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LEISHMANICIDAL ACTIVITY *in vivo* OF A MILTEFOSINE DERIVATIVE IN *Mesocricetus auratus*

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ABSTRACT

Visceral leishmaniasis (VL) is a chronic and systemic disease; if untreated, it can cause death in a large number of cases. The therapy is based on the use of antimonials, which have been used for over 50 years. However, cases of resistance have been reported in some countries. In this context, miltefosine (MIL) was introduced to treat antimonial unresponsive cases. Nonetheless, in recent years MIL unresponsive and relapse cases of VL have increasingly been reported. In the current study, the therapeutic potential of compound 5-(4-(3-methanesulfonatepropyl)-1*H*-1,2,3-triazol-1-yl)dodecyl methanesulfonate (C11), an MIL derivative, was assessed in an experimental VL hamster model. For this purpose, golden hamsters (*Mesocricetus auratus*) were infected with *Leishmania (L.) infantum chagasi* and treated daily for 10 days with C11 and MIL administered orally; in addition, Glucantime (GLU), peritoneal route, were administered at 15, 10, 50 mg/kg body weight/day, respectively. Twenty four hours after the end of treatment the animals were euthanatized; and the specimens were collected to evaluate the relative mRNA expression of cytokines IFN-γ, TNF-α, IL-17, TGF-β, IL-4 and IL-10 in fragments of the spleen and liver; moreover, the parasitism in these organs was evaluated as well as the main histopathological alterations. The C11-treated animals showed greater expression of IL-17 and TNF-α cytokines and reduced expression of IL-10 in the spleen in comparison to the infected untreated group (UTG) ($p < 0.05$). The C11 and GLU groups showed a significant reduction in the IgG levels in comparison to the UTG group ($p < 0.05$). Moreover, the C11-treated animals had fewer parasites in the spleen than the UTG animals (reduction of 95.9%), as well as a greater preservation of white pulp architecture in the spleen than the UTG, GLU and MIL groups ($p < 0.05$). For the liver, the animals from the C11 and MIL groups showed a significant increase in TNF-α relative expression in comparison to the UTG animals, which would explain the increase in the number of granulomas and the reduction in the parasitic load ($p < 0.05$). Combined, these findings indicate that C11 is an interesting compound that should be considered for the development of new drugs against VL, mainly due to its leishmanicidal effect and immunostimulating action.

1. Introduction

Visceral leishmaniasis (VL) is a disseminated infection that affects especially the liver, spleen and bone marrow in humans. Irregular episodes of fever, substantial weight loss, enlargement of the spleen and liver and anemia (which can be severe) are the main symptoms. The mortality rate in developing countries can reach 100% within 2 years when not treated (https://www.who.int/leishmaniasis/burden/en/). Hamsters (*Mesocricetus auratus* [*M. auratus*]) provides a good experimental model because the infection largely correlates with the human manifestation of the disease (*Gupta et al., 2012*).

There are still many gaps in the literature in relation to a specific protective response to *Leishmania*. Anyway, the protection predominantly depends on the mechanisms of cellular immunity based on

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macrophages, which are responsible for capturing the parasite at the site of the infection and presenting it to naive $CD4^+$T cells stimulating their differentiation and proliferation. An efficient presentation of antigens and the production of Interleukin 12 (IL-12) by dendritic cells (CD) lead to differentiation of naive T lymphocytes into Th1-type lymphocytes and to the production of IFN-γ (Khadem and Uzonna, 2014). This cytokine acts on macrophages by activating the enzymes induced by nitric oxide synthase (iNOs or NOs2), which leads to the production of nitric oxide (NO) and the consequent death of the phagocytosed parasites. In turn, the cytokine TNF-α is produced by macrophages, and it synergizes with IFN-γ, increasing the activation of iNOs (Maran et al., 2016).

Among the cytokines related to susceptibility, Interleukin 10 (IL-10) was identified in several cases from human VL through analysis of mRNA expression in the bone marrow, lymph nodes and spleen, as well as by detection of elevated serum levels (Gautam et al., 2011; Osorio et al., 2014). A response with high production of Th2 standard cytokines, such as Interleukin 4 (IL-4), Interleukin 5 (IL-5), IL-10 and Interleukin 13 (IL-13), in addition to polyclonal activation of B cells, can result in hyperglobulinemia, which would characterize susceptibility to disease (Guo et al., 2002; Osorio et al., 2014; Saha et al., 2011). TGF-β is also an important cytokine for VL susceptibility. Murine models have shown that TGF-β inhibits NO production by macrophages; thus, it has an important role in modulating the immune response (Rodrigues et al., 2009). Interleukin 17 (IL-17) is an inflammatory cytokine that plays a protective role against intracellular parasites. In VL, the presence of IL-17 has been correlated with resistance to the disease (Ghosh et al., 2013). According to Nascimento et al. (2015), IL-17 is present in the immune response of patients with VL, and their levels decrease with successful treatment. Thus, a positive regulation of the Th1 response is extremely important in order to obtain satisfactory results in the therapy to combat the parasite.

The therapeutic arsenal currently used for the treatment and control of leishmaniasis is restricted, and it has many limitations. Pentavalent antimonials, which are the first-choice drugs for the treatment of leishmaniasis, have been used for more than 50 years. These drugs have low oral absorption; thus, they are administered intramuscularly or intravenously. They also have high toxicity, which leads to the persistence of side effects as well as cases of resistance in some countries (Lockwood and Moore, 2010). In turn, miltefosine (MIL), an alkyphosphocoline, has been used since 2005 to treat cutaneous leishmaniasis in cases where there are contraindications or a non-response to pentavalent antimonial drugs (Fernández et al., 2014). To treat VL, the drug was registered in India in 2002, and it has been used due to refractiveness from conventional antimonial treatment (Sundar et al., 2002, 1998). It is the first oral use drug for the treatment of leishmaniasis; it has many benefits, such as a decrease in the need for hospitalization in VL cases, which has generated revenue for the state, a reduction in discomfort from injections, improvements in a patient's quality of life; and increasing adherence to treatment (Castelo Branco et al., 2016). Just as resistance to pentavalent antimonials has emerged, there is a concern regarding MIL due to its indiscriminate use in India. Thus, this drug must have a supervised public distribution system protecting it from irrational use (Sundar and Murray, 2005). However, the MIL is no more in use for treatment of VL because of Liposomal Amphotericin B has showed a better efficacy and safety for treatment of VL than MIL (Sundar et al., 2014). Besides, its effectiveness has not yet been proven for VL species in North and South America (Alvar et al., 2006).

To develop new low cost, effective drugs that have less aggressive adverse reactions to the host, new chemotherapeutic targets from *Leishmania* have been investigated. Thus, analogous compounds of MIL have been synthesised and tested for their leishmanicidal and cytotoxic activities *in vitro*; among the various analogues tested, the 5-(4-(3-methanesulfonatepropyl)-1*H*-1,2,3-triazol-1-yl) dodecyl methanesulfonate (C11) presented the best results to CI_{50}, CC_{50} e IS (Gontijo et al., 2015). Due to these *in vitro* results, the present work evaluated the compound

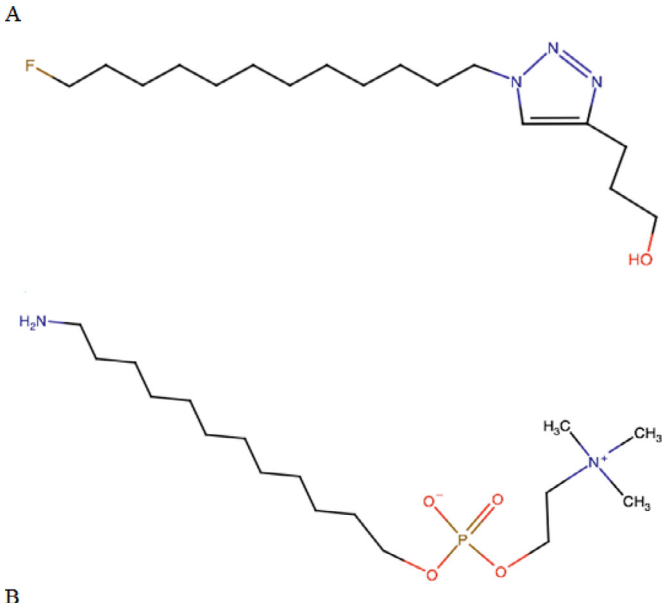

Fig. 1. A) Structure of Compound 11 (Alquiltriazol). Source: (Gontijo et al., 2015); B) Structure of miltefosine.

C11 for its leishmanicidal activities combined with its immunostimulating action in an *in vivo* context.

2. Materials and Methods

2.1. Compound

The compound 5-(4-(3-methanesulfonatepropyl)-1*H*-1,2,3-triazol-1-yl) dodecyl methanesulfonate (C11) was evaluated in this study (Fig. 1A). This compound is derivative from miltefosine (Fig. 1B). The synthesis and chemical characterisation have been described previously (Gontijo et al., 2015).

2.2. Experimental design

In the present study, eight-week-old male golden hamsters (*Mesocricetus auratus*) were infected intraperitoneally with 1×10^7 amastigotes from the spleen of an infected animal. Then, 50 days after the infection, the animals were divided into four (4) groups (n = 5/group), and subjected to one of the following treatments for 10 consecutive days: 0.5% carboxymethyl cellulose (vehicle) suspension, administered orally (untreated group [UTG]); 50 mg/kg/day of GLU, administered by intraperitoneal injection (GLU group; positive control); 10 mg/kg/day of MIL (MIL group) and 15 mg/kg/day of C11 (MIL derivative), administered orally as suspensions in 0.5% carboxymethyl cellulose (C11 group). After 10 days of treatment, the animals were sacrificed in a CO_2 chamber, and a specimens of the spleen and liver were removed, weighed and used for total RNA extraction, as previously described (Reimão et al., 2011). In addition, five healthy animals were used, denominated as the control group (CTL, n = 5) for blood and organ sampling for serological and histopathological testing. All the experimental procedures involving animals were approved by the Research Ethics Commission at Universidade Federal de Alfenas under project number 21/2017. All the experiments were performed according to the Guide for the Care and Use of Laboratory Animals (2011).

2.3. DNA/RNA extractions and cDNA synthesis

Standard curves of parasite DNA for use in quantitative real-time

polymerase chain reaction (qRT-PCR) experiments were produced, as described previously (Colombo et al., 2015). Specimens of liver and spleen (30 mg; weighed using sterile and disposable surgical material) were removed from the treated hamsters and placed in sterile microfuge tubes, then frozen immediately at -80°C in a storage buffer (RNAlater), to avoid RNA degradation. RNA extraction was performed 24 hours after removing the specimens, using the RNeasy Mini Kit (Qiagen®, Hilden, Germany), according to the manufacturer's instructions. The RNA specimens were frozen immediately after extraction. For reverse-transcription into cDNA, 1 µl of dNTPs mix (10 mM) and 1 µl of random primers (3 µg/µL) were added to 11 µl of the RNA specimen (containing 500 ng of RNA), and the specimens were incubated in a thermal cycler for approximately 5 min, at 65°C. Then, the tubes were placed on ice for 20 s, and 2 µl of DTT (100 mM) and 4 µl 5x buffer (Tris-HCl 250 mM, pH 8.3, containing 375 mM KCl, 15 mM MgCl2,) were added. The specimens were then incubated again in the thermal cycler for 20 s, at 37°C. Finally, 1 µl (200 U/µL) M-MLV RT enzyme was added, and the specimens were incubated for 50 min at 37°C, for cDNA synthesis. The purity of the cDNA specimen was confirmed by measuring the absorbance at 260/280 in a NanoDrop ND2000. Specimen integrity was verified by agarose gel electrophoresis and PCR. The specimens were frozen at -20°C for subsequent use in RT-qPCR.

2.4. Evaluation of the relative expression of cytokines by RT-qPCR

The assay to evaluate the gene expression of the cytokines (IL-10, IL-4, TGFβ, IFN-γ, IL-17 and TNFα) was performed using biological material obtained from the spleen and liver specimens of the animals (C11, MIL, GLU and UTG groups). Whole RNA was extracted and submitted to subsequent transformation to cDNA to perform the qRT-PCR analyses. TaqMan-type hydrolysis probes methodology, doubly labelled with the specific fluorophores, were used to quantitate the relative expression of the cytokines whose normalising gene was a hamster micro-globulin obtained from the GenBank gene sequence.

The primers were designed using Primer-BLAST software. The nucleotide sequences for the genes evaluated in this study were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The primers, which were produced by Integrated DNA Technologies (IDT), and their sequences are shown in Table 1.

The reactions were performed in a StepOne™ Real-Time PCR System (Applied Biosystems®, Foster City, California, USA), in a final volume of 10 µL per reaction. A volume of 0.5 µl of a mixture including the forward, reverse and TaqMan® probes, labelled with FAM for the cytokines and HEX for the constituent gene, were added using NFQ as a quencher on the plate. Then, a mix containing 5 µL of 2X TaqMan Universal PCR Master Mix and 3.5 µL of DNAse and RNAse-free water and 1 µL of cDNA from the samples was prepared and homogenized at the same time for all markers, so that they all had the same final concentration of cDNA. The mix was then placed into the plate.

A negative control was used for each marker. Amplifications occurred in an initial cycle of 50°C for 2 min. The second step was a cycle at 95°C for 10 min. In the third step, 40 cycles were performed at 95°C for 15 seconds and at 60°C for 1 min. The results were expressed using the cycle threshold (ΔCt) method, which consists of a relative quantification where the expression normalisation of each target is performed by subtracting the Ct value found for each cytokine by the Ct value of the constitutive gene.

2.5. Evaluations of anti-Leishmania IgG by enzyme-linked immunosorbent assay (ELISA)

Blood samples from all the animals were collected by intracardiac route, which was centrifuged to obtain the serum samples that were stored in a freezer for the serological tests.

An indirect ELISA test was performed according to the technique previously described (Voller et al., 1978), with some modifications (Colombo et al., 2011) to evaluate the total number of IgG anti-*Leishmania* antibodies.

Table 1
Primer **sequences used to evaluate the relative expression of the cytokines by RT-qPCR**

IL-10
Primer 1: CCA GCT GGA CAA CAT ACT C
Primer 2: CTG GAT CAT TTC TGA TAA GGT TTG G
Probe: /56-FAM/TG CAG GAC T/Zen/T TAA GGG TTA CTT GGG T/3IABkFQ/
IL-4
Primer 1: GAA CTC CAC GGA GAA AGAC
Primer 2: GGG TCA CCT CAT GTT GGA AAT A
Probe:/56-FAM/CT TCC CAG G/Zen/T GCT TCG CAA GTT T/3IABkFQ
TGF-β
Primer 1: GGC AGC TGT ACA TCG ACT TT
Primer 2: GAC AGA AGT TGG CGT GGT AG
Probe: /56-FAM/TG GAA GTG G/Zen/A TTC ACG AGC CCA AG/3IABkFQ/
IFN-γ
Primer 1: GAG CAT AGA CAC CAT CAA G
Primer 2: CCT GAA GGT CAT TTA CCG GAA T
Probe: /56-FAM/TC TTC AAC A/Zen/G CAG CAT GGA GAA ACT CA/3IABkFQ/
IL-17
Primer 1: CTC CAG CAA CTC TTC TT
Primer 2: TCT GTT GCT GGT CTC TTG
Probe: /56-FAM/CC AGC CAG G/Zen/G TTC TCA AGC TC/3IABkFQ/
TNF-α
Primer 1: GGT TTA CTC CCA GGT TCT CTT C
Primer 2: GGA CAG GAG GTT GAC GTT AT
Probe: /56-FAM/TC AGC CGC A/Zen/T TGC TGT GTC CTA /3IABkFQ/
MG (Microglobulin, a housekeeping gene from *M. auratus*)
Primer 1: GGT CTT TCT ATC TCT TGG CTC A
Primer 2: CTT GGG CTC CTT CAG AGT TAT G
Probe: /HEX/ACT GCG ACT/ Zen/ G ATA AAT ACG CCT GCA/3IABkFQ/

2.6. Parasite load estimation by Linj31 RT-qPCR in the liver and spleen specimens

To evaluate the anti-parasitic effect of C11, MIL and GLU in comparison to the UTG, the hamsters with an established leishmanial infection (60 days after parasite inoculation) were treated for 10 days, and the specimens from spleen and liver of the infected animals were collected for DNA extraction and qRT-PCR analysis. The number of amastigotes per gram of (spleen or liver) tissue was then calculated based on the linear regression parameters obtained from the standard curve (Colombo et al., 2017).

Then, qRT-PCR was performed using the TaqMan probe double-labelled with FAM at the 5′-end and a non-fluorescent quencher at the 3′-end, and the primers LINJ31 (Colombo et al., 2011). Reactions were performed using a StepOne Real-Time PCR System (Applied Biosystems), and the reaction mixtures contained 1 µL of DNA (control) or cDNA samples, 5 µL of 2X TaqMan Universal PCR Master Mix, 0.5 µl of a mixture of primers and probe, besides DNase/RNase-free water to a final volume of 10 µL. The following PCR conditions were used: one step of 50°C for 2 min, followed by one step of 95°C for 10 min and 40 cycles of 95°C for 15s and 60°C for 1 min.

The number of parasites per gram of spleen or liver tissue was calculated based on the linear regression data from the standard curve performed with promastigote parasites (Colombo et al., 2015). Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by the Mann-Whitney test (unpaired, two-tailed) to test to determine statistical significance ($p < 0.05$).

2.7. Histopathological study

The spleen and liver specimens were collected from the CTL, C11, MIL, GLU and UTG groups. The samples were packed in 10% buffered formalin solution, followed by the processing of usual histology techniques and staining with haematoxylin and eosin (HE). The spleen

slides were analysed to evaluate the white pulp and red pulp ratio, using Image-Pro Plus software to measure the areas relative to these pulps. The liver slides were analysed to measure the intensity of the periportal infiltrate and to count of the number of granulomas.

2.8. Statistical analysis

Statistical analyses were performed on GraphPad Prism 5.0 (Prism Software, Irvine, CA, USA). To test for normality, the data were submitted to the Shapiro-Wilk test, followed by one-way ANOVA for between-groups comparisons, followed by the Kruskal-Wallis test. The Mann-Whitney test was used to compare two groups (unpaired, two-tailed). The Chi-square test was used to compare the analysis of the white pulp and the red pulp of the spleen. In all analyses, statistical significance was considered when $p < 0.05$.

3. Results

3.1. Evaluation of the relative expression of the proinflammatory TNF-α, IFN-γ, IL-17 and anti-inflammatory cytokines IL-10, IL-4 and TGF-β in the spleen and liver specimens

The relative mRNA expression from the cytokines IL-17, IFN-γ, TNF-α, IL-4, IL-10 and TGF-β was quantitatively assessed using qRT-PCR in the spleen and liver from the experimentally infected and treated hamsters in comparison to the UTG group (infected and untreated control group). The results are represented by ΔCt of the relative mRNA/cytokines expression, shown in Fig. 2 and 3, respectively.

The spleens of the infected animals treated with C11 and GLU expressed higher levels of IL-17 in comparison to the spleens of the UTG animals ($p < 0.01$). A higher expression of TNF-α was found in the C11, MIL and GLU groups in comparison to the UTG group ($p < 0.05$). Only the GLU group showed an increase in IFN-γ expression in comparison to the UTG group ($p < 0.01$).

A significant reduction in the relative mRNA expression of the IL-10 cytokine was verified in the C11 group in comparison to the UTG group ($p < 0.05$). The IL-4 and TGF-β expressions increased in the C11, MIL and GLU groups in comparison to the UTG group.

The expression of TNF-α levels was higher in the liver of the infected animals treated with C11 in comparison to the UTG animals ($p < 0.05$). The IL-4 expressions increased in the C11 and MIL groups in comparison to the UTG group. Moreover, TGF-β expression increased in the MIL group in comparison to the UTG group.

3.2. Humoral immune response

The humoral immune response was evaluated in the serum from the CTL, MIL, C11, GLU and UTG groups. The infected hamsters treated with C11 and GLU showed a significant reduction in IgG levels in comparison to the UTG group ($p < 0.05$). The IgG levels were higher in the C11, MIL, GLU and UTG groups than the CTL group ($p < 0.05$) (Fig. 4).

3.3. Parasite burden evaluation

In the UTG group, the average number of parasites was 6.66×10^6 and 1.32×10^6 per gram of tissue, in the spleen and liver, respectively. This finding confirms that these animals had an established infection. GLU (50 mg/kg/day, intraperitoneal route) was the most effective compound at reducing the parasite burden in the infected animals,

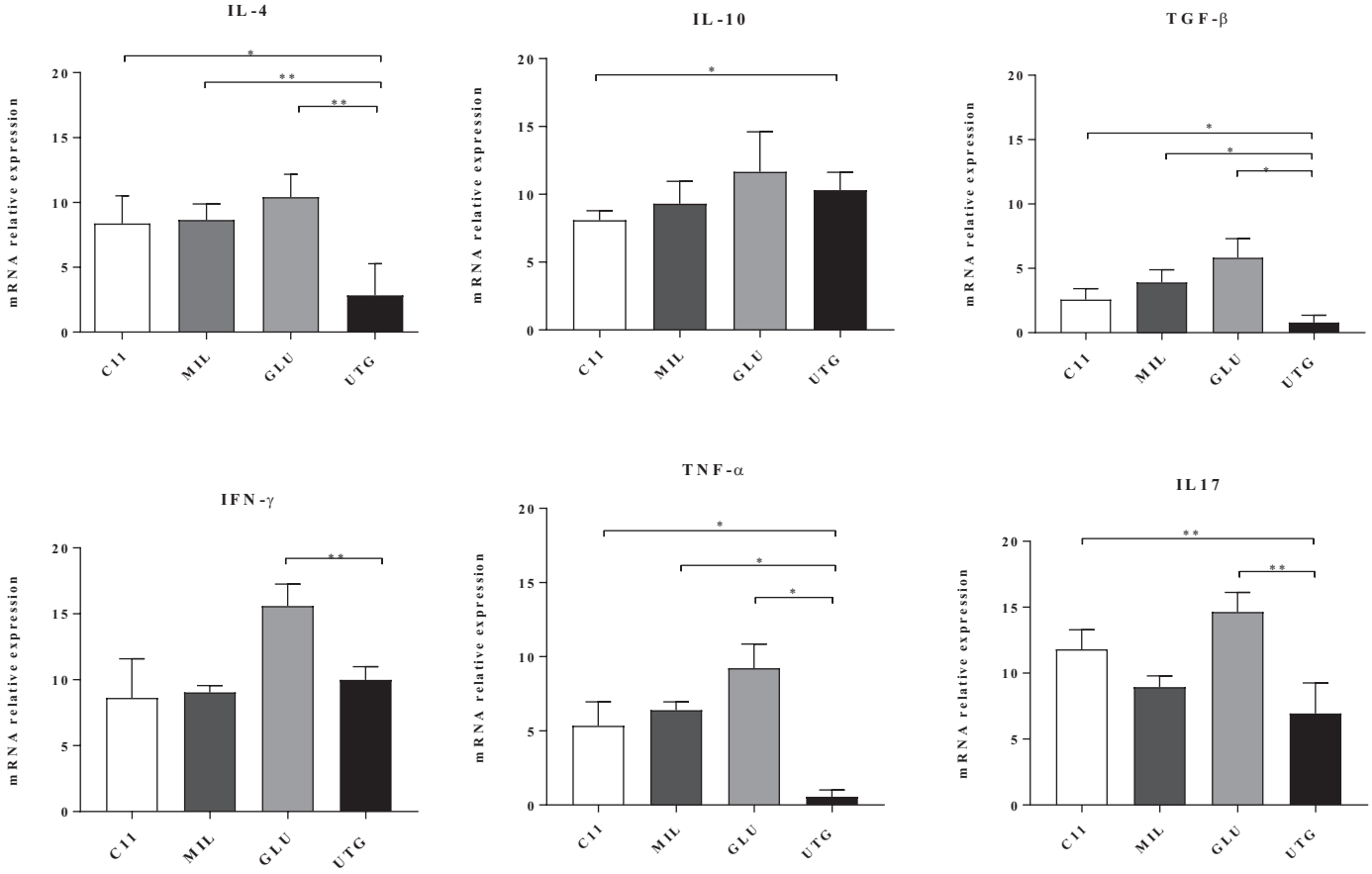

Fig. 2. Relative mRNA expression of cytokines in the hamster spleen specimens. The results are expressed as median +/- standard deviation. Significant differences based on the Mann-Whitney test (* = $p < 0.05$; ** = $p < 0.01$).

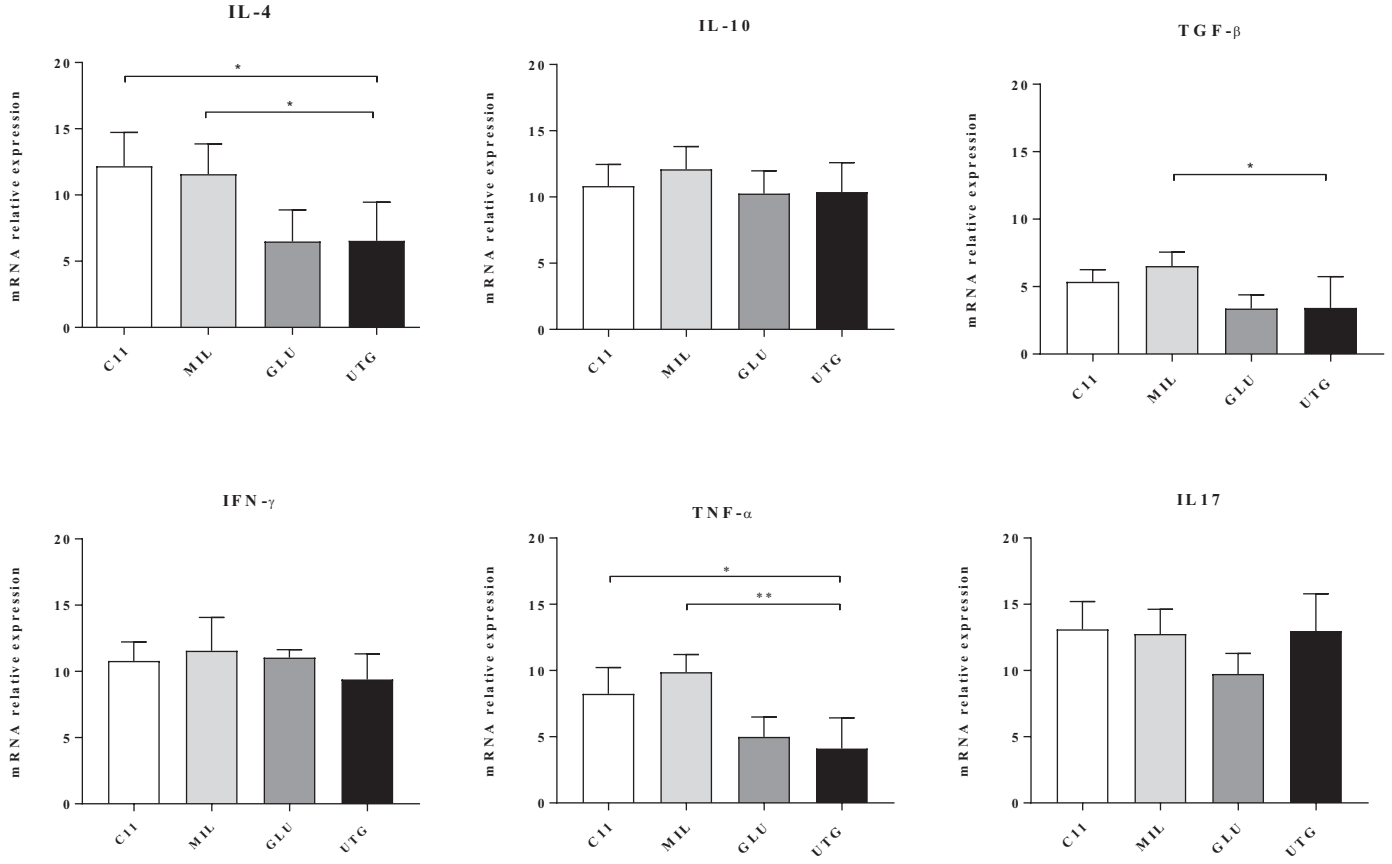

Fig. 3. Relative mRNA expression of cytokines in the hamster liver fragments. The results are expressed as mean +/- standard deviation. Significant differences based on the Mann-Whitney test (* = $p < 0.05$; ** = $p < 0.01$).

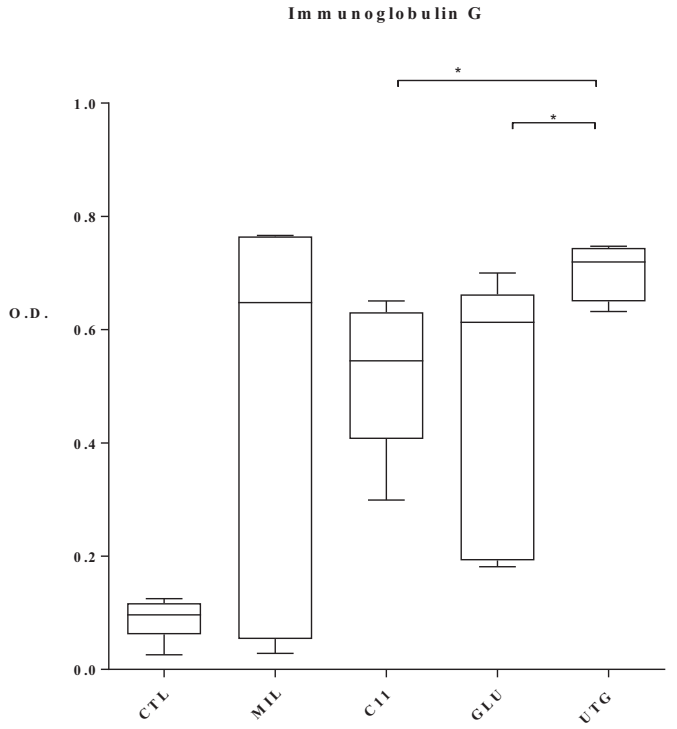

Fig. 4. Distribution of optical density (OD) results obtained from the VL ELISA test. Total IgG reactivity in the hamster serum from the GLU, C11, control (CTL) and UTG groups. The results are expressed as OD values.

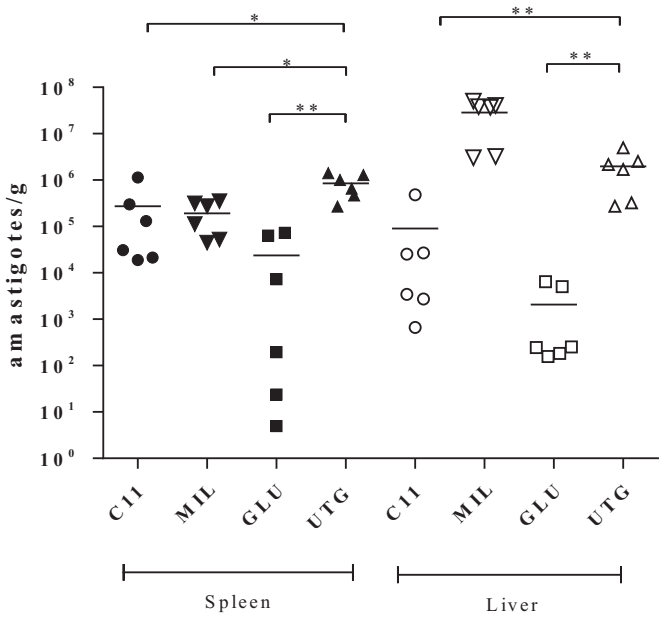

Fig. 5. Quantification of parasite burden in the spleen and liver from hamsters infected with *L. infantum chagasi*, as quantified by qRT-PCR to detect the Linj31marker. The number of parasites (amastigotes) per gram of tissue was estimated based on a RT-qPCR standard curve using promastigotes. Data are represented as group medians and individual values for each animal (* = $p < 0.05$; ** = $p < 0.01$) Mann-Whitney test.

since treatment in the GLU group led to a reduction in the number of amastigotes to 2.40×10^4 and 2.06×10^3, in the spleen and liver, respectively (Fig. 5). Treatment with C11 significantly reduced the number of amastigotes in the spleen (to 2.73×10^5) and in the liver (9.07×10^4) in comparison to the UTG group ($p < 0.05$) (reduction of 95.9%). The number of amastigotes was reduced in the spleen (to 1.91×10^5; 97.13%) in the MIL group in comparison to the UTG group ($p < 0.05$), but these differences were not statistically significant in relation to liver (2.87×10^7) (Fig. 5).

3.4. Histopathological alterations

The presence of vessels with periportal infiltrates, other than granulomas, was observed as the main histological changes in the liver from the UTG, C11, MIL and GLU groups. In the UTG group, an intense periportal infiltrate, few granulomas and fibrosis tissue were observed. Conversely, in the C11, MIL and GLU groups, a greater preservation of the periportal spaces was observed. The infiltrate noted in these groups was less extensive than the infiltrate seen in the UTG groups. Moreover, the samples from the animals in the C11 group contained many granulomas, whereas periportal infiltrates were not found in the CTL group.

The spleen specimens from the CTL animals, which histological architecture was preserved, were used to compare them with the alterations from the other groups. In the analyses from the UTG group, the lymphoid follicles were replaced by infected macrophages; that is, there was a loss of organ architecture, and the white pulp (hypoplasia) was replaced by the red pulp (Fig. 6). A higher preservation of white pulp was found for the C11, MIL and GLU-treated animals in comparison to the UTG animals (Fig. 6).

4. Discussion

VL is a major public health problem in Brazil, and around the world, because therapeutic resources are limited and there is an increase in the resistance to the available drugs used to treat it. Moreover, there is no effective vaccine against it. Thus, while it is necessary to identify new compounds with leishmanicidal action, the discovery of new drugs requires the use of *in vivo* models that reproduce the main clinical symptoms of infection. Toward that end, the present study used a golden hamster (*M. auratus*) experimental model for *in vivo* leishmanicidal assays of compounds similar to MIL in order to reduce its toxicity and increase its selectivity. According to Moreira et al., (2012), *M. auratus* reproduces several characteristics of human VL, such as clinical manifestations, as well as haematological, biochemical, histological and immunological alterations. (Melby et al., 2001) showed that the clinical-pathological characteristics and immunopathological mechanisms of VL in *M. auratus* are remarkably similar to human disease, but they are different from the commonly-used murine model, which does not develop the disease progressively. Moreover, it is possible to control the infection by generating NO. This effective mechanism does not have a clear role in the antimicrobial function of human macrophages (Melby et al., 2001).

In the present work, the evaluation of the cellular immune response by mRNA expression of proinflammatory (TNF-α, IFN-γ and IL-17) and anti-inflammatory cytokines (IL-4, IL-10 and TGF-β) in the spleen and liver samples collected from hamsters contributed to identifying the immune response after different treatments (Fig. 2 and 3). In an earlier study, performed with *L. donovani*-infected hamsters, Th1 and Th2 immune response profiles were observed in both the infected and untreated groups, with a significant increase in IL-4, IL-10 and TGF-β mRNA expression and a decrease in TNF-α, INF-γ and IL-12 (Gupta et al., 2012). Moreira et al. (2012), also analysed hamster infection by *L. infantum chagasi* and observed that the immune response profile showed a high expression of IL-10 and TGF-β, which could suggest a suppression of this response from animals, allowing for the establishment of the active infection and parasite proliferation. Corrêa et al., (2007) demonstrated that, in canine VL, symptomatic animals presented greater expression of TGF-β and IL-10 in both the liver and spleen, which would relate to the progression of the disease. These findings are consistent with the present study's results for the spleen (Fig. 2), which showed a significant increase in IL-10 expressions from the UTG group in comparison to the C11 group (MIL derivative). Moreover, the TNF-α expression increased in the GLU, C11 and MIL groups in comparison to the UTG group, which would characterise the disease progression and the hamster's susceptibility to infection.

In VL, the humoral immune response shows a high production of antibodies; however, while there is no known protective effect of these immunoglobulins against infection, they have been associated with

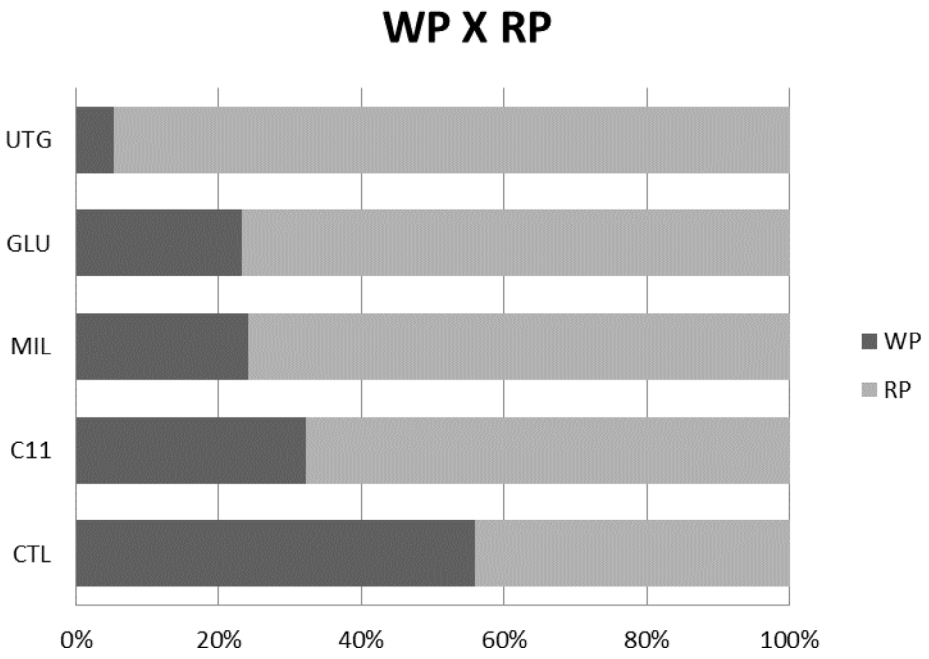

Fig. 6. Percentage ratio of white pulp (WP) and red pulp (RP). Analysis of loss of spleen morphology after infection by means of evaluating the percentage of WP and RP in comparison to the healthy control (CTL). Preservation comparison in WP: CTL > C11 = MIL = GLU > UTG ($p < 0.05$ Chi-square test).

progression and susceptibility to disease (Goto and Lindoso, 2004). Thus, the reduction of IgG levels (Fig. 4) observed in the C11-treated group in comparison to the UTG group is shown to be a good prognosis of the treatment.

In hamsters, dogs and humans, VL induces a humoral and cellular immune response. However, this response cannot control the disease process; consequently, there is an increase in the parasitic load in different organs. This evolution may be related to the increased expression of Th2-type cytokines known to be related to susceptibility to disease. Conversely, in the resistance to infection, a Th1 response is expected with the production of INF-γ, TNF-α and IL-17 cytokines (Trinchieri, 2007). Gupta et al. (2012) showed that MIL-treated VL hamsters had a remarkable increase in the expression of Th1 (INF-γ, TNF-α) and a significant decrease in Th2-type (IL-4, IL-10 and TGF-β) cytokines, showing a restoring effect of immunity, which would benefit the resistance to the disease. In the present study, the C11 compound was also found to benefit immunity because it contributed to an increase in the mRNA expression of IL-17 and TNF-α (Th1) and a reduction of IL-10 (Th2) cytokines in the spleen.

Nascimento et al. (2015) analysed the immune response profile to VL by *L. infantum chagasi*, since the C57BL/6 knockout mice for IL-17 had a higher parasite burden in the organs targeted by the infection, such as the liver and spleen. Nascimento et al. (2015) also concluded that the IL-17 cytokine may be involved in host resistance, which is related to a negative regulation of IL-10 production, with increased lymphoproliferative capacity and enhancement of NO production. This relationship between IL-17 elevation and IL-10 reduction was observed in the present study when the C11 compound was used; moreover, a decrease in IL-17 and an increase in IL-10 were observed in the UTG group.

In an experimental VL in hamsters, the liver and spleen are the main organs that are affected, besides being specific organs for development of the immune response (Laurenti et al., 1990). In the present study, the main histopathological changes observed in the liver were the presence of a periportal infiltrate and granulomas. The animals treated with C11, as well as those treated with GLU and MIL, had a moderate degree of infiltrate in comparison to the animals in the UTG group, which were found to have an intense inflammatory infiltrate. The MIL- and GLU-treated animals were found to have a significant reduction in the number of granulomas. It is believed that this might be related to the time of the analysis, since the parasite load had already been reduced. Moreover, the main anti-inflammatory cytokines from liver and spleen organs, such as TGF-β and IL-4, were more expressed in MIL and C11 groups in relation to UTG, acting as regulators of the immune response to control the pro-inflammatory response (Novoa et al., 2011). The C11-treated group showed a large number of granulomas, which are important for containing the infection and reducing the parasite load. The expression of pro-inflammatory cytokines, such as IL-17 and TNF-α, was greater in the C11-treated group, which favours the inflammatory process needed to control the infection (Murray, 2001).

The development of granulomas in the liver is closely linked with the attempt of the immune response to control the *Leishmania* infection (Vianna et al., 2002). Thus, the increase in the number of granulomas observed in the C11 group demonstrates that the compound was able to effectively control the infection. The animals in the C11 group showed a significant increase in the relative expression of TNF-α in the liver (Fig. 3), in comparison to the UTG group ($p < 0.05$). This would explain the increase in the number of granulomas, since their development is stimulated by TNF-α (Murray, 2001). The UTG group was found to have a lower number of granulomas, but fibrosis and necrosis were observed, due to non-resolution and progression of the disease, as described by Mangoud et al. (1997). Thus, Mangoud et al. (1997) showed that the evolution of disease in hamsters infected with *L. infantum chagasi* leads to an increase in the number of granulomas with the subsequent formation of fibrosis and necrosis.

Furthermore, the main changes observed in the spleen were white pulp depletion and red pulp hyperplasia, characterised by a loss of the normal architecture of this organ, which is partially due to the replacement of white pulp with cells from the red pulp. These findings were observed at a lower intensity in the C11-treated group in comparison to the UTG, MIL and GLU groups, showing that C11 is better able to preserve the organ architecture than MIL or GLU (Fig. 6). These changes were also described in canine VL, where the animals had granulomas, follicular depletion of white pulp, hypercellularity of red pulp, with the presence of lymphocytes, macrophages and neutrophils in the liver, as well as granulomas and periportal infiltrates (Moreira et al., 2016). The reduction of the lymphocyte area can lead to immunosuppression and aggravate the disease (Rodrigues et al., 2016). In the present study, a great preservation of white pulp was observed in the C11-treated animals in comparison to the animals in the other groups, which could suggest a better immune response from the host after the treatment (Fig. 5). The C11 was designed to reduce toxicity compared to MIL, which has a significant toxicity. In addition it was sought more selective compounds with high membrane permeability and that could be administered orally. And to achieve the goal of searching for new potential drugs to be used for the treatment of leishmaniasis it was developed synthetically through a short and efficient route.

The parasite load, detected by RT-qPCR, was significantly reduced in the spleen ($p = 0.04$) and liver ($p = 0.008$) of the C11-treated animals in comparison to the UTG group, showing *in vivo* leishmanicidal activity of the tested compound (Fig. 5). It is suggested that a possible increase in the concentration of C11 associated with a higher dosage regimen may result in better outcomes in both the liver and spleen. Gupta et al. (2012) observed an almost complete cure with MIL after 45 days of treatment in hamsters infected by *L. donovani* in comparison to untreated infected animals. A correlation between parasitic load, histopathological and immunological changes is essential for understanding the evolution of VL; and, similar to the present study many papers have reported that correlation (Corrêa et al., 2007; Gupta et al., 2012; Liew and O'Donnell, 1993; Melby et al., 2001; Moreira et al., 2012; Pitta et al., 2009; Stanley and Engwerda, 2007).

In conclusion, in this study, it was observed that treating the animals with C11 resulted in a reduction of the parasite load in the spleen and liver. Moreover, after treatment with C11, the spleen architecture was better, the periportal spaces of the liver were preserved and the profile of cytokine expression was more favorable to resistance to VL with increased expression of IL-17 and TNF-α and a reduction of IL-10. In turn, the GLU-treated animals were found to have a decrease in the parasitic load, possibly due to the increase in INF-γ (Th1) expression. However, it was not possible to maintain the architecture of the organ, showing a large loss of nodules from the white pulp in the spleen. Considering the increase in the reports of failure of VL treatment, it is extremely important to understand the immune response to the parasite since the modulation of the immune response of the host may be a strategy to determine the efficacy of a future drug that could lead to the death of the parasite and to immunity against the disease.

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Conflict of interest

The authors have no conflicts of interest to declare.

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