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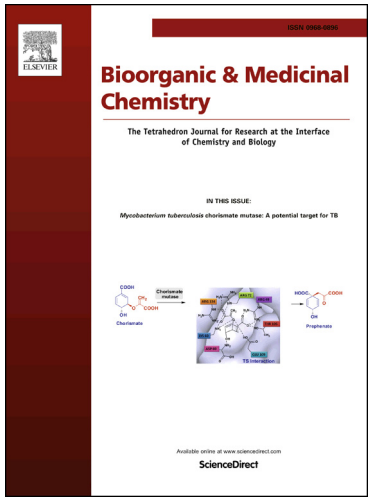

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Effect of 1,2,3-Triazole salts, non-classical bioisosteres of miltefosine, on *Leishmania amazonensis*.

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Abstract

Here, we report the effect of new non-classical bioisoteres of miltefosine on *Leishmania amazonensis*. Fifteen compounds were synthesized and the compound **dhmtAc**, containing an acetate anion, a side chain of 10 carbon atoms linked to N-1 and a methyl group linked to N-3, showed high and selective biological activity against *L. amazonensis*. On the intracellular amastigotes, stages of the parasite related to human disease, the IC_{50} values were near or similar to the 1.0 μM (0.9, 0.8 and 1.0 μM on *L. amazonensis*-WT, and two transgenic *L. amazonensis* expressing GFP and RFP, respectively), being more active than miltefosine. Furthermore, **dhmtAc** did not show toxic effects on human erythrocytes and macrophages (CC_{50} = 115.9 μM) being more destructive to the intracellular parasites (selectivity index > 115). Promastigotes and intramacrophage amastigotes treated with **dhmtAc** showed low capacity for reversion of the effect of the compound. A study of the mechanism of action of this compound showed some features of metazoan apoptosis, including cell volume decreases, loss of mitochondrial membrane potential, ROS production, an increase in the intracellular lipid bodies, *in situ* labeling of DNA fragments by TUNEL labeling and phosphatidylserine exposure to the outerleaflet of the plasma membrane. In addition, the plasma membrane disruption, revealed by PI labeling, suggests cell death by necrosis. No increase in autophagic vacuoles formation in treated promastigotes was observed. Taken together, the data indicate that the bioisotere of miltefosine, **dhmtAc**, has promising antileishmanial activity that is mediated via apoptosis and necrosis.

Keywords: 1,2,3-triazoles; *Leishmania*; miltefosine; antileishmanial activity; apoptosis; necrosis.

1. Introduction

Leishmaniasis is a complex vector-borne protozoan disease caused by over 20 species of the genus *Leishmania* that are pathogenic to humans [1]. In South American countries, *L. amazonensis* is one of the main etiological agents of leishmaniasis and capable of producing a wide spectrum of manifestations including localized cutaneous, mucocutaneous which is characterized by latency and chronicity, diffuse cutaneous which is commonly refractory to available treatments and most severe and potentially fatal if left untreated visceral leishmaniasis [2-4].

The current chemotherapy options for leishmaniasis include pentavalent antimonials, pentamidine, amphotericin B and its lipoidal formulations, but these are associated with limited effectiveness, a long-term treatment, toxicity, and significant side effects [5].

Miltefosine (Figure 1), a member of a class of alkyl-phosphocholines, is the first orally antileishmanial drug which was originally developed as an anticancer drug, and has been recently used to treat visceral leishmaniasis in India [5]. Several similar mechanisms of action have been described for the drug in both human tumor cells and *Leishmania* parasites [6-8]. However, its exact mechanism of action is not completely understood. At present, the drug is reported to trigger apoptosis effects such as mitochondrial dysfunction, externalization of phosphatidylserine, cell-cycle arrest at the sub-G*0*/G*1* phase and DNA fragmentation in *Leishmania* species [7, 8], impaired phospholipid sterol metabolism in the parasite as well as acting as an immunomodulator [9], promoting macrophage activation [10], and lipid-dependent cell signaling in tumor cells [11]. Miltefosine shows important drawbacks including gastrointestinal side effects, occasional hepato- and nephrotoxicity and potential teratogenicity [12]. Another limitation of this drug is emerging drug resistance, the occurrence of relapses in immunocompetent patients and variation in its sensitivity between American cutaneous leishmaniasis species [13, 14].

Non-classical bioisosteres include the replacement of entire functional groups, exchanging cyclic and non-cyclic forms and the inversion of functional groups (retroisosteres); this powerful concept has inspired rational drug design in medicinal chemistry [15]. Based on this concept, the aim of the present study was to investigate whether the replacement of the phosphocholine functional group on miltefosine by the

1,2,3-triazole and the size of the carbon chain (Figure 1) could maintain or even increase the biological activity of miltefosine. The triazole nucleus is a well known a class of five-membered heterocyclic compounds of great importance for the design of new drugs with broad and potent biological properties. In this work, fifteen compounds were synthesized, being three as 1,4-disubstituted-1,2,3-triazole precursors molecules (**bht**, **hht** and **dht**) and twelve salts of 1,4-disubstituted-1,2,3-triazole derivatives (Figure 1). Preliminary biological studies were performed with all triazole derivatives (data not shown). Serendipitously, only the compound **dhmtAc**, containing an acetate anion and a side chain of 10 carbon atoms, showed high selective biological activity against *Leishmania* parasites without any toxic effect on mammalian cells. These results showed that the non-classical bioisosterism proved advantageous since the compound **dhmtAc** was more effective on *L. amazonensis* than the miltefosine prototype. In addition, this work also showed that the action of **dhmtAc** on the parasite involves biochemical and cellular changes related to apoptosis and necrosis, similar to the previously related to mechanism of action of miltefosine.

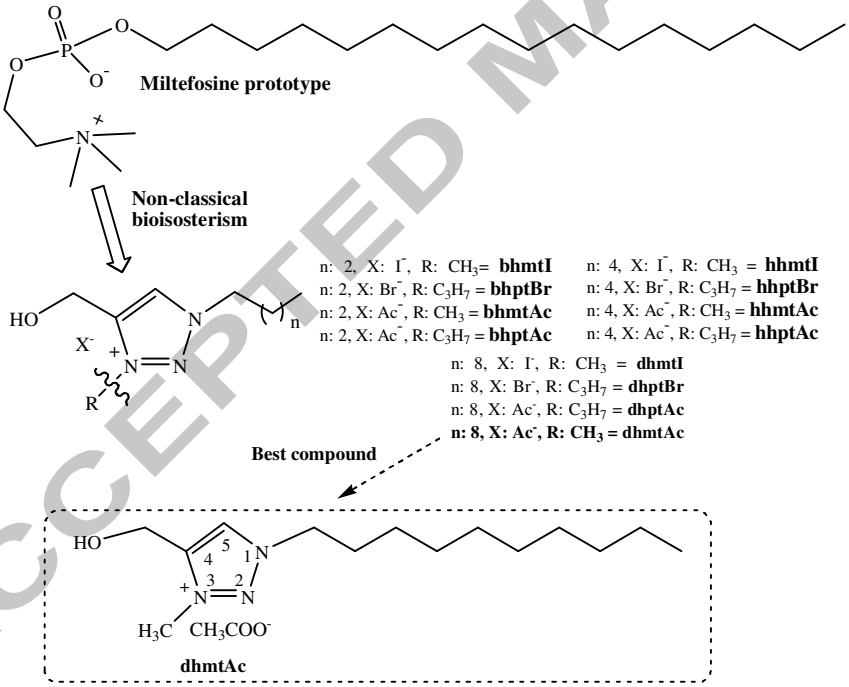
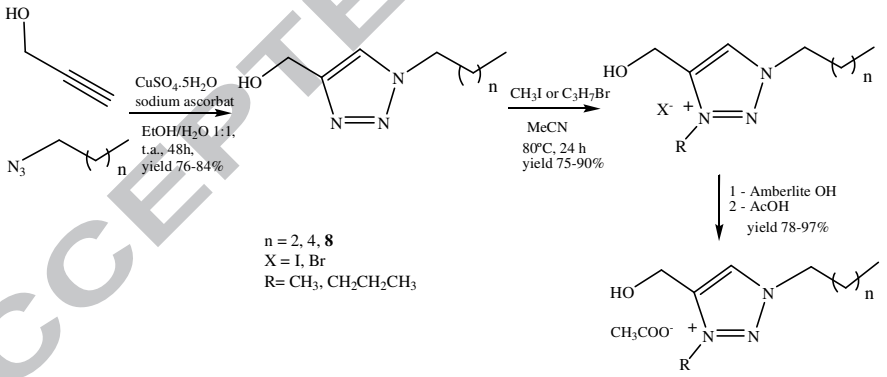


Figure 1: Chemical Structure of Miltefosine prototype and non-classical bioisosteres.

2. Results

2.1 Chemistry

The synthesis of the 1,4-disubstituted-1,2,3-triazole derivatives and their salts were realized in two steps, and after the salt of acetate was obtained by metathesis reaction. The synthesis of the starting material began by preparing alkyl azides azidobutane, azidohexane and azidodecane via an azidation reaction of the alkyl halides 1-bromobutane, 1-bromohexane and 1-bromodecane in the presence of sodium azide and a mixture 1:1 of ethanol:deionized water [16]. Subsequently, the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) provided regiospecifically the 1,4-disubstituted-1,2,3-triazoles **bht**, **hht** and **dht** after 48 h reaction time. Addition reaction of 1,2,3-triazoles with alkylhalide (iodomethane and 1-bromopropane), in acetonitrile at 80°C during 24 h to give the salts 4-(hydroxymethyl)-3-alkyl-1-alkyl-1*H*-1,2,3-triazole-3-ium halide. The acetate salt **dhmtAc** was previously reported in the patent literature [17]. The salts with the ion acetate were obtained passing the corresponding salts halide by anion exchange resin and subsequently neutralizing species formed according to the literature [18] (Scheme 1). The 1,2,3-triazoles were properly purified, partially characterized and the structure elucidation of the new compounds was obtained by the aid of ^{1}H,^{13}C NMR, and Mass spectrometry (Supplementary Figure - S1).



Scheme 1. Synthesis of 1,4-disubstituted-1,2,3-triazole derivatives and their salts.

2.2 Biological essays

*2.2.1 In vitro antileishmanial activity and mammalian cytotoxicity of **dhmtAc***

An initial screening with twelve non-classical bioisosteres of miltefosine on murine macrophages and promastigotes of *L. amazonensis* was done. SAR studies showed that among the salts 1,4-disubstituted-1,2,3-triazole derivatives, only compounds containing then carbons atoms linked to N-1 (**dhmtI**, **dhmtAc**, **dhptBr** and **dhptAc)** exhibited activity against promastigotes of *L. amazonensis* (IC_{50} values of 14.3 μM, 2.6 μM, 0.70 μM and 0.73 μM, respectively). All other compounds did not show antileishmanial activity at maximum concentration tested (IC_{50} > 100.0 μM). Among the four active compounds, two compounds **dhptBr** and **dhptAc**, containing a propyl group linked to N-3, showed a strong toxic effect on macrophages (CC_{50} values of 0.0039 μM and 4.6 μM, respectively). Finally, the compound **dhmtAc**, containing the acetate anion showed to be 5.5 times more active against promastigotes of *L. amazonensis* than the compound **dhmtI**, possessing the iodide anion. So, this compound was chosen for more studies about its antileishmanial activity and mechanism of action.

On promastigote forms, the proliferative insect vector-borne stage of *Leishmania*, the compound **dhmtAc** showed high activity with IC_{50} values below 16.0 μM (2.6, 3.3 and 15.6 μM, against *L. amazonensis*-WT, *L. amazonensis*-GFP and *L. amazonensis*-RFP, respectively) (Table 1). Next, we tested the effect of compound on intracellular amastigotes, which are the pathogenic stage of the parasite in mammalian host. Our results showed that sensitivity to the compound **dhmtAc** among various *Leishmania* used in this work was uniform. In addition, the antileishmanial activity was very expressive, with IC_{50} values near or similar to the 1.0 μM (0.9, 0.8 and 1.0 μM against *L. amazonensis*-WT, *L. amazonensis*-GFP and *L. amazonensis*-RFP, respectively), being more active than miltefosine, a control standard drug. The IC_{50} values for miltefosine were 4.2, 6.6 and 12.7 μM, for *L. amazonensis*-WT, *L. amazonensis*-GFP and *L. amazonensis*-RFP, respectively. These are promising results, given that the compound **dhmtAc** proved to be markedly more potent that miltefosine against both proliferative stages of this parasite.

Table 1 also shows that the compound **dhmtAc** presented a low cytotoxic effect on murine macrophages with CC_{50} of 115.9 μM. The selectivity index (SI) was also calculated and the compound was much more destructive to the parasitic form than to the macrophages, exhibiting an appreciable SI of 128.8, 144.9 and 115.9 for *L. amazonensis*-WT, *L. amazonensis*-GFP and *L. amazonensis*-RFP, respectively.

Table 1: Effect of **dhmtAc** on *L. amazonensis*, murine macrophages and selectivity index.

	L. amazonensis (IC_{50} µM)[a]									
	Promastigotes			Amastigotes			Macrophages	Selectivity index (SI)[#]		
	WT[c]	GFP[d]	RFP[e]	WT[c]	GFP[d]	RFP[e]	(CC_{50}µM)[b]	WT[c]	GFP[d]	RFP[e]
dhmtAc	2.6 ±0.1	3.3 ±0.7	15.6 ±0.1	0.9 ±0.6	0.8 ±0.1	1.0 ±0.5	115.9 ±1.1	128.8	144.9	115.9
Miltefosine*	22.0 ±0.5	13.2±0.8	18.9 ±0.1	4.2 ±0.2	6.6 ±1.8	12.7 ±0.9	143.5 ±9.1	34.2	21.7	11.3

[a]Values of inhibitory concentration of 50% of parasites (IC_{50}). [b]Values of cytotoxic concentration of 50% of macrophages (CC_{50}). [c]WT (*L. amazonensis*-Wild type; IFLA/BR/1967/PH8 strain); [d]GFP (*L. amazonensis* transfected with Green Fluorescent Protein; WHOM/BR/75/Josefa strain); [e]RFP (*L. amazonensis* transfected with Red Fluorescent Protein; IFLA/BR/1967/PH8 strain). *Miltefosine was used as reference drug. The data are expressed as the means from three experiments performed in duplicate. [#]Selectivity Index (SI) was calculated as the ratio between the CC_{50} for macrophages and the IC_{50} against intracellular amastigotes.

To determine whether the inhibitory effect in multiplication on promastigotes and intracellular amastigotes of *L. amazonensis* induced by **dhmtAc** was reversible, the cells were treated with different concentration of this compound. Promastigotes were treated with 20.0, 10.0, 5.0 or 2.5 µM compound (corresponding to 8, 4, 2 and 1 times of IC_{50} value) for 72 h, and after this time the cells were washed and reincubated for another 72 h without the compound **dhmtAc**. The results showed that promastigotes treated with 20.0, 10.0 and 5.0 µM **dhmtAc** presented low proliferate capacity when the compound was removed from the culture in the time analyzed, which reflected in their interruption of cell proliferation and reduced re-adaptation to the medium conditions (Figure 2A). For intramacrophage amastigotes, the infected macrophages were treated for 72 h with compound **dhmtAc** at 3.6, 1.8 and 0.9 µM (corresponding to 4, 2 and 1 times of IC_{50} value) and after this time, the cells were washed, placed in a medium without the compound for 72 h and we investigated the ability of these intracellular stages to differentiate into promastigotes. As shown in the Figure 2B, the treatment with **dhmtAc** at concentrations of 3.6; 1.8 and 0.9 µM reduced the capacity of the remaining amastigotes to differentiate into promastigotes by 83.1, 69.0 and 61.9%, respectively, as compared to the untreated parasites.

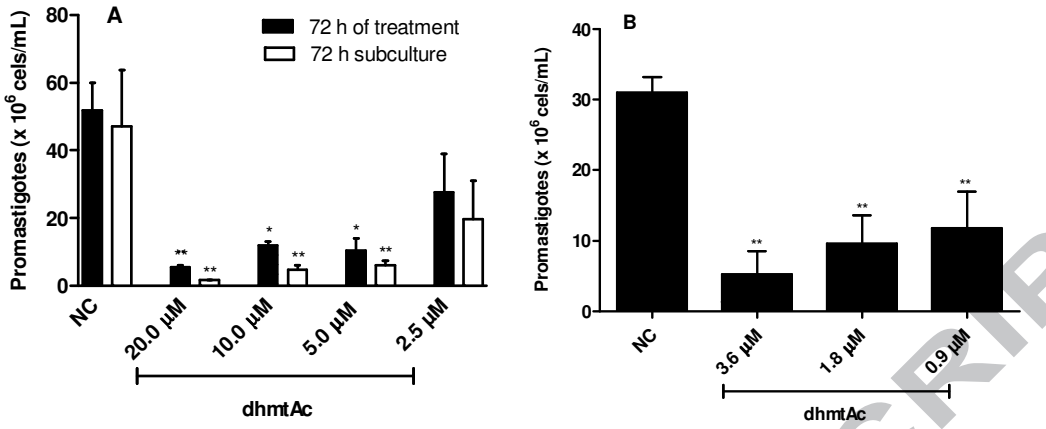


Figure 2: Reversibility of the effect of dhmtAc on proliferation of promastigotes and intracellular amastigotes of *L. amazonensis*. **(A)** Promastigote forms of *L. amazonensis* were treated with the indicated concentrations of **dhmtAc** for 72 h at 25 ºC. Parasite were counted, washed and incubated for more 72 h at 25 ºC without the compound. (**B)** After 72 h of the treatment, the infected macrophages were washed twice and incubated with Warren's medium and 10% fetal bovine serum at 25 ºC for more 72 h and the promastigotes were counted. $P<0.001$ (**), significant difference compared with the negative control (NC) The data were expressed as the means of three different experiments performed in duplicate.

To test the safety of the compound on human erythrocytes, the ability of **dhmtAc** to lyse erythrocytes was also assessed. The treatment did not cause significant damage to human erythrocytes, exhibiting hemolysis levels of less than 5% at all concentrations tested (Supplementary Figure - S2).

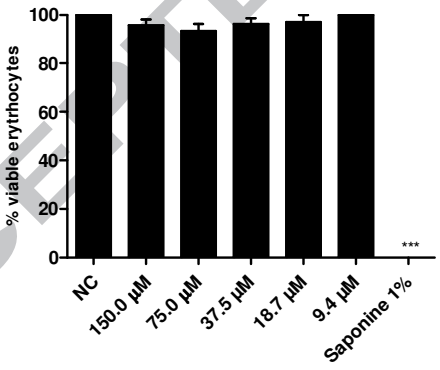


Figure S2: Cytotoxicity of dhmtAc against human erythrocytes. To evaluate the cytotoxicity to human erythrocytes, cells were exposed to the compound **dhmtAc** as described in materials and methods. NC (negative control). Saponine (1%) was used as positive control. $P< 0.0001$ (***), significant difference compared with the negative control. The data were expressed as the means of three different experiments performed in triplicate.

Since a significant effect of the non-classical bioisotere of miltefosine on *L. amazonensis* was observed, our next question was whether **dhmtAc** could also trigger

the programmed cell death of parasites, similar to that observed for miltefosine [7, 8, 12]. As compound **dhmtAc** is a new drug, preliminary studies were performed by using promastigote forms of *L. amazonensis*-WT.

2.2.2 ***DhmtAc*** *induces collapses of mitochondrial membrane potential*

The mitochondrial function of the parasites was first analyzed by the determination of mitochondrial membrane potential (Δψm) using the fluorescent marker JC-1 [19]. Fluorometric data presented in Figure 3 illustrate that the treatment of parasites with the compound for 24 h resulted in a marked reduction in the Δψm at all concentrations evaluated. In this sense, promastigotes treated with **dhmtAc** at 20.0, 10.0, 5.0 or 2.5 µM was capable of reducing the Δψm at all concentrations (41.5, 32.5, 20.0 and 37.5%, respectively). Promastigotes treated with FCCP, a classic protonophore uncoupler, exhibited a strong decrease of the Δψm (87.2%).

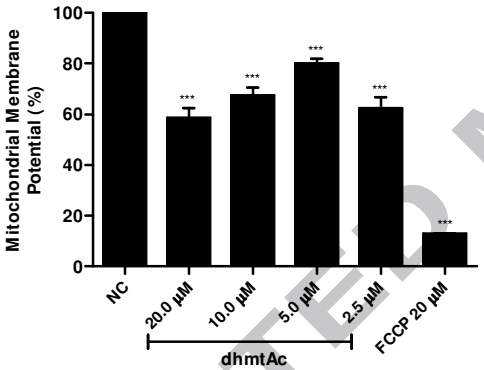


Figure 3: Changes in the Δψm of *L. amazonensis* promastigotes following treatment with dhmtAc. Parasites were untreated (negative control) or treated with compound at 2.5, 5.0, 10.0 or 20.0 µM and after 24 h were probed with JC-1. Fluorescence was measured fluorometrically. Negative control was regarded as 100% and the results expressed as a percentage in relation of the control. FCCP (20.0 µM) was used as positive control. $P < 0.0001$ (***), significant difference compared with the negative control. The data were expressed as the means of three different experiments performed in triplicate.

2.2.3 ***DhmtAc*** *increases total reactive oxygen species (ROS) in promastigotes of L. amazonensis*

The alterations of Δψm induced in *Leishmania* by a variety of molecules has been associated with the generation of ROS, which triggers damage to the components of the electron transport chain, collapses mitochondrial functionality and culminates in

apoptosis-like cell death [20]. Figure 4 shows a significant increase in the ROS production at all of the concentrations tested compared with untreated parasites (negative control). The treatment with 20.0, 10.0, 5.0 and 2.5 µM caused significant increases in the ROS levels of 75.5, 54.0, 40.7 and 38.4%, respectively. Promastigotes treated with miltefosine (22.0 µM), used as a positive control, also increased the ROS production of 66.7%.

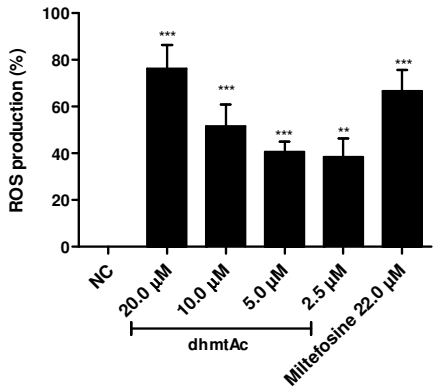


Figure 4: **ROS production in *L. amazonensis* promastigote forms treated with dhmtAc.** Parasites were untreated (negative control-NC) or treated with the compound at 2.5, 5.0, 10.0 and 20.0 µM for 72 h, and after this time the cells were probed with H_2DCFDA. Miltefosine (22.0 µM) was used as positive control. Fluorescence was measured fluorometrically and the data were expressed as the fluorescence intensity in arbitrary units (A.U.) of means of three independent experiments. $P < 0.0001$ (***) and 0.001 (**), significant difference compared with the negative control.

*2.2.4 Analysis of lipid bodies accumulation in promastigotes of L. amazonensis treated with **dhmtAc***

Considering the effect of compound **dhmtAc** on the induction of mitochondrial stress in promastigotes by **(a)** reduction of membrane potential and, **(b)** increase of ROS generation, we determined whether this compound also induces the formation of lipid inclusions, which is strongly related to mitochondrial dysfunction [21, 22]. To evaluate the effect of **dhmtAc** on the accumulation of neutral lipid bodies, we performed fluorometric assay by using the Nile Red stain. Nile red is a fluorescent compound used to localize and quantify lipids, particularly neutral lipid droplets within cells [23]. Fluorimetry analysis indicated that treatment with the compound in the highest concentrations tested (20.0 and 10.0 µM) for 24 h was able to induce a significant increase in the accumulation of lipid bodies (2.5 and 1.9 times, respectively), compared with the untreated parasites. On the other hand, the treatment with 5.0 and 2.5 µM

compound did not show significant alteration in the formation of lipid inclusions (Figure 5).

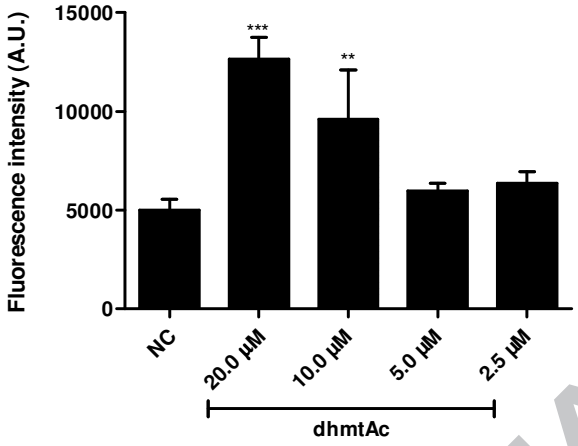


Figure 5: Lipid inclusions in *L. amazonensis* promastigote forms treated with compound dhmtAc. Parasites were untreated (negative control-NC) or treated with **dhmtAc** at 2.5, 5.0, 10.0 or 20.0 µM and after 24 h were probed with Nile Red. Fluorescence was measured fluorometrically and the data were expressed as the fluorescence intensity in arbitrary units (A.U.) of three independent experiments. *P*< 0.0001 (***) and 0.001 (**) significant difference compared with the negative control. Data were expressed as the means of three different experiments performed in triplicate.

*2.2.5 Effect of **dhmtAc** on plasma membrane permeability of L. amazonensis*

Plasma membrane rupture is a key event of necrotic cell death [24]. In order to investigate whether the strong mitochondrial collapse marked by loss of ΔΨm could be associated with the ability of compound to alter the plasma membrane permeability of parasite, we evaluated the internalization of the fluorescent dye PI into the cytoplasm of promastigotes previously treated with **dhmtAc** (20.0, 10.0, 5.0 and 2.5 µM) for 24 h. Figure 6 shows that the intensity of PI fluorescence was higher at the maximum concentration used (20.0 µM) than in the untreated cells, indicating permeability on the parasite plasma membrane. All others concentrations tested caused no interference on the percentage of PI-labelled parasites and probably did not undergo a necrotic process. The positive control (parasites heated) induced strong permeabilization of the parasite membrane (Figure 6).

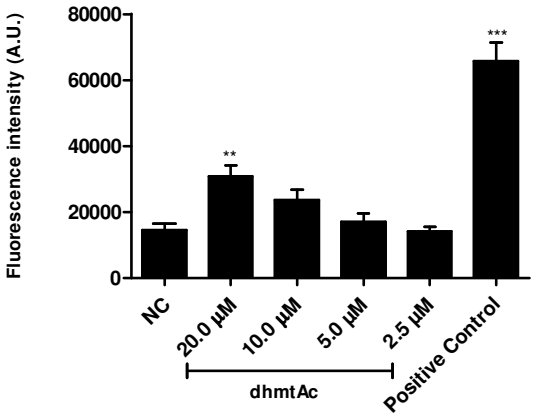


Figure 6: Cell membrane integrity of *L. amazonensis* promastigote forms treated with dhmtAc. Parasites were untreated (negative control) or treated with compound **dhmtAc** at 2.5, 5.0, 10.0 or 20.0 µM for 24 h and stained with PI. Fluorescence was measured fluorometrically and the data were expressed as the fluorescence intensity in arbitrary units (A.U.) of means of three independent experiments. $P< 0.0001$ (***) and $P< 0.001$ (**), significant difference compared with the negative control. Positive control: cells heated at 65 ºC for 10 min.

*2.2.6 **DhmtAc** induces phosphatidylserine exposure in promastigotes of L. amazonensis*

Apoptosis is marked by biochemical alterations, including phosphatidylserine exposure to the outerleaflet of the plasma membrane. Annexin V, a Ca^{+2}-dependent phospholipid-binding protein with an affinity for phosphatidylserine, is routinely used to label the externalization of phosphatidylserine. Since annexin V can also label necrotic cells, a PI stain was used to differentiate among apoptotic cells (annexin V^+/PI), necrotic cells (annexin V^-/PI^+) and normal cells (annexin V^-/PI^-) [25]. As shown in Figure 7, a significant percentage (> 28%) of promastigote forms that were treated for 24 h with **dhmtAc** were annexin-V positive, compared with only 5.3% in the untreated parasites (negative control), indicating phosphatidylserine exposure. The histogram analysis also showed that promastigotes treated at 20.0 and 10.0 µM were double-stained with annexin V and PI (16.9 and 11.2%, respectively). Parasites treated with miltefosine (44.0 µM), used as a positive control, exhibited similar behavior: promastigotes annexin V-positive (53.2%); double-stained with annexin V and PI (12.2%) and only PI-positive (7.1%).

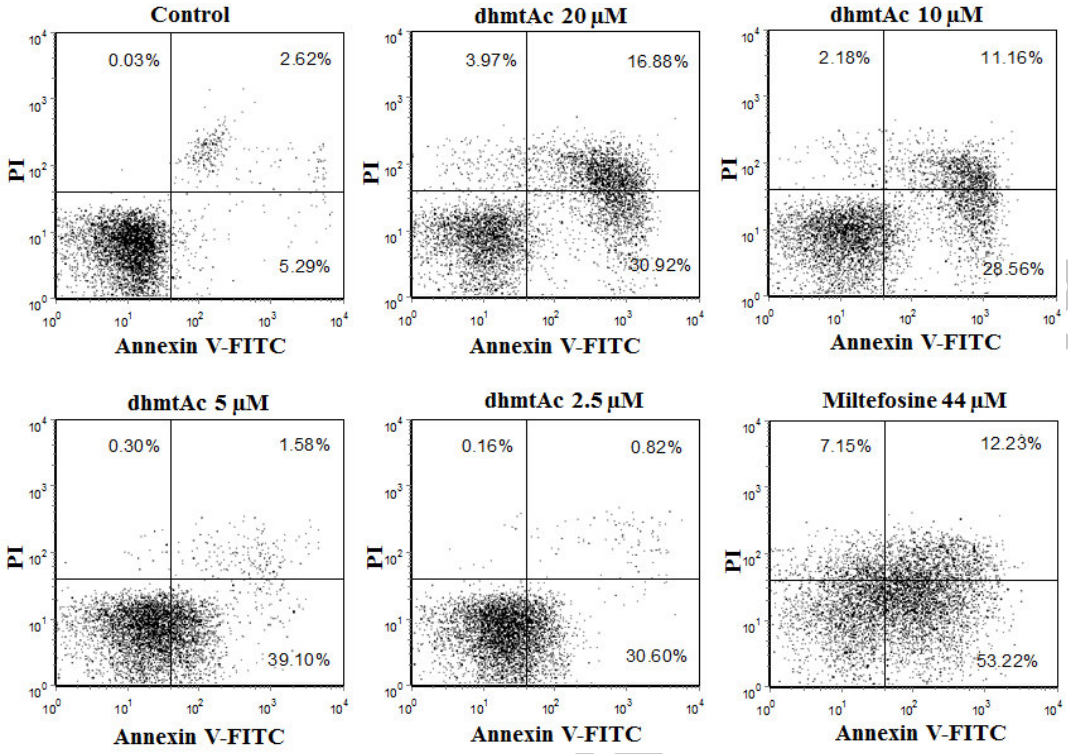


Figure 7: Phosphatidylserine exposure on the cell membrane in promastigote forms of *L. amazonensis* treated with dhmtAc. Promastigotes were incubated with 2.5, 5.0, 10.0 or 20 µM **dhmtAc** for 24 h. After, the cells were stained with the fluorescent probes annexin V-FITC and PI and analyzed by flow cytometer. Promastigotes treated with miltefosine (44.0 µM), were used as a positive control. Histograms are representative of three independent experiments, with 10,000 parasites analyzed. The percentage of annexin V-positive cells are shown in the upper and lower right quadrants. The percentage of PI-positive cells are shown in the upper and lower left quadrants.

*2.2.7 **DhmtAc** decreases cell volume of parasite*

Morphological changes were evaluated by optical microscopy. The untreated parasites exhibited typical features of the promastigote forms, with an elongated cell body and a free flagellum. In contrast, parasites that were treated with 2.5, 5.0, 10.0 and 20.0 µM **dhmtAc** exhibited an altered shape, a size reduction, and a rounded cell body (Supplementary Figure - S3).

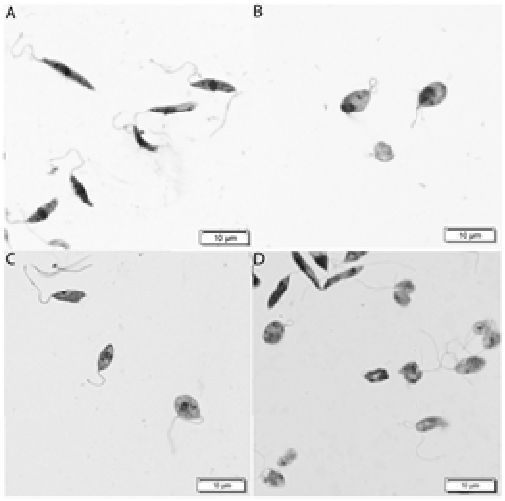


Figure S3: Morphological analysis of promastigotes of *L. amazonensis* treated with dhmtAc. Promastigotes were untreated or treated with 5.0, 10.0 and 20.0 µM **dhmtAc** for 24 h and were stained with Giemsa. **(A)** Untreated parasites; **(B)** Promastigotes treated with 20 µM **dhmtAc**; **(C)** Promastigotes treated with 10.0 µM **dhmtAc; (D)** Promastigotes treated with 5.0 µM **dhmtAc.** Scale bars=10 µm.

2.2.8 ***DhmtAc*** *induces DNA fragmentation in promastigotes of L. amazonensis*

DNA fragmentation is a typical hallmark of cells undergoing the final outcome of the apoptosis-like death and is strongly related to the oxidative imbalance [25]. Thus, the TUNEL assay was performed in order to verify whether the compound was able to induce nuclear DNA and/or mitochondrial DNA fragmentation in promastigotes of *L. amazonensis*. Promastigotes that were treated with the compound **dhmtAc** at 5.0 and 10.0 µM for 24 h exhibited bright fluorescence, indicating DNA double-strand ruptures compared with untreated parasites (Figure 8). Bright fluorescence was also observed with the promastigote treated with the reference compound, miltefosine (22.0 µM). DAPI fluorescence revealed nuclei and kinetoplasts in both untreated control and treated promastigotes with **dhmtAc** and miltefosine.

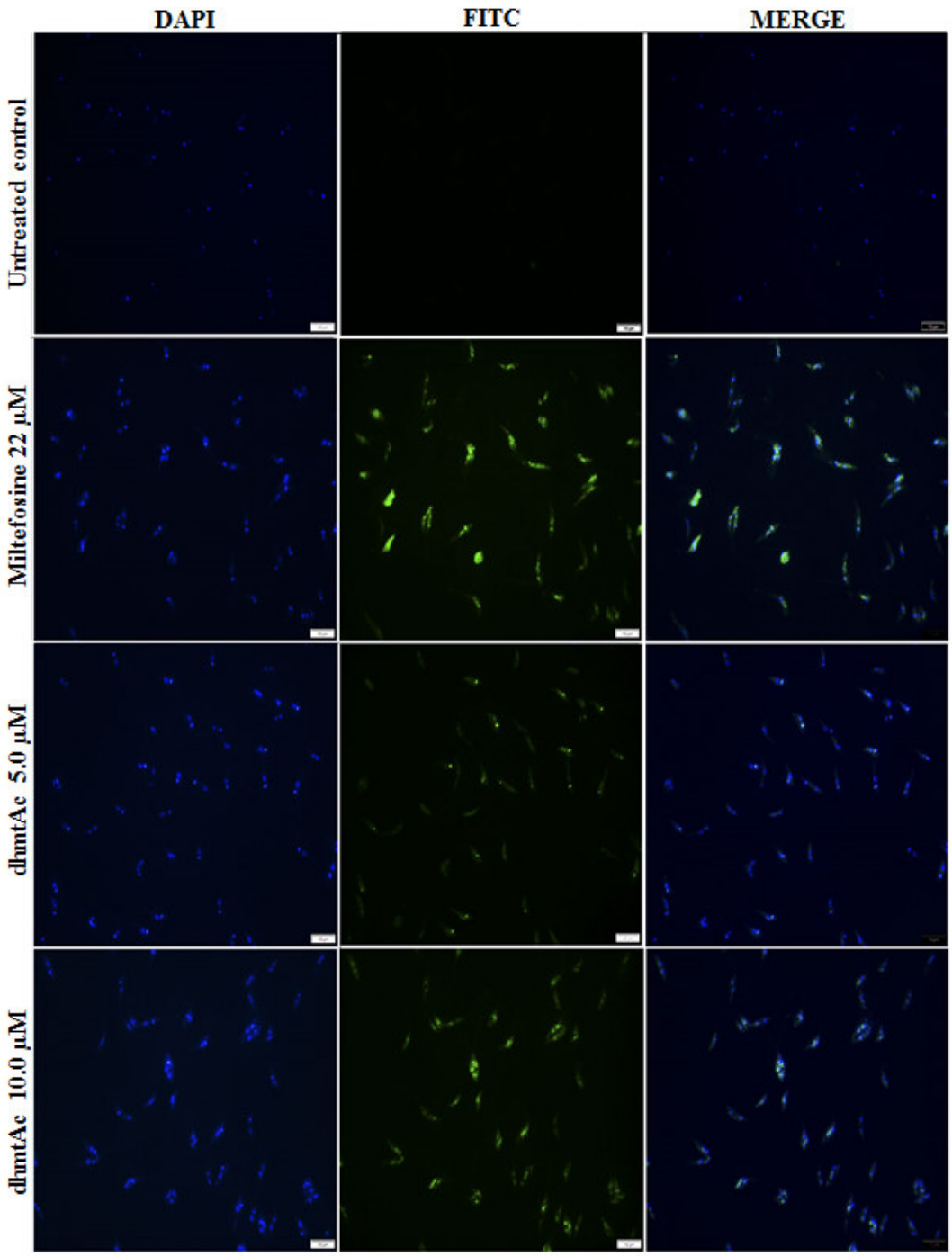


Figure 8: DNA fragmentation in promastigote forms of *L. amazonensis*. Parasites were treated with the compound **dhmtAc** (5.0 and 10.0 µM) for 24 h and stained with TUNEL kit and examined by fluorescence microscopy at 100X. Control group (untreated parasites). Promastigotes incubated with Miltefosine at 22.0 µM were utilized as positive control. Green fluorescence indicates DNA fragmentation. Blue fluorescence of DAPI show nuclei and kinetoplasts in both control and treated promastigotes. Merged images revealed the localization of the nuclei and kinetoplasts inside the parasites. Scale bars=10 µm.

*2.2.9 Promastigotes treated with **dhmtAc** did not exhibited increase of the autophagic vacuoles*

Finally, to evaluate the hypothesis that treatment with compound can also induce autophagy death of the parasite, promastigotes treated with the compound **dhmtAc** were labeled with MDC, a selective marker for the presence of autophagosomes [26]. Promastigotes that were treated with **dhmtAc** (2.5, 5.0 and 10.0 µM) displayed a non-significant increase in MDC fluorescence in relation to the untreated parasites. Interestingly, the fluorescence by parasites treated with compound at 20.0 µM was significantly lower (49.8%) than that observed in untreated parasites (Supplementary Figure - S4). These results show that treatment with compound did not result in the formation of autophagic vacuoles in *L. amazonensis* promastigotes.

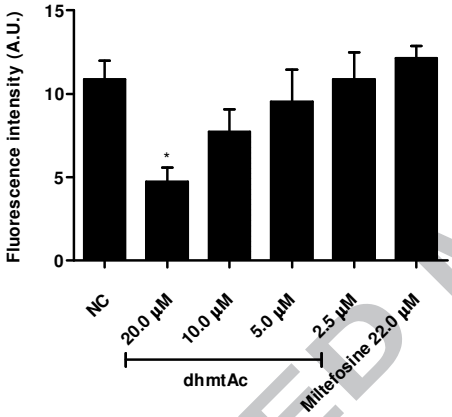


Figure S4: Evaluation of autophagic vacuoles by MDC labeling of *L. amazonensis* promastigotes treated with compound dhmtAc. Parasites were untreated (negative control-NC) or treated with compound **dhmtAc** at 2.5, 5.0, 10.0 or 20.0 µM for 24 h and stained with MDC. Reading was performed in a microplate reader using wavelengths of 335 and 460 nm for excitation and emission, respectively. The data were expressed as the fluorescence intensity in arbitrary units (A.U.) of means of three independent experiments. $P < 0.01$ (*) significant difference compared with the negative control.

3. Discussion

In this work, we show that **dhmtAc**, a non-classical bioisotere of miltefosine, showed a strong effect on promastigote and amastigote forms of *L. amazonensis,* being more effective than the alkylphosphocholine miltefosine prototype. Miltefosine exhibits variation in activity against several *Leishmania* species, laboratory strains, and also between the same species from different endemic areas [13, 27]. Furthermore, the New World *Leishmania* species are considered less susceptible to miltefosine action,

suggesting that higher doses of miltefosine would be needed to treat patients infected with *Leishmania* species prevalent in Brazil [27]. Therefore, it was interesting to note that the compound **dhmtAc** showed IC_{50} values near the 1.0 μM against all *L. amazonensis* amastigotes tested in this work. As can be noted, amastigotes of *L. amazonensis*-GFP (Josefa strain) and *L. amazonensis*-RFP (PH8 strain) presented IC_{50} values very close to the IC_{50} value of *L. amazonensis*-WT (PH8 strain), highlighting the antileishmanialeffect of **dhmtAc**. Furthermore, **dhmtAc** exhibited low cytotoxicity for murine macrophages (CC_{50} value of 115.9 μM), being much more destructive to the intracellular parasites of all *Leishmania* strains analyzed (SI = 115.0) than to the host cell. These are promising results, given that this compound proved to be more selective to the amastigotes than miltefosine. For *Leishmania* sp, an SI > 20 is considered adequate for subsequent *in vivo* studies [28]. In addition, the ability of **dhmtAc** to cause red blood cell lysis was also assessed and this compound did not present a significant effect on the erythrocyte viability at the highest concentration tested. It is known that miltefosine has high hemolytic activity and could not be administered intravenously [29].

Preliminary studies about the mechanism of action of the compound **dhmtAc** were performed using promastigote forms of *L. amazonensis* and the mitochondria organelle was initially the target chosen for the studies. The single mitochondria is vital for the survival of *Leishmania* parasite and alterations in this organelle can to induce the loss of cell viability and represents an important target for drugs [20, 30]. In this sense, our results strongly suggest that **dhmtAc** induces mitochondrial deregulation with marked reduction of the $\Delta\psi m$ and increase in the ROS production. Also, promastigotes of *L. amazonensis* treated with this compound exhibited an increase in the intracellular lipid bodies. Lipid body (LP) formation can be caused by different perturbations to the parasite cellular functions and LPs are a hallmark of cellular stress can be caused by mitochondrial dysfunction and constitute a characteristic of apoptotic cell death [21, 22, 31].

Since we observe in this work that the miltefosine bioisostere affected the mitochondria, and several works show that miltefosine induces most of the features associated with metazoan apoptosis in *Leishmania* sp [7, 8, 32, 33], our next question was whether the treatment of *L. amazonensis* with **dhmtAc** could also be associated with biochemical features related to apoptosis. We found that **dhmtAc** was able to induce nuclear DNA and/or mitochondrial DNA fragmentation using TUNEL staining in promastigotes, and findings suggest that this compound might also interact with DNA

parasite (i.e., by binding to DNA or intercalating DNA), thus leading to structural changes in DNA, reflected by an increase in the population of cells in the sub-G_0/G_1 phase (data not shown). DNA fragmentation can disturb the parasite duplication, leading to cell cycle arrest, culminating in apoptosis [34].

In a parallel step, we studied the effect of **dhmtAc** on the plasma membrane of promastigotes of *L. amazonensis*. The study of the alterations of the plasma membrane provides us with important data to distinguish between apoptosis and necrosis, considering the rupture of the plasma membrane as a morphological feature of the necrosis and the exposure of phosphatidylserine residues on the external leaflet of the parasite membrane has been associated with apoptosis [24]. By using Annexin V/FITC staining, we demonstrated the exposure of phosphatidylserine residues on the external leaflet of the parasite membrane after treatment with **dhmtAc**. Also, parasites treated with **dhmtAc** at the maximum concentrations tested (20.0 and 10.0 μM) caused disruption of the plasma membrane, revealed by PI labeling. Altogether, these results indicate that the target of this molecule is partially related to the plasma membrane of the parasites and not exclude the possibility that death occurred through classic necrosis.

De Macedo-Silva et al. [23] related the alterations of lipid composition resulting from treatment with drugs, which were also revealed here by Nile red accumulation in treated promastigotes, and showed that they could interfere with plasma membrane integrity, culminating in parasite death by necrosis. Interestingly, in this work, the treatment of parasites with miltefosine (44.0 μM) also altered the percentage of cells labeled with PI, indicating the possibility of necrosis-like death in the later stages. This result is not surprising and previous studies showed that miltefosine induces necrosis in *Leishmania* [8].

Autophagy is another form of programmed cell pathway which has been well described for trypanosomatids, and involves degradation of unnecessary cellular components through the actions of lysosomes [35]. Although apoptosis and autophagy should be considered as two distinct processes, they are closely related to mitochondrial dysfunction associated with loss of membrane potential and an increase in ROS formation in treated promastigotes [34]. Besides these effects observed on the promastigotes mitochondria, our data clearly demonstrated that **dhmtAc** did not induce the formation of autophagic vacuoles in *L. amazonensis* promastigotes at the time and concentrations tested, thus ruling out the possibility of cell death by autophagy.

Our results also showed that this compound had a strong effect on intramacrophage amastigotes and the intracellular parasites showed a low capacity towards reversion of the treatment, reflecting the efficacy of the treated and infected-macrophages in eliminate these stages of parasite. It has been suggested that miltefosine presents direct effect on the intracellular parasite since this drug retain their antileishmanialeffect on immunodeficient mice [10]. Since the compound **dhmtAc** is an amphiphilic molecule and is positively charged, we hypothesized that it could interact with both negatively charged proteins and membrane phospholipids and be able to cross membranes, reaching the parasite within the parasitophorous vacuole [36].

It has been reported in literature that the miltefosine unresponsive strain *Leishmania* resists formation ROS [37, 38]. Regarding this, the new compound **dhmtAc** may be seen as an alternative against miltefosine unresponsive strain.

Finally, our findings provide clear evidence that, like miltefosine, **dhmtAc** also induces promastigote cell death involving a cascade of biochemical events which display major hallmarks of apoptosis-like such as mitochondrial depolarization, ROS production, lipid body accumulation, phosphatidylserine externalization in plasma membrane and DNA fragmentation. However, necrosis-like death in later stages can also be involved in the mechanism of action of this miltefosine bioisostere on *L. amazonensis*.

4. Conclusions

In this work, we showed the successful rational drug design using the bioisosterism concept. A new miltefosine bioisostere showed superior activity over the prototype on *L. amazonensis* along with an appreciable selectivity index and therefore makes it a strong candidate for further investigations towards the development of new antileishmanial drugs. Furthermore, the present data provide insights into the mechanism of action of this compound. *In vivo* activity in murine models, as well *in vitro* studies with other *Leishmania* species are now being conducted in order to better establish the antileishmanial effect of this compound.

5. Experimental

5.1 Chemicals

Infrared (FT-IR) spectra were recorded with an Alpha Bruker FT-IR Eco-ATR spectrometer, in the region 4000 - 600 cm^{-1}. It was used a 4 cm^{-1} spectral resolution and an average of 24 scans. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance III HD 500 at 500.13 MHz. The chemical shifts (δ) are given in parts per million (ppm) relative to tetramethylsilane. Spectra were acquired in DMSO-d_6, CD_3OD and $CDCl_3$. High resolution mass spectra were obtained with a Bruker MicrOTOF II ESI mass spectrometer. The mass of the molecular ion was identified through analysis by (MALDI-TOF MS) using a pulsed nitrogen ultraviolet laser (λ = 337 nm) of an AXIMA Performance MALDI-TOF MS (Shimadzu Biotech). All reagents of analytical grade were obtained from commercial suppliers and were used without previous purification.

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5.1.1 General procedures for the preparation of alkyl azides

In a flask, sodium azide (15.8 g, 0.243 mol) in 40 mL of (EtOH) and 40 mL of deionized water was added. Alkyl halide (1-bromobutane, 1-bromohexane and 1-bromodecane) (0.243 mol) was added to sodium azide at room temperature with stirring. The reaction was allowed to proceed at 60°C and monitored by ^{1}H NMR analysis of aliquots. The alkyl azides were not isolated, following cycloaddition reaction *in situ.*

*5.1.2 General procedure for the preparation of triazols (**bht** [39], **hht** and **dht**)*

Propargyl alcohol (78 mmol), $CuSO_4.5H_2O$ (3 mmol), and sodium ascorbate (24 mmol) were added in the solution of EtOH/H_2O with the alkyl azides (60 mmol). The reactions were stirred at room temperature for 48 h with monitoring by ^{1}H NMR analysis of aliquots. Filtration and washing with EtOH afforded 84% of (**bht**), 76% of (**hht**) and 81% of (**dht**). A brown oil was formed from the reactions (**bht** and **hht**) and a brown solid was formed to the **dht**.

bht

Yield 84%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.88 (t, 3H, *J*= 7.3, CH_3), 1.23 (sex, 2H, *J*= 7.5, CH_3), 1.77 (qui, 2H, *J*= 7.2, CH_2), 4.32 (t, 2H, *J*= 7.0, CH_2), 5.16 (s, 2H,

CH_2OH), 7.95 (s, 1H, H-triazole); ^{13}C NMR (125 MHz, DMSO- d_6) δ 13.3, 19.1, 31.8, 48.9, 55.0, 122.6, 147.9; IR νmax (cm^{-1}): 3322, 2955, 1638, 1460.

hht

Yield 76%; ^1H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, 3H, *J*= 6.9, CH_3), 1.23 (m, 6H), 1.90 (qui, 2H, *J*= 7.0, CH_2), 4.34 (t, 2H, *J*= 7.2, CH_2), 4.78 (s, 2H, CH_2OH), 7,62 (s, 1H, H-triazole); ^{13}C NMR (125 MHz, CDCl3) δ 14.1, 22.6, 26.3, 30.4, 31.3, 50.7, 56.5, 122.9, 149.6; IR νmax (cm^{-1}): 3307, 3137, 2925, 1661, 1459.

dht

Yield 81%; ^1H NMR (500 MHz, $CDCl_3$) δ 0.89 (t, 3H, *J*= 7.2, CH_3), 1.38 (m, 14H), 1.90 (m, 2H, CH_2), 4.41 (t, 2H, *J*= 7.2, CH_2), 4.67 (s, 3H, CH_3), 8.14 (s, 1H, H-triazole); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.6, 23.8, 27.5, 30.2, 30.5, 30.7, 31.3, 31.8, 33.1, 37.1, 51.8, 56.9, 140.4, 165.0; IR νmax (cm^{-1}): 3326, 2917, 1653, 1463.

*5.1.3 General procedure for the preparation halide salts (**bhmtI**, **hhmtI**, **dhmtI**, **bhptBr**, **hhptBr** and **dhptBr**)*

In a flask, a mixture of the 1,4-dissubstituted-1,2,3-triazole (**bht**, **hht** and **dht**) (50 mmol) and alkyl halide (iodomethane and 1-bromopropane) (200 mmol) was stirred at 80°C for 24 h in 10 mL of acetonitrile. The reaction mixture was concentrated in vacuo to afford **bhmtI** (75%), **hhmtI** (81%), **dhmtI** (90%), **bhptBr** (87%), **hhptBr** (88%) and **dhptBr** (82%). A brown oil was formed from the reactions, except for the **dhmtI** (brown solid).

bhmtI

Yield 75%; ^1H NMR (500 MHz, CD_3OD) δ 1.00 (t, 3H, *J*= 7.30, CH_3), 1,43 (sextet, 2H, *J*= 7.30, CH_2), 2.01 (quintet, 2H, *J*= 7.3, CH_2), 4.32 (s, 3H, CH_3), 4.64 (t, 2H, *J*= 7.20, CH_2), 4.87 (s, 2H, CH_2OH), 8.70 (s, 1H, H-triazole); ^{13}C NMR (125 MHz, CD_3OD) δ 13.8, 20.5, 32.4, 39.0, 53.9, 54.9, 129.9, 145.4; MALDI-TOF MS m/z, calcd for [M]+ 170.1293, found 170.1280; IR νmax (cm^{-1}): 3282, 3084, 2955, 1581, 1454.

hhmtI

Yield 81%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.89 (t, 3H, *J*= 7.20, CH_3), 1.34 (m, 6H), 2.01 (quintet, 2H, *J*= 7.30, CH_2), 4.39 (s, 3H, CH_3), 4.64 (t, 2H, *J*= 7.30, CH_2), 5.04 (s, 2H, CH_2OH), 8.95 (s, 1H, H-triazole); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 22.5, 26.0, 29.5, 31.1, 39.7, 53.0, 54.6, 130.0, 143.9; MALDI-TOF MS m/z, calcd for [M]+ 198.1606, found 198.1613; IR νmax (cm^{-1}): 3281, 3078, 2924, 1582, 1452.

dhmtI

Yield 90%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.82 (t, 3H, *J*= 7.25, CH_3), 1.27 (m, 14H), 1.97 (m, 2H, CH_2), 4.34 (s, 3H, CH_3), 4.58 (t, 2H, *J*= 7.2, CH_2), 4.98 (s, 2H, CH_2OH), 8.91 (s, 1H, H-triazole); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.2, 22.7, 26.3, 28.9, 29.3, 29.4, 29.5, 29.6, 31.9, 39.7, 52.9, 54.5, 129.9, 143.8; MALDI-TOF MS m/z, calcd for [M]+ 254.2232, found 254.2228; IR νmax (cm^{-1}): 3302, 2918, 1592, 1465.

bhptBr

Yield 87%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.98 (t, 3H, *J*= 7.30, CH_3), 1.03 (t, 3H, *J*= 7.40, CH_3), 1.42 (sex, 2H, *J*= 7.50, CH_2), 2.02 (qui, 2H, *J*= 7.50, CH_2), 2.07 (sex, 2H, *J*= 7.30, CH_2), 4.64 (t, 2H, *J*= 7.30, CH_2), 4.70 (t, 2H, *J*= 7.30, CH_2), 4.97 (s, 2H, CH_2OH), 9.18 (s, 1H, H-triazole); ^{13}C NMR (125 MHz, $CDCl_3$) δ 11.0, 13.4, 19.5, 22.7, 31.3, 52.4, 53.8, 54.0, 130.0, 143.7; MALDI-TOF MS m/z, calcd for [M]+ 198.1606, found 198.1600; IR νmax (cm^{-1}): 3236, 2958, 1579, 1460.

hhptBr

Yield 88%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, 3H, *J*= 7.0, CH_3), 1.03 (t, 3H, *J*= 7.3, CH_3), 1.35 (m, 6H, $3CH_2$), 2.05 (m, 4H, $2CH_2$), 4.62 (t, 2H, *J*= 7.3, CH_2), 4.67 (t, 2H, *J*= 7.3, CH_2), 4.98 (s, 2H, CH_2OH), 9.15 (s, 1H, H-triazole); ^{13}C NMR (125 MHz, $CDCl_3$) δ 11.0, 14.1, 22.5, 22.7, 26.0, 29.5, 31.1, 52.6, 53.9, 54.4, 130.1, 143.9; MALDI-TOF MS m/z, calcd for [M]+ 226.1918, found 226.1910; IR νmax (cm^{-1}): 3297, 2928, 1630, 1459.

dhptBr

Yield 82%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, 3H, *J*= 7.5, CH_3), 1.03 (t, 3H, *J*= 7.5, CH_3), 1.25 (m, 16H), 2.03 (m, 2H, CH_2), 4.63 (m, 4H, $2CH_2$), 4.97 (s, 2H, CH_2OH), 9.12 (s, 1H, H-triazole); ^{13}C NMR (125 MHz, $CDCl_3$) δ 10.9, 14.1, 22.7, 22.9,

26.2, 28.9, 29.1, 29.3, 29.4, 29.5, 29.6, 31.9, 52.5, 53.8, 54.3, 130.0, 143.7; MALDI-TOF MS m/z, calcd for [M]+ 282.2545, found 282.2541; IR νmax (cm^{-1}): 3335, 2924, 1626, 1465.

*5.1.4 General procedure for the preparation acetate salts (**bhmtAc**, **hhmtAc**, **dhmtAc**, **bhptAc**, **hhptAc** and **dhptAc**)*

Ethanolic solution of the salts of 1,2,3-triazolium cations containing hydroxide as anion were prepared from 1,2,3-triazolium halide (**bhmtI**, **hhmtI**, **dhmtI**, **bhptBr**, **hhptBr** and **dhptBr**) using anion exchange resin AMBERLITE IRA400OH (Sigma). To these solutions were added equimolar amounts of acetic acid in ice bath. The mixture was stirred for 16 h at room temperature. Then solvent was evaporated to give the product as yellow oil. **bhmtAc** (78%), **hhmtAc** (96%), **dhmtAc** (85%), **bhptAc** (97%), **hhptAc** (91%) and **dhptAc** (86%).

bhmtAc

Yield 78%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.97 (t, 3H, *J*= 7.30, CH_3), 1.40 (sextet, 2H, *J*= 7.50, CH_2), 1.87 (s, 3H, CH_3COO^-), 1.98 (qui, 2H, *J*= 7,30, CH_2), 4.34 (s, 3H, CH_3), 4.60 (t, 2H, *J*= 7.40, CH_2), 4.93 (s, 2H, CH_2OH), 9.23 (s, 1H, H-triazole); ^{13}C NMR (125 MHz, $CDCl_3$) δ 13.5, 19.6, 24.9, 31.4, 38.4, 52.5, 53.8, 130.2, 145.2, 178.1; MALDI-TOF MS m/z, calcd for [M]+ 170.1293, found 170.1288; IR νmax (cm^{-1}): 3375; 3080; 2957; 1566; 1387.

hhmtAc

Yield 96%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, 3H, *J*= 7.2, CH_3), 1.32 (m, 6H, $3CH_2$), 1.86 (s, 3H, CH_3COO^-), 1.97 (qui, 2H, *J*= 7,2, CH_2), 4.31 (s, 3H, CH_3), 4.60 (t, 2H, *J*= 7.3, CH_2), 4.90 (s, 2H, CH_2OH), 9.03 (s, 1H, H-triazole); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 22.5, 25.1, 26.0, 29.4, 31.4, 38.2, 52.4, 54,0, 130.0, 145.0, 177.7; MALDI-TOF MS m/z, calcd for [M]+ 198.1606, found 198.1605; IR νmax (cm^{-1}): 3358; 3095; 2925; 1565; 1391.

dhmtAc

Yield 85%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, 3H, *J*= 7.0, CH_3), 1.31 (m, 14H), 1.90 (s, 3H, CH_3COO^-), 1.98 (qui, 2H, *J*= 7.4, CH_2), 4.33 (s, 3H, CH_3), 4.56 (t, 2H, *J*=

7.4, CH_2), 4.91 (s, 2H, CH_2OH), 9.20 (s, 1H, H-triazole); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 22.6, 24.4, 26.2, 28.8, 29.2, 29.3, 29.4, 29.4, 31.8, 38.1, 52.2, 53.8, 130.1, 145.2, 177.5; MALDI-TOF MS m/z, calcd for [M]+ 254.2232, found 254.2241; IR νmax (cm^{-1}): 3363, 3090,2923, 1566, 1390.

bhptAc

Yield 97%; ^1H NMR (500 MHz, $CDCl_3$) δ 0.97 (t, 3H, *J*= 7.3, CH_3), 1.01 (t, 3H, *J*= 7.5, CH_3), 1.38 (sex, 2H, *J*= 7.4, CH_2), 1.90 (s, 3H, CH_3COO^-), 1.99 (qui, 2H, *J*= 7.4, CH_2), 2.03 (sex, 2H, *J*= 7.3, CH_2), 4.57 (t, 2H, *J*= 7.4, CH_2), 4.60 (t, 2H, *J*= 7.3, CH_2), 4.92 (s, 2H, CH_2OH), 9.36 (s, 1H, H-triazole); ^{13}C NMR (125 MHz, $CDCl_3$) δ 10.8, 13.3, 19.4, 22.6, 24.7, 31.2, 52.2, 53.1, 53.6, 130.4, 145.1, 177,7; MALDI-TOF MS m/z, calcd for [M]+ 198.1606, found 198.1615; IR νmax (cm^{-1}): 3372, 3088, 2961, 1566, 1388.

hhptAc

Yield 91%; ^1H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, 3H, *J*= 7.0, CH_3), 1.01 (t, 3H, *J*= 7.2, CH_3), 1.31 (m, 6H, $3CH_2$), 1.89 (s, 3H, CH_3COO^-), 2.03 (m, 4H, $2CH_2$), 4.57 (t, 2H, *J*= 7.5, CH_2), 4.59 (t, 2H, *J*= 7.2, CH_2), 4.91 (s, 2H, CH_2OH), 9.24 (s, 1H, H-triazole); ^{13}C NMR (125 MHz, $CDCl_3$) δ 10.9, 14.0, 22.5, 22.7, 24.8, 25.9, 29.4, 31.1, 52.4, 53.3, 54.0, 130.4, 145.0, 177.8; MALDI-TOF MS m/z, calcd for [M]+ 226.1918, found 226.1928; IR νmax (cm^{-1}): 3350, 3111, 2927, 1565, 1391.

dhptAc

Yield 86%; ^1H NMR (500 MHz, $CDCl_3$) δ 0.86 (t, 3H, *J*= 7.1, CH_3), 0.99 (t, 3H, *J*= 7.3, CH_3), 1.23 (m, 14H, $7CH_2$), 1.86 (s, 3H, CH_3COO^-), 1.99 (m, 4H, $2CH_2$), 4.55 (m, 4H, $2CH_2$), 4.88 (s, 2H, CH_2OH), 9.09 (s, 1H, H-triazole); ^{13}C NMR (125 MHz, $CDCl_3$) δ 11.0, 14.3, 22.7, 22.8, 24.5, 26.3, 29.0, 29.4, 29.5, 29.5, 29.6, 32.0, 52.4, 53.3, 54.1, 130.3, 144.8, 178,0; MALDI-TOF MS m/z, calcd for [M]+ 282.2545, found 282.2559.

5.2 Biological Assays

5.2.1 Reagents

Miltefosine was supplied by Cayman Chemical Company (Michigan, USA). Propidium Iodide (PI), 9-diethylamino-5H-benzo[alpha] phenoxazine-5-one (Nile Red), monodansylcadaverin (MDC), poly-L-lysine, Hank´s Balanced Sal Solution (HBSS), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), geneticin (G418), dimethylsulfoxide (DMSO), hemin, saponin, folic acid and 3-[4,5-dimethylthiazol-2-yl]-2,5-100 diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), RPMI 1640 medium, penicillin G and streptomycin were purchased from Cultilab (Campinas, São Paulo, Brazil); brain heart infusion (BHI) was purchased from Himedia (Mumbai, Indian). The terminal deoxyribonucleotidyltransferase (TdT)-mediated dUTP nick end labelling (TUNEL) kit was supplied by Promega, Madison, WI, USA. Annexin V-FITC was obtained from Invitrogen (Eugene, OR, USA). 4',6-Diamidino-2-Phenylindole, Dihydrochloride (Dapi) was acquired from Santa Cruz Biotechnology (Dallas, TX, USA).

5.2.2 Parasite culture

Promastigotes of *L. amazonensis* wild-type (IFLA/BR/1967/PH8), *L. amazonensis* (IFLA/BR/1967/PH8) transfected with the gene of red fluorescent protein (RFP) [40] and *L. amazonensis* (WHOM/BR/75/Josefa) transfected with the gene of green fluorescent protein (GFP) [41] were used. *Leishmania amazonensis*-WT, *L. amazonensis*-GFP and *L. amazonensis*-RFP were used in antipromastigote and antiamastigote assays, and *L. amazonensis*-WT was used in the study of action mechanism. The promastigotes of *L. amazonensis*-WT, *L. amazonensis*-GFP and *L. amazonensis*-RFP were cultured as previously reported [42,43]. The parasites were routinely isolated from cutaneous lesions of infected BALB/c mice and passaged as promastigote forms in culture. All protocols were approved by the Ethical Committee for Animal Research of Federal University of Juiz de Fora (#055/2013 -CEUA).

5.2.3 Antipromastigote assay

The promastigotes of *L. amazonensis*-WT, *L. amazonensis*-GFP and *L. amazonensis*-RFP ($2x10^6$ cells/mL) from logarithmic phase culture were not exposed (negative control) or exposed to an increasing concentration of the compound (1.6-100.0 µM) for 72 h at 25 °C in a BOD incubator. The viability of the promastigotes was determined by using MTT colorimetric assay [42,43]. The results were expressed as the

concentration inhibiting parasite growth by 50% (IC_{50}). Miltefosine (6.2-100.0 µM) was used as an antileishmanial reference drug. Results were obtained from three independent experiments performed in duplicate.

5.2.4 Reversibility effect of dhmtAc on promastigote proliferation

To determine whether the antipromastigote effect of **dhmtAc** was reversible, promastigotes of *L. amazonensis*-WT were incubated with this compound (2.5-20.0 µM) for 72 h at 25 ºC in BOD incubator, and the number of parasites was then counted. The parasites were centrifuged, washed twice in PBS and the number of cells was adjusted ($10x10^6$ cells/mL) and incubated with Warren's medium plus 10% SBF without the compound **dhmtAc.** After 72 h at 25 ºC in BOD incubator, the number of viable promastigotes was counted in a Neubauer chamber [44]. Results were obtained from three independent experiments performed in duplicate.

5.2.5 Viability assay against murine macrophages

Peritoneal macrophages ($2x10^6$ cells/mL) were obtained from mice in a protocol already described [45]. Adherent macrophages were incubated with increasing concentrations of **dhmtAc** (9.4-150.0 µM) or with RPMI 1640 medium for 72 h at 33 ºC and 5% CO_2. Viability of the macrophages was determined by MTT assay. The cytotoxic concentration of the compound to reduce 50% of viable macrophages (CC_{50}) was obtained from three independent experiments performed in duplicate. The Selectivity Index (SI) of this compound was calculated as the ratio between the CC_{50} for macrophages and the IC_{50} against intracellular amastigotes [44]. All protocols were approved by the Ethical Committee for Animal Research (#054/2013-CEUA).

5.2.6 Antiamastigote activity

Murine macrophages obtained according to protocol previously described [45], were infected with promastigotes of *L. amazonensis* in the stationary growth phase and *L. amazonensis*-macrophages were treated with different concentrations of the compound **dhmtAc** (0.78-100.0 µM) diluted in sterile deionized H_2O. The parasite burden was evaluated by counting the intracellular amastigotes, uninfected and infected macrophages (at minimum 200 macrophages) in treated and untreated cultures [45].

The antiamastigote assays using *L. amazonensis*-GFP and *L. amazonensis*-RFP were performed as previously described [42, 43]. The infected macrophages were treated with different concentrations of the compound **dhmtAc** (0.78-100.0 µM) and after 72 h of treatment, the fluorescence intensity of the cultures was measured using a plate-reader fluorometer (FLx800, BioTek Instruments, Inc., Winooski, VT, USA) set at 540 nm excitation/600 nm emission for *L. amazonensis*-RFP [43] and at 485 nm excitation/528 nm emission for *L. amazonensis*-GFP [42]. Miltefosine (1.6-25.0 µM) was used as the reference drug. The results were obtained from three independent experiments performed in duplicate.

5.2.7 Analysis of the reversibility of the effect of dhmtAc on the intracellular parasite proliferation

The macrophages infected with *L. amazonensis*-WT were treated with **dhmtAc** (0.45; 0.9; 1.8 and 3.6 µM) for 72 h at 33 ºC in 5% CO_2. After this time, the infected macrophages were washed twice in PBS and incubated with Warren's medium supplemented with 10% SBF, without the compound, at 25 ºC in a BOD incubator for more 72 h. After, the parasite viability was quantified by counting the viable promastigotes under the microscope [44]. Results were obtained from three independent experiments performed in duplicate. All protocols were approved by the Ethical Committee for Animal Research of Federal University of Juiz de Fora (#0056/2013-CEUA).

5.2.8 Erythrocytes lysis assay

The hemolytic activity was determined by incubation of a suspension of fresh human red blood cells in PBS with different concentrations of dhmtAc (9.4-150.0 µM) as previously described [46].

5.2.9 Determination of mitochondrial membrane potential

The promastigotes of *L. amazonensis*-WT ($1x10^7$ cells/mL) in the exponential phase were untreated or treated with different concentrations starting from IC_{50} of the compound **dhmtAc** (2.5, 5.0, 10.0 or 20.0 µM) for 24 h at 25 °C in a BOD incubator. Cells were harvested, washed, incubated with JC-1 and the fluorescence was spectrofluorometrically measured (FLx800, BioTek Instruments, Inc., Winooski, VT,

USA) at both 485/528 nm and 540/600 nm wavelength [42]. The promastigotes treated with FCCP (20.0 µM) for 30 minutes were utilized as positive control. Relative $\Delta\psi m$ value was quantified using the ratio between the reading at 600 nm and the reading at 528 nm [42].

5.2.10 Detection of reactive oxygen species (ROS) production

The detection of ROS production was done by using the fluorescent probe H_2DCFDA [42]. Briefy, *L. amazonensis*-WT promastigotes in the exponential phase were untreated or treated with compound **dhmtAc** (2.5, 5.0, 10.0 or 20.0 µM) for 24 h at 25 °C in a BOD incubator. Parasite concentration was adjusted to $2x10^7$ promastigotes in PBS and H_2DCFDA was added, incubated for 30 min in dark at room temperature and the ROS generation were measured using an excitation and emission wavelengths of 485 and 528 nm, respectively, in a fluorometer microplate reader [42]. Promastigotes incubated with miltefosine (22.0 µM) were used as positive control.

5.2.11 Detection of formation of lipid droplets

Exponential phase *L. amazonensis*-WT promastigotes ($1x10^7$ cells/mL) were cultured for 24 h in the absence or presence of the compound **dhmtAc** (2.5, 5.0, 10.0 or 20.0 µM), at 25 ºC in a BOD incubator (Fanem, São Paulo, SP, Brazil). For detection of lipids droplets, the parasites were incubated with Nile Red and the fluorescence was spectrofluorometrically measured [47].

5.2.12 Cell membrane integrity

Promastigotes of *L. amazonensis*-WT ($1x10^7$ cells/mL) in the exponential phase were untreated or treated with 2.5, 5.0, 10.0 or 20.0 µM **dhmtAc** for 24 h at 25 °C in BOD incubator. After washed, the parasites were incubated with PI at 1.0 µg/mL for 15 min in dark at room temperature [42]. Fluorescence was spectrofluorometrically measured (excitation wavelength of 540 nm and an emission wavelength of 600 nm). As positive control, cells heated at 65°C for 10 min were used[45]. Alterations in the PI fluorescence were quantified as the percentage of increase in the fluorescence compared with the untreated promastigotes.

5.2.13 Detection of phosphatidylserine exposure by annexin-V-FITC labeling

Exponential phase *L. amazonensis*-WT promastigotes ($1x10^7$ cells/mL) were cultured for 24 h in the absence or presence of 2.5, 5.0, 10.0 or 20.0 µM **dhmtAc** , at 25 ºC in a BOD incubator (Fanem, SP, Brazil). Phosphatidylserine exposure was evidenced using annexin V-FITC and propidium iodide (PI) assay as already described [44]. Promastigotes treated with miltefosine (44.0 µM) were used as positive control. Data acquisition and analysis were performed using a FACsCanto II flow cytometer (Becton Dickinson, Rutherford, NJ, USA) equipped with DIVA software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA). A total of 10,000 events were analysed per sample in the region that was previously established as the one that corresponded to the parasites.

5.2.14 Evaluation of morphology of promastigotes

Promastigotes of *L. amazonensis* ($1x10^7$ cells/mL) in the exponential phase were treated with 2.5, 5.0, 10.0 or 20.0 µM **dhmtAc** for 24 h at 25 °C in BOD incubator. After, the parasites were harvested, resuspended in PBS, smeared on glass slides, fixed with absolute ethanol and stained with Giemsa and visualized using an Olympus BX53 microscopy.

5.2.15 DNA Fragmentation Assay

Exponential phase *L. amazonensis*-WT promastigotes ($1x10^7$ cells/mL) were cultured for 24 h in the absence or presence of 5.0 or 10.0 µM **dhmtAc** at 25 ºC in a BOD incubator (Fanem, São Paulo, SP, Brazil). DNA fragmentation was analysed using a terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) apoptosis detection system, as previously reported [48]. Promastigotes treated with miltefosine (22.0 µM) were used as positive control. The slides were observed using an Olympus BX53 fluorescence microscope, and images were captured with an Olympus camera DP73.

5.2.16 Evaluation of autophagic vacuoles

Leishmania amazonensis-WT promastigotes ($1x10^7$ cells/mL) in the exponential phase were treated with the compound **dhmtAc** (2.5, 5.0, 10.0 and 20.0 µM) or untreated (negative control) for 24 h, at 25 ºC in a BOD incubator (Fanem, SP, Brazil).

After washed, the cell concentration was adjusted to $10x10^6$ cells/mL and incubated in the dark with 100 µM of monodansyl cadaverine (MDC) for 1 h at 25 ºC. The fluorescence was analyzed in a microplate reader using a 335 and 460 nm for excitation and emission, respectively [49]. Promastigotes treated with miltefosine (22.0 µM) were used for comparison.

5.2.17 Statistical Analysis

The 50% inhibitory concentration, i.e., the compound concentration that inhibited 50% of the survival of the *Leishmania* (IC_{50}) and cytotoxic concentration of 50% of macrophages (CC_{50}) were obtained by using GraFit Version 5 software (Erithacus Software Ltd., Horley, U.K). The data presented in the graphs were expressed as the mean ± standard deviation obtained from three independent experiments performed in triplicate. Data were statistically analyzed using One-way-ANOVA followed by Dunnett post-test to compare all the groups to the negative control using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Results were regarded as statistically significant when $p < 0.0001$ (***), $p < 0.001$ (**) and $p < 0.01$ (*).

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Salts of 1,2,3-Triazole as non-classical bioisosteres of miltefosine. Effect on *Leishmania amazonensis* is related to apoptosis and necrosis.

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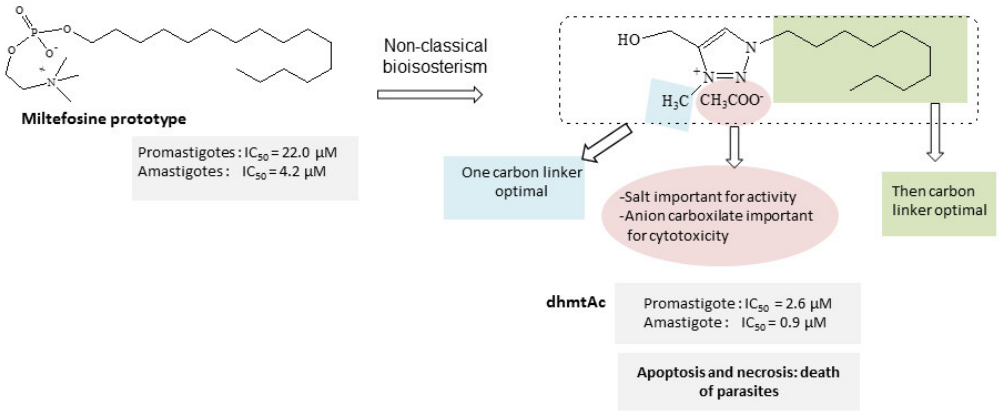

Highlights

Some new non-classical bioisoteres of miltefosine were synthesized and assayed against *Leishmania amazonensis*.

DhmtAc, with an acetate anion and a side chain of 10 carbon atoms, showed selective biological activity.

DhmtAc was more effective than the miltefosine prototype.

The anti-leishmanial activity is mediated via apoptosis and necrosis**.**