. B	3.74	1.37	5.62
BSF-H	NP	NP	NP
BSF-Cl	NP	NP	NP
BSF-NO_2	NP	NP	NP
BSF-CH_3	NP	NP	NP
BSF-Butil	NP	NP	NP

AV: Annexin V positive; AV + PI: Annexin v and propidium iodide positive; PI: propidium iodide positive; CN: negative control - promastigotes untreated; CP: promastigotes induced apoptosis with Camptothencin; NP: not performed.

the most active antileishmanial drugs ever described (Sesana et al., 2011; Lucero et al., 2015; Yesilova et al., 2015), but other drugs with lower antileishmanial activity are widely used in the treatment of leishmaniasis, such as miltefosine (Kaur et al., 2015; Sundar et al., 2015; Vakil et al., 2015).

With the demonstrated action against promastigotes and the selectivity index already demonstrated, we set out to assess the possible action of the compounds on promastigotes. It is known that drugs used in the treatment of leishmaniasis induce parasite death by apoptosis (Vincent et al., 2013), therefore we evaluated whether our compounds induce the parasite apoptotic death. Thereunto, we assessed the expression of phosphatidylserine, which is a marker of cell death by apoptosis. In our tests, nitro-heterocyclic compounds induced the externalization of phosphatidylserine from *L. L. infantum* promastigotes after 48 h of treatment, and the result showed that the BSF-H compound showed 71.2% of labeled cells annexin V and propidium iodide at a concentration of 100 uM, compared with amphotericin B, which presented 62.3% of labeled cells in 100 μM. This double marking indicates the late phase of apoptosis (Kulkarni et al., 2009). This result suggests that the permeability of the plasma membrane is altered when *L. L. infantum* promastigotes are incubated with nitro-heterocyclic compounds, allowing propidium iodide internalization and binding to stain nucleic acids. As *in vitro* experimental model, we used THP-1 cells and set the best cell infection would condition the use of 10 parasites concentration for each cell. Our results show that the infection rate was reduced when these cells were incubated with the compounds, mainly with BSF-Cl and BSF-NO_2. This reduction in the percentage of infected cells and number of intracellular parasites was similar to that observed with amphotericin B. Thus, our compounds showing activity against promastigotes, also had activity against intracellular amastigotes, which confirms the hypothesis that they are potential drug candidates against *Leishmania*. Interestingly, with respect to the toxicity of compounds in THP-1 cells infected with *L. L. infatum*, we observed that they were more resistant to the action of the compounds, and the toxicity was lower when compared to uninfected cells. This result may be due to the compounds exhibiting greater selectivity against parasites, however the mechanism by which the compounds were active against intracellular amastigotes is unclear and may be either by direct action on the parasite by inhibiting reductase trypanothione, which would leave the parasite susceptible to the action of cationic radicals, or by indirect action, inducing higher nitric oxide production by the host cell. The anti-Leishmania activity probably makes it more susceptible to the action of cationic radicals. Romão et al., 2006 showed that the parasites become more susceptible to the action of reactive species of nitrogen and oxygen. In order to determine this point, we evaluated the observed NO production and increased production of NO by the cells when incubated with the compounds. These findings suggest that resting cells probably do not produce NO, since there is no stress situation which would induce NO production with the aim of eliminating a possible external agent from the cell. One hypothesis is that the compounds of the BSF group could act as a NO donor, in a cellular stress situation, as with cells infected with *L. L. infantum* in our experiment. The mechanism NO exerts on cytotoxicity activity is not well understood, but it may be by inhibition of mitochondrial respiration, glycolysis, peroxidation of membrane lipids, inactivation of peroxidases and arginases (Fonseca-Silva et al., 2015; Ghosh et al., 2015; da Silva et al., 2015; Giudice et al.,, 2007; Grandoni and Ascendi, 2004; Vickers et al., 2004).

Our results suggest activity of the compounds on promastigotes, inducing death by apoptosis, action against intracellular

Fig. 3. Representation in dot plot of the evolution of phosphatidyl serine externalization of Leishmania (L) infantum promastigotes incubated for 48 h with nitro heterocycle compounds. Cells were stained with annexin V FITC and propidium iodide, and analyzed by BD LRS Fortessa cytometer (Beckton Dickson®). The results were analyzed by FlowJo software (Tree Star ®, Inc.), and 10,000 events were analyzed per compound and for each concentration.

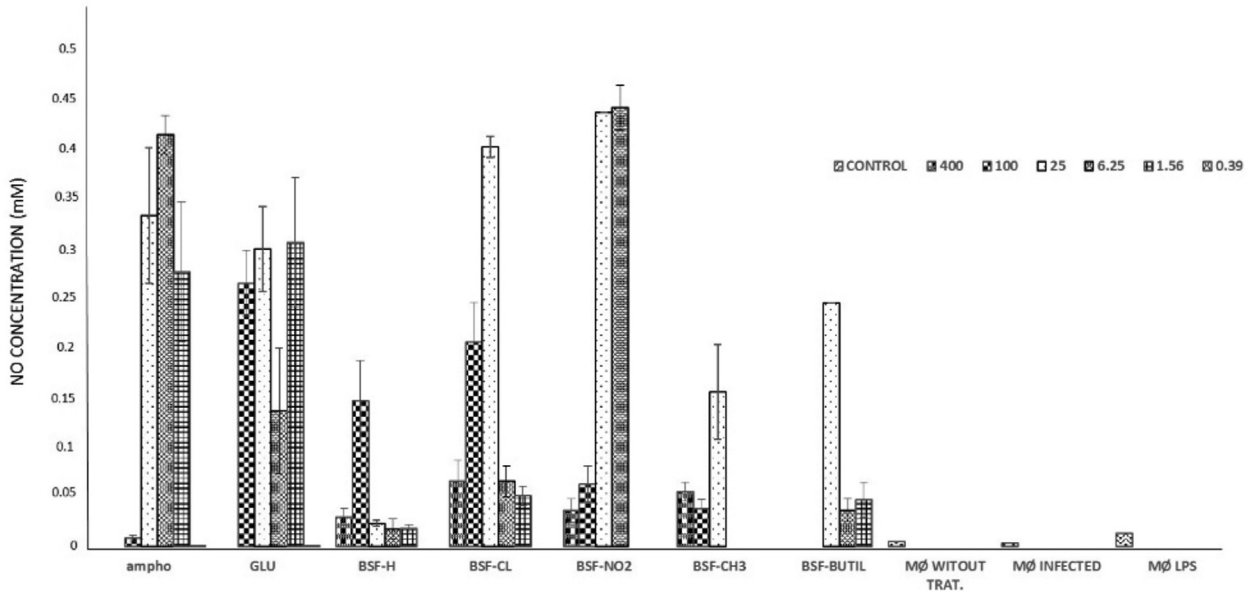

Fig. 4. Nitric oxide levels measured by Griees' method in supernatant of untreated infected-macrophages, macrophages upon treatment with LPS and infected-macrophages treated with nitro heterocyclic series. The results are expressed in concentration of nitric oxide (mM).

amastigotes and inducing increased production of NO by infected cells. We hypothesized that the synthetic derivatives of BSF series nitro-heterocyclic compounds could act in two ways: by inhibiting amastigote defense mechanism, or by enhancing nitric oxide production by infected cells, leading to the death of intracellular parasites.

Finally, our results indicate that the BSF series compounds seem to be promising candidates for studies of anti-*Leishmania* activity, and that out of the five compounds investigated, BSF-Cl and BSF-NO_2 compounds have shown potential candidates for further *in vivo* studies.

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