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TREATMENT AND PROPHYLAXIS - ORIGINAL PAPER

Biological activity of Morita-Baylis-Hillman adduct homodimers in *L. infantum* and *L. amazonensis*: anti-*Leishmania* activity and cytotoxicity

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Abstract

This study is a report on the anti-*Leishmania* activity of Morita-Baylis-Hillman (MBH) homodimers adducts against the promastigote and axenic amastigote forms of *Leishmania* (*Leishmania*) *infantum* and *Leishmania* (*Leishmania*) *amazonensis* and on the cytotoxicity of these adducts to human blood cells. Both studied homodimers, MBH 1 and MBH 2, showed activity against the promastigote forms of *L. infantum* and *L. amazonensis*, which are responsible for visceral and cutaneous leishmaniasis, respectively. Additionally, the homodimers presented biological activity against the axenic amastigote forms of these two *Leishmania* species. The adducts exhibited no hemolytic activity to human peripheral blood mononuclear cells or erythrocytes at the tested concentrations and achieved higher selectivity indices than amphotericin B. Evaluation of cell death by apoptosis revealed that the homodimers had better apoptosis/necrosis profiles than amphotericin B in the promastigote forms of both *L. infantum* and *L. amazonensis*. In conclusion, these Morita-Baylis-Hillman adducts had anti-*Leishmania* activity in an in vitro model and may thus be promising molecules in the search for new drugs to treat leishmaniasis.

Keywords *Leishmania* spp. · Anti-*Leishmania* activity · Treatments · Morita-Baylis-Hillman

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Introduction

Leishmaniasis is a complex of tropical endemic diseases caused by parasites of the genus *Leishmania* that are classified phylogenetically in the order *Kinetoplastida* and the family *Trypanosomatidae* (Akhoundi et al. 2016). The diseases in the complex are considered neglected tropical diseases and are endemic in 98 countries, with over 700,000 new cases worldwide and a mortality rate of 20,000 people per year (WHO 2017). A wide spectrum of cutaneous forms of leishmaniasis exists, including nonulcerative, ulcerative, and nodular forms. Additionally, leishmaniasis may reach mucosal and cartilage tissues in its mucocutaneous form, leading to increased morbidity associated with cutaneous disease (Chacon-Vargas et al. 2017). The species *Leishmania* (*Leishmania*) *infantum* and *Leishmania* (*Leishmania*) *amazonensis* are associated with the visceral and cutaneous manifestations of leishmaniasis, respectively, in Latin America (Scorza et al. 2017).

The drugs currently used to treat leishmaniasis are expensive and highly toxic with numerous side effects. Additionally, some patients may present drug resistance during treatment (Singh et al. 2016). Pentavalent antimonials such as meglumine antimoniate (Glucantime®) and sodium stibogluconate (Pentostan®) have been the first-line medications for leishmaniasis treatment since 1945. These drugs are parenterally administered to patients and cause local and systemic side effects (Mendonça et al. 2017). When patients do not respond adequately to these first-line drugs, second-line drugs, such as amphotericin B, pentamidine, paromomycin, and miltefosine, must be used. Unfortunately, second-line drugs also have drawbacks, such as toxicity and high costs (Torres-Guerrero et al. 2017). Considering the number of individuals who are infected or are at risk of infection, the search for novel compounds to treat leishmaniasis is justified by the negative effects of the currently available drugs.

Morita-Baylis-Hillman (MBH) adducts are produced by a coupling reaction between alkenes (or alkynes) and aldehydes, with tertiary amines as catalysts (Sousa et al. 2017). This reaction is an important chemical transformation process that enables the synthesis of new molecules with promising biological activity (Da Silva Wagner et al. 2016). Many pharmacological properties of MBH adducts have been reported, such as antifungal and antibacterial activity (Narender et al. 2006) and anti-inflammatory activity (Faheina-Martins et al. 2017), and MBH adducts are promising drugs for treating neglected diseases such as leishmaniasis (Souza et al. 2007; Lima-Junior et al. 2009; Junior et al. 2010; Barbosa et al. 2011; Silva et al. 2011; Xavier et al. 2016), schistosomiasis (Vasconcellos et al. 2006), malaria (Narender et al. 2005), and Chagas disease (Sandes et al. 2010, 2014).

In a previous study, our research group synthesized two new MBH adducts, MBH 1 and MBH 2 (Fig. 1), and assessed their anti-*Leishmania* activity against *L. donovani* (Da Silva Wagner et al. 2016). Given that *L. infantum* and *L. amazonensis* are the parasites responsible for the visceral and cutaneous clinical forms of leishmaniasis, respectively, it is important to determine whether MBH 1 and MBH 2 can be used to treat infection by these species.

In this context, the present study evaluated the anti-*Leishmania* activity of these two MBH homodimers (MBH 1 and MBH 2) against the promastigote and axenic amastigote forms of *L. infantum* and *L. amazonensis*. In addition, the effects of these homodimers on human peripheral blood mononuclear cells (PBMCs) and erythrocytes were evaluated to provide a better understanding of their cytotoxicity. Our results indicate that cell death by apoptosis should be deeply investigated in order to understand better the mechanisms about cell death process caused by MBH in *Leishmania* species.

Materials and methods

Morita-Baylis-Hillman adducts

Two MBH adducts (MBH 1 and MBH 2; Fig. 1) were synthesized according to the protocol described by Da Silva Wagner et al. (2016) and were diluted in dimethylsulfoxide (DMSO) to obtain stock solutions (20 mg/mL). To obtain the final concentrations of the drugs in the assays, the stock solutions were diluted at least two times in culture medium, and the final concentration of DMSO in the highest concentration–working solution of each adduct was below 1%. Therefore, the final concentration of DMSO in each working solution was below the threshold for *Leishmania* toxicity.

Ethics statement

All experiments were performed in compliance with the relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki. Written informed consent was obtained from patients, and the study was approved by the Ethics Committee of the Federal University of Paraíba (process number: 2.560.067 and CAAE: 82944118.5.0000.5188).

Leishmania culture conditions

The promastigote forms of *L. infantum* (MHOM/BR/2008/RN-05) and *L. amazonensis* (IFLA/BR/1967/PH8) were maintained in vitro at 26 °C and pH 7.0 in Schneider's medium supplemented with 20% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/L streptomycin. Extracellular axenic amastigote forms of *L. infantum* and *L. amazonensis* were obtained based on the methods described

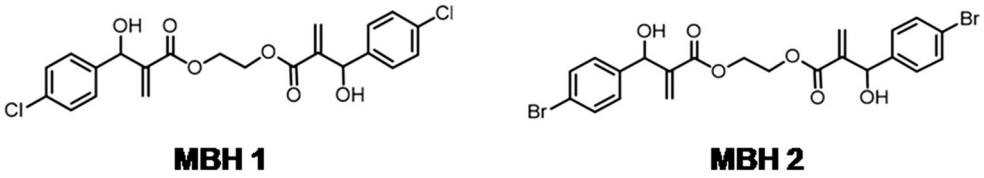

Fig. 1 Chemical structures of the MBH adducts

by Debrabant et al. (2004) and Ueda-Nakamura et al. (2006), respectively. Promastigote forms in the stationary growth phase were differentiated into axenic amastigotes by pH (pH 5.5) and temperature (37 °C for *L. infantum* and 32 °C for *L. amazonensis*) treatment.

Anti-*Leishmania* activity

A promastigote growth inhibition assay was performed as previously described by Rodrigues et al. (2015). Briefly, promastigotes (1×10^6 cells per well) were harvested in the exponential growth phase and incubated in Schneider's medium (96-well plates) in the presence or absence of different concentrations (400, 200, 100, 50, 25, 12.5, 6.25, and 3.13 μg/mL) of MBH adducts (MBH 1 and MBH 2) or reference drugs such as meglumine antimoniate and amphotericin B. The plates were incubated at 26 °C for 72 h in a biological oxygen demand (BOD) incubator. The inhibitory effects of the drugs were then evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. First, 10 μL of MTT (5 mg/mL; Amresco, OH, USA) was added, and after 4 h of incubation, 10% sodium dodecyl sulfate (SDS) was added to dissolve the formazan crystals. The absorbance at 540 nm was measured using a plate reader (Biosystems model ELx800; Curitiba, Paraná, Brazil). The viability of *L. infantum* and *L. amazonensis* axenic amastigotes after exposure to different concentrations (400, 200, 100, 50, 25, 12.5, 6.25, 3.13 μg/mL) of MBH 1, MBH 2, or the reference drugs (meglumine antimoniate and amphotericin B) was evaluated by MTT assay as described for the promastigote form, with three modifications: first, the treatment time was 24 h for both species; second, the test temperature and pH for *L. infantum* were 37 °C and 5.5, respectively; and third, the test temperature and pH for *L. amazonensis* were 32 °C and 5.5, respectively. After statistical analysis, the 50% inhibitory concentration (IC_{50}) and 50% effective concentration (EC_{50}) values were calculated using the probit regression model (SPSS program, version 13.0, Chicago, IL, USA).

In vitro cytotoxicity to peripheral blood mononuclear cells

PBMCs (peripheral blood mononuclear cells) were obtained from blood samples from healthy volunteers ($n = 9$) using a Ficoll gradient (Maciel et al. 2014), and cell viability was assayed by MTT assay. Approximately 1×10^6 PBMCs per well were seeded into 96-well plates containing RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 mg/L streptomycin in the presence (at 1 × or 4 × the half-maximal inhibitory concentration (IC_{50}) for *L. infantum*) or absence of MBH 1, MBH 2, or amphotericin B at 37 °C and 5% CO_2 for 24 h. The choice to use concentrations of 1 × and 4 × IC_{50} for *L. infantum* to evaluate PBMC toxicity was based on the premise that these concentrations of adducts are capable of killing both *L. infantum* and *L. amazonensis* but should not negatively affect human cells. After incubation, cytotoxicity was evaluated by adding 10 μL of MTT (5 mg/mL), incubating the cells for 4 h, adding SDS (10%, *w*/*v*), and measuring the absorbance at 540 nm using a plate reader (Biosystems model ELx800).

Erythrocyte lysis assay

The hemolytic activity of MBH 1, MBH 2, meglumine antimoniate, and amphotericin B was determined using human erythrocytes ($n = 9$) according to the method described by Jain et al. (2015). Briefly, 80 μL of a 5% erythrocyte/phosphate-buffered saline (PBS) suspension was mixed with 20 μL of a series of concentrations (400, 200, 100, 50, 25, 12.5, 6.25, 3.13 μg/mL) of adducts or reference drugs. After incubation at 37 °C for 1 h, 200 μL of PBS (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136.9 mM NaCl, and 2.6 mM KCl, pH 7.2) were added to stop hemolysis, and the samples were centrifuged for 10 min at 1000×*g*. The supernatants were collected, and hemolysis was measured spectrophotometrically at 540 nm. The percent hemolysis was determined as $[(Abs_{sam}–Abs_{con})/(Abs_{tot}–Abs_{con}) \times 100]$, where Abs_{sam} is the absorbance of the sample, Abs_{con} is the absorbance of the blank control (without drugs), and Abs_{tot} is the absorbance of a sample with total hemolysis (suspension solution replaced with an equal volume of Milli-Q water). After statistical analysis, the 50% haemolytic concentration (HC_{50}) values were calculated using the probit regression model (SPSS program, version 13.0, Chicago, IL, USA).

Apoptosis/necrosis profiling with annexin V/PI staining

The promastigote forms of *L. infantum* and *L. amazonensis* (1×10^6) in the logarithmic growth phase were incubated with

MBH adducts or amphotericin B at 1 × or 4 × IC_{50} values for 24 h. After incubation, the promastigotes were washed three times in cold PBS, resuspended in binding buffer (10-mM HEPES, 140-mM NaCl, and 2.5-mM $CaCl_2$, pH 7.4), and stained using a FITC Annexin V/Dead Cell Apoptosis Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. The stained cells were diluted in annexin V binding buffer (BD Biosciences, San Jose, CA, USA), and the suspended cells were subjected to flow cytometry. Annexin V-FITC/propidium iodide (PI)–stained cells were analyzed using a BD FACSCanto® II flow cytometer (BD Biosciences, San Jose, CA, USA). In total, 30,000 cells were analyzed per measurement. The data were analyzed using FlowJo 10.0.7 software (TreeStar Inc., Ashland, OR, USA).

Calculation of selectivity index and data analysis

Selective index (SI) of the MBHs and amphotericin B were calculated by the dividing the 50% haemolytic concentration (HC_{50}) by the half-maximal inhibitory (IC_{50}), for the promastigote form, or half-maximal effective concentration (EC_{50}), for the amastigote form. One-way ANOVA followed by Tukey's test was used to analyze differences between groups with a p value < 0.05 as the threshold for statistical significance. GraphPad Prism® software version 6.0 and SPSS version 13.0 (GraphPad Software, San Diego, CA, USA) were used for statistical analysis.

Results

Anti-*Leishmania* activity of MBH 1 and MBH 2

The anti-*Leishmania* activity of MBH 1 and MBH 2 was evaluated in promastigote and axenic amastigote forms of *L. infantum* and *L. amazonensis*. For *L. infantum* axenic amastigotes, the IC_{50} values for MBH 1, MBH 2 and amphotericin B were 5.6 ± 1.5 μg/mL (12.4 μM), 5.3 ± 1.1 μg/mL (9.9 μM), and 0.372 ± 0.1 μg/mL (0.40 μM), respectively, and the EC_{50} values were 9.08 ± 1.3 μg/mL (20.2 μM), 10.76 ± 1.1 μg/mL (20.0 μM), and 1.6 ± 0.26 μg/mL (1.73 μM), respectively. The IC_{50} values for *L. amazonensis* promastigotes were 13.2 ± 3.5 μg/mL (29.3 μM), 13.4 ± 3.5 μg/mL (24.9 μM), and 0.722 ± 0.6 μg/mL (0.78 μM) for MBH 1, MBH 2, and amphotericin B, respectively, whereas the EC_{50} values for the axenic amastigote forms were 13.4 ± 4.2 μg/mL (29.8 μM), 10.4 ± 3.2 μg/mL (19.3 μM), and 0.719 ± 0.1 μg/mL (0.78 μM), respectively.

Cytotoxicity of MBH 1 and MBH 2 to human blood cells

The adducts were less toxic to human erythrocytes and PBMCs than to the promastigote and axenic amastigote forms of *L. infantum* and *L. amazonensis*. Hemolytic assays demonstrated that MBH 1 and MBH 2 did not exhibit toxicity to human erythrocytes, as the HC_{50} values were greater than 400 μg/mL. Amphotericin B, the reference drug, had an HC_{50} of 10.72 ± 0.7 μg/mL. Selectivity index of MBH 1 and MBH 2 were 2.47- and 2,63-folds higher than amphotericin B (Table 1). MBH 1 and MBH 2 at 22.4 and 21.2 μg/mL, respectively, which is 4 × the IC_{50}, were not toxic to PBMC, whereas amphotericin at 1.48 μg/mL was toxic to PBMC.

Apoptosis/necrosis profiling in *L. infantum* and *L. amazonensis* promastigotes treated with MBH 1, MBH 2, or amphotericin B

Flow cytometry with annexin V-FITC and PI labeling was used to identify cell death stages in parasites exposed to MBH 1 or MBH 2. These markers can identify cells are neither undergoing apoptosis nor necrosis (annexin V-/PI-), cells undergoing early apoptosis (annexin V+/PI-), cells undergoing late apoptosis or early necrosis (annexin V+/PI+), and cells undergoing necrosis (annexin V-/PI +) according to the loss of plasma membrane integrity (Misra et al. 2008). After exposure to MBH adducts at either concentration, the percentage of *L. infantum* promastigote cells in the initial apoptotic stage was not different from that of control cells (Fig. 2). Treatment with MBH 2 at either concentration increased the percentage of promastigotes in the late stage of apoptosis. However, this result did not occur with MBH 1 treatment. No differences in necrotic cell death were observed in the treated groups compared with the control group, and the reference drug amphotericin B did not increase the percentages of cells undergoing early apoptosis and necrosis compared with the control treatment. However, the reference drug significantly increased the number of cells undergoing late apoptosis at the 4× IC_{50} concentration (Table 2). No differences were observed in cell death rate or the percentage of cells in the initial apoptotic stage after treatment with MBH 1, MBH 2, or amphotericin B (Table 2). On the other hand, treatment with MBH 2 at the 4 × IC_{50} concentration increased the percentage of cells in the late stage of apoptosis compared with the other drug treatments and the control treatment. A similar cell death profile was observed for treatment with amphotericin B. Furthermore, the percentage of cells undergoing late apoptosis was increased more by MBH 2 treatment than by MBH 1 treatment at both tested concentrations.

Representative dot plots (Fig. 3) show the distribution of annexin V-FITC/PI-stained *L. amazonensis* promastigotes after treatment with MBH 1, MBH 2, and amphotericin B. A

Table 1 Anti-*Leishmania* activity, cytotoxic effects against human blood cells and the selectivity indexes of MBH 1, MBH 2 and amphotericin B

	Cytotoxicity	Anti-*Leishmania* activity							
	Erythrocytes	Promastigotes				Axenic amastigotes			
		L. infantum		*L. amazonensis*		*L. infantum*		*L. amazonensis*	
Compounds	HC_{50} (μg/mL)	IC_{50} (μg/mL)	SI_{rb}	IC_{50} (μg/mL)	SI_{rb}	EC_{50} (μg/mL)	SI_{rb}	EC_{50} (μg/mL)	SI_{rb}
MBH 1	> 400	5.6	> 71.4	13.2	> 30.3	9.08	> 44.05	13.4	> 29.9
MBH 2	> 400	5.3	> 75.8	13.4	> 29.8	10.76	> 37.17	10.4	> 38.5
Amphotericin B	10.72 ± 0.7	0.372	28.82	0.722	14.72	1.6	6.7	0.719	15.1

SI_{rb} (selectivity index for erythrocytes) = HC_{50}/IC_{50} or HC_{50}/EC_{50}

higher percentage of cells in early apoptosis was observed among cells treated with 4× IC_{50} MBH 1 or MBH 2 than among control cells. Similarly, the percentage of cells in late apoptosis was higher among cells treated with 4× IC_{50} MBH 1 or MBH 2 than among control cells. No differences in necrosis rate were observed among treatments. However, the

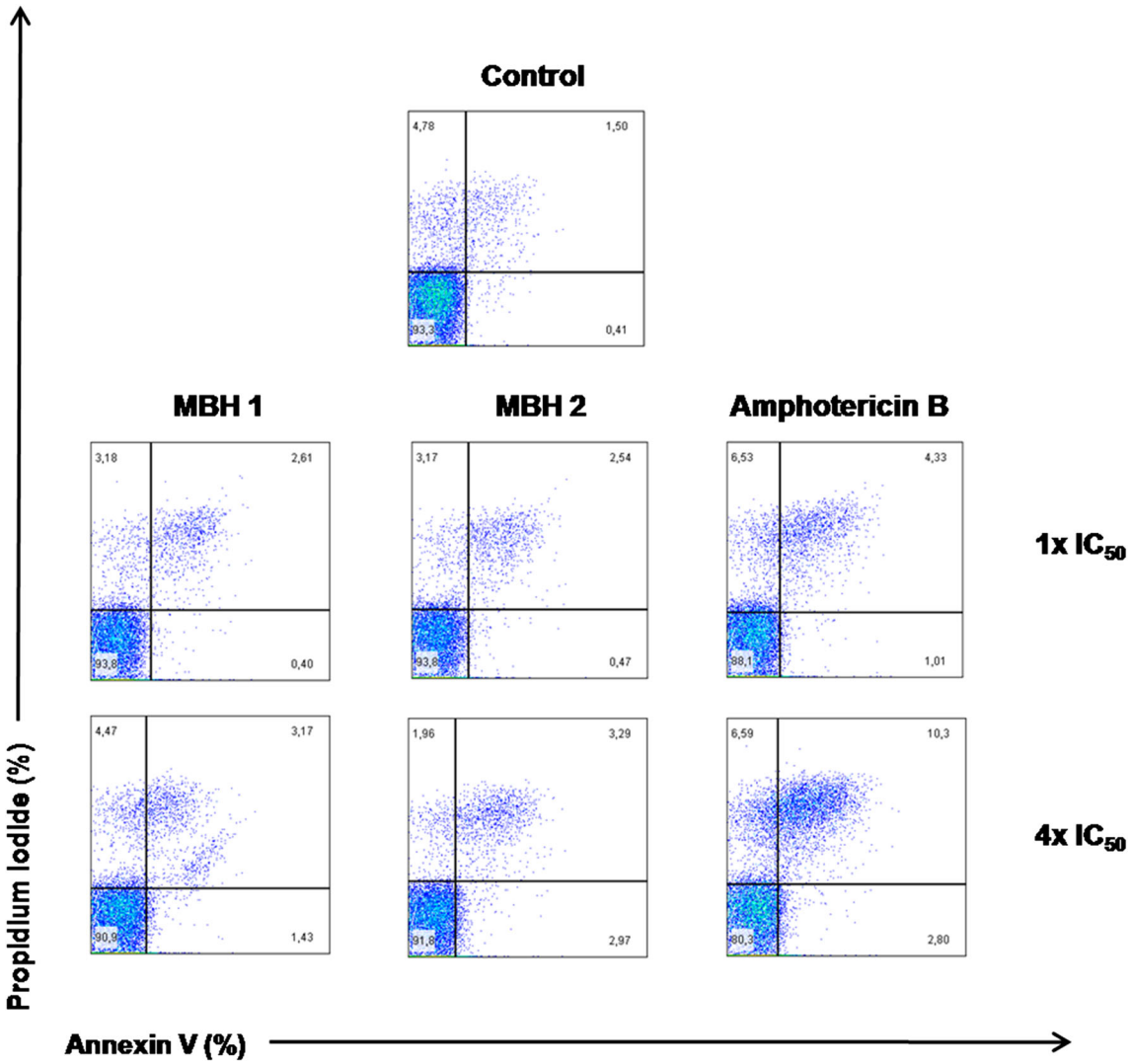

Fig. 2 Representative dot plots showing staining of *L. infantum* promastigotes. *L. infantum* promastigotes were incubated at 26 °C for 24 h in the absence or presence of MBH adducts or amphotericin B at concentrations of 1× or 4× IC_{50}. Annexin V/propidium iodide staining was performed, and the samples were analyzed by flow cytometry

Table 2 Flow cytometry analysis of *L. infantum* promastigotes after treatment with MBH 1, MBH 2, or amphotericin B

	Cells (%)		
	Annexin	Annexin/PI	PI
Control	0.43 ± 0.02	1.55 ± 0.05	3.94 ± 0.85
MBH 1 1× IC_{50}	0.38 ± 0.04	2.77 ± 0.08[d,e]	3.26 ± 0.06
MBH 1 4× IC_{50}	1.62 ± 0.75	2.98 ± 0.31	3.20 ± 0.85
MBH 2 1× IC_{50}	0.33 ± 0.09	5.11 ± 0.40[b,c]	2.95 ± 0.35
MBH 2 4× IC_{50}	2.61 ± 0.37	10.60 ± 0.30[b,d,e]	3.00 ± 1.04
Amphotericin B 1× IC_{50}	3.02 ± 1.12	2.12 ± 0.37[d]	8.03 ± 2.77
Amphotericin B 4× IC_{50}	1.84 ± 0.96	4.01 ± 0.72[a]	6.45 ± 0.14

Percentage of cells positive for annexin V and/or propidium iodide (PI). The results are presented as the mean ± SEM of three experiments performed in triplicate

[a] $p < 0.01$ vs. control; [b] $p < 0.001$ vs. control; [c] $p < 0.05$ vs. amphotericin B 1× IC_{50}; [d] $p < 0.05$ vs. amphotericin B 4× IC_{50}; [e] $p < 0.01$ vs. MBH 2 1× IC_{50}; [f] $p < 0.01$ vs. MBH 2 1× IC_{50}

percentage of cells in the late apoptotic and necrotic stages was substantially higher among cells treated with 1× IC_{50} amphotericin B than among control cells, and the percentage of cells in late apoptosis was higher among cells treated with 4× IC_{50} amphotericin B than among control cells. However, initial apoptosis levels were not different between amphotericin B-treated cells and control cells (Table 3).

After comparing treated cells with control cells, we evaluated the differences in apoptosis/necrosis among the cells treated with MBH 1, MBH 2, and amphotericin B (Table 3). The percentage of cells in early apoptosis was higher after treatment with 4× IC_{50} MBH 1 or MBH 2 than after treatment with 1× IC_{50} MBH 1 or MBH 2. In addition, MBH 2 and amphotericin B both increased the percentage of cells in the initial apoptotic phase at concentrations of 4× IC_{50}. Both MBH adducts elicited higher percentages of cells in late apoptosis at 4× IC_{50} than at 1× IC_{50}. Amphotericin B at 1× and 4× IC_{50} induced higher percentages of cells in late apoptosis than did MBH 1 and MBH 2 at the same concentrations. Furthermore, amphotericin B induced more necrosis at 1× IC_{50} than did MBH 1 and MBH 2 at the same concentration.

Discussion

Several studies have shown the pharmacological activity of MBH adducts (Junior et al. 2010; Barbosa et al. 2011; Sandes et al. 2014; Xavier et al. 2016; Faheina-Martins et al. 2017), including their activity against the promastigote forms of *L. amazonensis* (Souza et al. 2007; Silva et al. 2011; Xavier et al. 2016), *L. chagasi* (Lima-Junior et al. 2009; Barbosa et al. 2011), and *L. braziliensis* (Amorim et al. 2013; Xavier et al. 2014). The adducts studied here (MBH 1 and MBH 2) also show activity against the promastigote forms of *L. donovani* (Da Silva Wagner et al. 2016). Therefore, MBH adducts represent a class of promising bioactive compounds against *Leishmania* strains.

The current study on MBH 1 and MBH 2 began with an assessment of their biological potential to inhibit the growth of *L. infantum* and *L. amazonensis* promastigotes. Our results showed that MBH 1 and MBH 2 had different IC_{50} values for anti-*Leishmania* activity against the promastigote forms of *L. infantum* and *L. amazonensis* (Table 1). *L. infantum* causes visceral leishmaniasis in the New World, while *L. amazonensis* is responsible for the cutaneous form of the disease (Torres-Guerrero et al. 2017). The investigation of these two adducts is justified because they were tested against two different species of *Leishmania* with molecular diversity that may influence final treatment responses.

In addition to their effects on promastigote forms, the effects of the adducts on axenic amastigote forms obtained from alternative in vitro methods were assessed (Rodrigues et al. 2015). Given the EC_{50} values obtained in the present study, MBH 1 and MBH 2 show promising pharmacological potential as anti-*Leishmania* drugs to treat infections caused by *L. infantum* and *L. amazonensis*. MBH adducts also have anti-*Leishmania* activity against amastigote forms of *L. braziliensis* that parasitize murine macrophages (Amorim et al. 2013). However, the literature lacks information on the effects of MBH adducts on the axenic amastigote form of *Leishmania.* Such information is important to understand whether these drugs can in fact kill the parasites in their amastigote form. Our study shows that MBH 1 and MBH 2 are indeed capable of killing the amastigote form of *L. infantum* and *L. amazonensis* in vitro.

The effects of the adducts on parasite cell death were compared with those of the reference drug amphotericin B, which shows marked anti-*Leishmania* activity in vitro against promastigotes and axenic amastigotes. As expected, amphotericin B presented better anti-*Leishmania* promastigote and axenic amastigote activity than the MBH 1 and MBH 2 adducts. However, amphotericin B treatment causes many side effects in patients with leishmaniasis (Barbosa et al. 2011). Therefore, it is important to discover new drugs without these side effects.

Toxicity assays are essential for any new drug, and we have shown that MBH adducts are not toxic to human PBMCs at the concentrations evaluated. These results are important because they demonstrate that the adducts were not toxic to human PBMCs at the assessed concentrations and indicate that MBH 1 and MBH 2 target parasite cells.

Amorim et al. (2013) found that one MBH adduct exhibited 3.6-fold greater toxicity to *L. braziliensis* promastigotes than to murine macrophages. Additionally, another study observed low cytotoxicity of racemic and pure MBH enantiomers to murine macrophages compared with their effects on

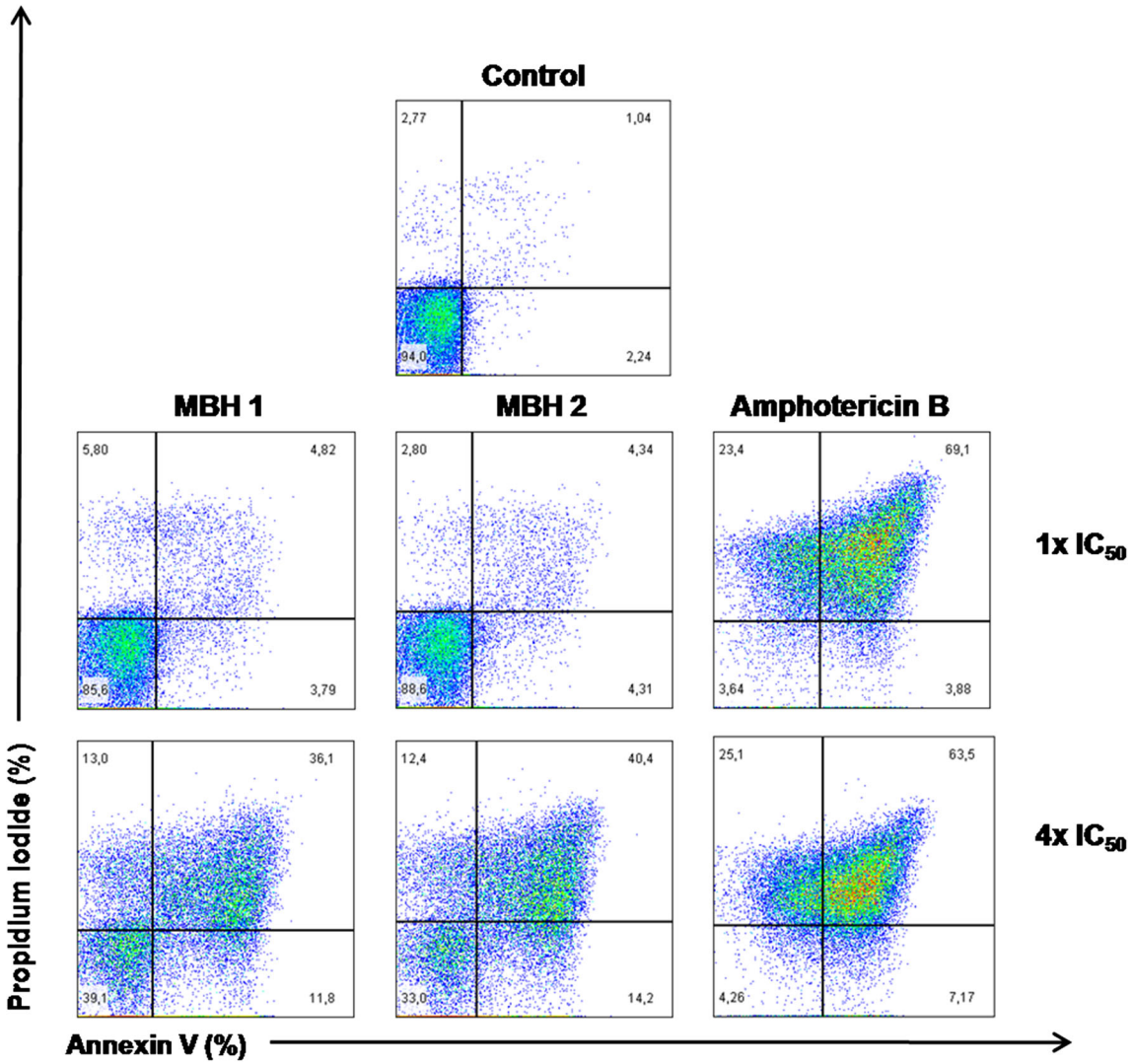

Fig. 3 Representative dot plots showing staining of *L. amazonensis* promastigotes. *L. amazonensis* promastigotes were incubated at 26 °C for 24 h in the absence or presence of MBH adducts or amphotericin B at concentrations of 1× or 4× IC_{50}. Annexin V/propidium iodide staining was performed, and the samples were analyzed by flow cytometry

L. braziliensis promastigotes (Xavier et al. 2014). Some authors have reinforced the importance of evaluating drug cytotoxicity to macrophages and monocytes when studying biological anti-*Leishmania* activity because these are the main cells parasitized by *Leishmania* spp. in humans (Tripathi et al. 2007).

On the other hand, the safety of a substance must also be evaluated for nontarget cells such as erythrocytes. Such evaluation involves assessment of the effects of these substances on the mechanical stability of the erythrocyte plasma membrane. The tested substances may generate changes in the membrane, leading to cell death due to permeability-altering modifications such as pore formation or total membrane rupture (Costa-Lotufo et al. 2002). The high HC_{50} values for MBH 1 and MBH 2 obtained in the current study indicate the safety of these compounds in vitro. Additionally, the actual values could be even higher because the adducts were not evaluated above concentrations of 400 μg/mL. This finding may well indicate a superior level of safety for human erythrocytes. The reference drug amphotericin B showed almost tenfold greater toxicity to human erythrocytes than the MBH adducts in this study, demonstrating that the adducts were not harmful to erythrocytes. Furthermore, MBH 1 and MBH 2 showed high selectivity for *L. infantum* and *L. amazonensis*, as revealed by their SI, in relation to their effect on human erythrocytes. Comparison of SIs reveals the safety levels of the compounds because the SI is the ratio between the cytotoxicity to mammalian cells (HC_{50}) and the anti-*Leishmania* activity (IC_{50} or EC_{50}). The safety levels of the MBH adducts were higher than that of the reference drug amphotericin B.

However, current understanding of anti-*Leishmania* action mechanisms is very rudimentary, especially concerning

Table 3 Flow cytometry analysis of *L. amazonensis* promastigotes after treatment with MBH 1, MBH 2, or amphotericin B

	Cells (%)		
	Annexin	Annexin/PI	PI
Control	2.29 ± 0.14	1.09 ± 0.09	2.66 ± 0.16
MBH 1 1× IC_{50}	5.85 ± 1.07	6.88 ± 1.82[f]	5.03 ± 1.06
MBH 1 4× IC_{50}	12.57 ± 0.71[b,c]	35.03 ± 0.67 [b,c,e]	11.53 ± 0.87
MBH 2 1× IC_{50}	3.54 ± 0.57	3.90 ± 0.51	2.81 ± 0.06
MBH 2 4× IC_{50}	12.17 ± 1.10 [b,d]	37.17 ± 1.70 [b,d,e,f]	12.83 ± 1.03
Amphotericin B 1× IC_{50}	4.61 ± 2.27	74.03 ± 5.08 [b]	18.22 ± 6.36 [a,c,d]
Amphotericin B 4× IC_{50}	5.99 ± 0.83[e]	69.90 ± 3.30 [b]	20.73 ± 2.67

Percentage of cells positive for annexin V and/or propidium iodide (PI). The results are presented as the mean ± SEM of three experiments performed in triplicate

[a] $p < 0.01$ vs. control; [b] $p < 0.001$ vs. control; [c] $p < 0.05$ vs. MBH 1 1× IC_{50}; [d] $p < 0.05$ vs. MBH 2 1× IC_{50}; [e] $p < 0.05$ vs. amphotericin B 4× IC_{50}; [f] $p < 0.05$ vs. amphotericin B 1× IC_{50}

adducts derived from the MBH reaction. One possible mechanism of toxicity to parasite cells could involve connection of the two unsaturated α, β-carbonyl portions of the homodimers to the proteases of the *Leishmania* parasite via a covalent bridge. Previous studies have classified MBH adducts as agents that attach to thiol groups (-C-SH-) in cysteine residues in proteases. This binding inhibits the metabolic processes of the parasite, leading to its death (Da Silva Wagner et al. 2016). However, this mechanism does not fully explain the bioactivity of these homodimeric molecules. Thus, further studies concerning the mechanisms of MBH adducts toxicity to *Leishmania* species are necessary.

The functions and mechanisms of action may vary according to their chemical structures. Therefore, it is believed that no single biological mechanism applies to all adducts obtained from the reaction from the MBH. Because of the structures of these substances, these adducts may have several therapeutic mechanisms (pharmacodynamics) and metabolic pathways (pharmacokinetics), making it difficult to elucidate the biological mechanisms of the adducts. Thus, in vitro experiments were performed in the present study to understand how MBH 1 and MBH 2 may cause cell death (apoptosis/necrosis). Apoptosis/necrosis profiles were observed through double staining with annexin V-FITC and PI. Annexin V is generally used to verify the presence of phospholipids on the membrane, as it binds with high affinity to phosphatidylserine. This binding is dependent on Ca^{2+} and indicates that cells are in the apoptotic stage. Distinction between apoptotic and necrotic cells is usually made via double staining with annexin V-FITC and PI. PI is a DNA intercalator that crosses only damaged membranes (Misra et al. 2008). However, it is possible that cells in late apoptosis or necrosis cannot be differentiated using this method since in late necrosis, annexin V may bind to phosphatidylserine on the inner membrane of damaged cells. Our results showed that MBH homodimers adducts behaved differently against the promastigote forms of *L. infantum* and *L. amazonensis*. For *L. infantum*, it was possible to ascertain that only MBH 2 induced cell death through apoptosis at the two concentrations tested. Regarding *L. amazonensis*, both adducts induced apoptosis. Amphotericin B is also known to cause cell death by apoptosis in several *Leishmania* species (Paris et al. 2004). In the study, amphotericin B was used as a positive control; unlike the adducts, the reference drug caused cell death by both apoptosis and necrosis. These results show that the evaluated adducts cause a cell death profile by apoptosis, which may be a possible mechanism of action. Therefore, the results suggest that studies related to adducts of MBH homodimers should be thoroughly investigated using macrophages in an animal model in vivo to better understand the mechanisms on the process of cell death caused by MBH.

Discovery and development of new effective drugs for leishmaniasis treatment are challenging endeavors because of factors such as the different clinical manifestations of the disease, different drug pharmacokinetics, and the resistance of the diseases to current drugs (Andrews et al. 2014). Although the biological action a mechanism of MBH adducts remain to be elucidated, this study has demonstrated the potential anti-*Leishmania* activity of these compounds. Other studies have demonstrated that modifying enzymes such as histone deacetylase and carbonic anhydrase are possible targets of the derivatives of MBH adducts (Andrews et al. 2014). Therefore, MBH adducts may have the potential to advance the development of new medicines for leishmaniasis treatment.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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