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Antileishmanial activity of a new chloroquine analog in an animal model of *Leishmania panamensis* infection

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ABSTRACT

Leishmania panamensis is a relevant causative agent of tegumentary leishmaniasis in several Latin American countries. Available antileishmanial drugs have several limitations including relatively high toxicity, difficult administration, high production costs and the emergence of resistance in circulating strains. Therefore, the identification of new molecules as potential therapeutics for leishmaniasis is of great relevance. Here, we developed a murine model of *L. panamensis* infection and evaluated the effect of a new compound *in vivo*. After treatment of animals with the compound, we observed a significant reduction of inflammation and parasite load at the inoculation site, in a dose-dependent manner. We observed a reduction in IL-10 production by popliteal lymph nodes cells of infected mice. These results pave the way for future evaluation of this compound as a potential antileishmanial drug or as a suitable scaffold for lead optimization strategies.

1. Introduction

Leishmaniasis is a disease caused by flagellate protozoa of the genus *Leishmania*. This disease is endemic in 98 countries around the world, affecting mainly tropical and subtropical regions. *Leishmania* species that are pathogenic to humans belong to two subgenera, *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*). Species from the subgenus *Viannia* are exclusively present in Central and South America and cause mainly cutaneous leishmaniasis, with occasional atypical or exacerbated presentations (Ehrlich et al., 2014; Hashiguchi et al., 2016; Muñoz and Davies, 2006). *L.* (*Viannia*) parasites can disseminate to the nasopharyngeal area, causing destructive lesions of the nasal, oral and hypopharyngeal mucosa, in a clinical presentation known as mucocutaneous leishmaniasis (Achtman et al., 2016; Mitropoulos et al., 2010; Varela-M et al., 2009).

Since the 1940s, the first-line treatment for leishmaniasis has been the pentavalent antimonials, with relatively few drugs available as second-line treatment, including miltefosine, pentamidine and amphotericin B (Tiuman et al., 2011). All the available antileishmanial drugs have several limitations, including serious side effects, large treatment doses, high production costs and the emergence of resistance in circulating parasite strains (Jain et al., 2012; Varela-M et al., 2012). Due to these limitations, huge efforts have been made to find new antileishmanial drugs. A promising line is the development of synthetic compounds with the potential to become novel, cost-effective, therapeutic agents. Due to the intracellular nature of the parasite and its capacity to disseminate to other locations, researchers have to overcome several difficulties to turn these molecules into effective drugs (Tiuman et al., 2011).

Murine animal models are key to understand the mechanisms underlying the immunopathogenesis of *Leishmania* and at the same time, provide a platform to test potential antileishmanial drugs (Lipoldová and Demant, 2006; Rojas et al., 1993). Development of murine models of infection for *L.* (*Viannia*) species was initially hampered by the general

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belief that mouse was a non-permissive host for these species, due to the poor infectivity observed in several laboratory mice strains (Castilho et al., 2010; Neal and Hale, 1983; Samuelson et al., 1991). Rojas et al. (1993) studied the differences in susceptibility of the BALB/c and C57BL/6 mouse strains to *L. panamensis* infection, reporting a similar pattern to that previously described for *L. major*, being BALB/c more susceptible than C57BL/6 mice.

In addition to the development of a proper animal model, a critical step in the drug discovery workflow is to obtain lead compounds with the expected biological activity and other features desirable for a future drug (Pink et al., 2005). Synthetic compounds are an alternative to create new molecules targeted for the treatment of leishmaniasis, reducing time and cost in the process of screening natural compounds. *N*-heterocycles are important structural scaffolds for bioactive compounds (Larionov et al., 2014). Quinoline derivatives are a major class of heterocycles, with a ring structure that occurs frequently in natural products. Its chemical scaffold is used in the design of many pharmacologically-active synthetic compounds (Kouznetsov et al., 2005). In a previous study, we observed that the chloroquine analog N^4-(7-chloro-2-cyclohexanylquinolin-4-yl)-N^1,N^1-diethylpentane-1, 4-diamine (here called compound **1**) inhibited the growth of *L. panamensis* amastigotes *in vitro* with a half maximal inhibitory concentration (IC_{50}) of 1.07 ± 0.51 μM and with the concomitant inhibition of the production of IL-10 by infected murine macrophages (Herrera et al., 2016). Here, we studied the *in vivo* effect of this compound on a murine model of *L. panamensis* infection. We first optimized an infection model in the hind footpad, comparing the infection in the mouse strains BALB/c and C57BL/6. The BALB/c strain was then used to characterize the effect of the compound, due to its higher susceptibility to *L. panamensis* infection. Compound **1** reduced, in a dose-dependent manner, the inflammation and the parasite load at the inoculation site. Compound **1** also modulated the production of IL-10 *in vivo*.

2. Materials and methods

2.1. Mice

Female BALB/c and C57BL/6 mice, 8 weeks of age, were provided by INDICASAT's animal facility. Animals were maintained with a 12 h light/dark cycle, at a constant temperature of 24 °C with free access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee of INDICASAT (IACUC-14-002) and were based on the strict observance of the ethical guidelines related to the handling of laboratory animals, in accordance with international regulations and those established by INDICASAT.

2.2. Parasites and infection

We used the PSC-1 strain of *L. panamensis* (MHOM/PA/94/PSCI-1), a strain originally isolated from a cutaneous leishmaniasis patient from Panama and used as a reference in previous studies from our group (Llanes et al., 2015; Restrepo et al., 2013). Promastigotes were cultured at 25 °C in Schneider medium (Sigma-Aldrich, St. Louis, MO) supplemented with 20% fetal bovine serum (FBS) (Gibco, Waltham, MA). Parasite virulence was maintained by inoculating stationary phase promastigotes in the hind footpad of golden hamsters, for the initial activation of the strain's infectivity. Four weeks later, parasites were recovered from the lesion site and cultured until they reached the stationary phase. These hamster-derived stationary phase promastigotes were adapted to mice by serial passages in the hind footpad of BALB/c for a period of two years.

Virulent *L. panamensis* promastigotes (10^5) from stationary phase culture were inoculated subcutaneously in the hind footpad (30 μL) of BALB/c and C57BL/6 mice ($n = 5$ per group), using a 30-gauge needle. Lesion development was evaluated weekly with a digital caliper (SE 784 EC) (Sona Enterprises, Santa Fe Springs, CA), measuring the thickness of the inoculated footpad.

2.3. Compound *1* treatment

Treatment started at 6 weeks after infection. Compound **1** was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) at a concentration of 20 mg/mL. This solution was then diluted with 0.9% saline injectable solution and administered intralesional (10 μL) at doses of 10 or 50 mg/kg every four days for three weeks. The control group was treated with the same concentration of DMSO in saline as the treatment group. We used chloroquine (50 mg/kg) as a control drug, administered to the animals following a procedure similar to that described for compound **1**.

2.4. Parasite load quantification

Tissue of the hind footpad was aseptically excised, weighted and homogenized with a tissue grinder in cold PBS with penicillin (100 U/mL)/streptomycin (100 μg/mL) (Sigma-Aldrich). The homogenate was washed with 1 mL of cold PBS (NaCl 137 mM, Phosphate 10 mM, KCl 2.7 mM, pH = 7.2) ten times, centrifuged at 430 x g and resuspended in 1 mL of PBS. The parasite load was measured by using a limiting dilution analysis as previously reported (Titus et al., 1985). Parasite growth was monitored for ten days.

2.5. Lymph node cells culture and cytokine quantification

Popliteal lymph nodes were recovered at 8 weeks post infection and transferred to nylon cell strainers of 70 μm (Corning). Cells were isolated by mechanical disruption and viable cells were counted by trypan blue (Sigma-Aldrich) exclusion. Cell suspensions were plated in 48-well plates at a concentration of 5×10^5 cells per well, in 500 μL of RPMI supplemented with 10% FBS and penicillin (100 U/mL)/streptomycin (100 μg/mL). Cells were treated with soluble *L. panamensis* antigen (SLA) (20 μg/mL) prepared by ten cycles of freezing and thawing, followed by sonication in an ice bath. Supernatants were harvested after 72 h of incubation at 37 °C in 5% CO_2. Cytokines concentrations (IL-4, IL-10, IL-13 and IFN-γ) were measured by ELISA (DuoSet Kit) (R&D System, Inc., Minneapolis, MN) as described by manufacturer. We used stimulation with concanavalin A as positive internal control.

2.6. Statistical analysis

Analyses were performed with GraphPad Prism version 6.00, (GraphPad Inc., La Jolla, CA). Data is presented as mean ± SEM. Statistical differences between groups were evaluated by using a one-way ANOVA followed by Bonferroni's or Holm-Sidak's multiple comparisons tests. A p-value < 0.05 was considered statistically significant.

3. Results and discussion

In a previous study, we identified chloroquine analog **1** as an inhibitor of *L. panamensis* growth *in vitro* (Herrera et al., 2016). Herein, we developed a simple model of *L. panamensis* infection in mice to evaluate the *in vivo* effect of this and other compounds with a suspected anti-leishmanial effect. Infection of different mouse strains with different *Leishmania* species has long been known to exhibit distinct outcomes (Afonso and Scott, 1993). Some species of *Leishmania*, including *L. major* and *L. amazonensis*, have been well characterized experimentally *in vivo*. However, the results of these studies should not be extrapolated to other species such as those from the *L.* (*Viannia*) subgenus, which differ largely from those of the *L.* (*Leishmania*) subgenus (McMahon-Pratt and Alexander, 2004). Taking these differences into account, we used both the BALB/c and C57BL/6 mouse strains during the development of the animal model of infection. A first pass was done in hamsters to reactivate the virulence of the strain, although we later found that the infectivity was still poor in BALB/c mice. Thus, four additional serial passages were

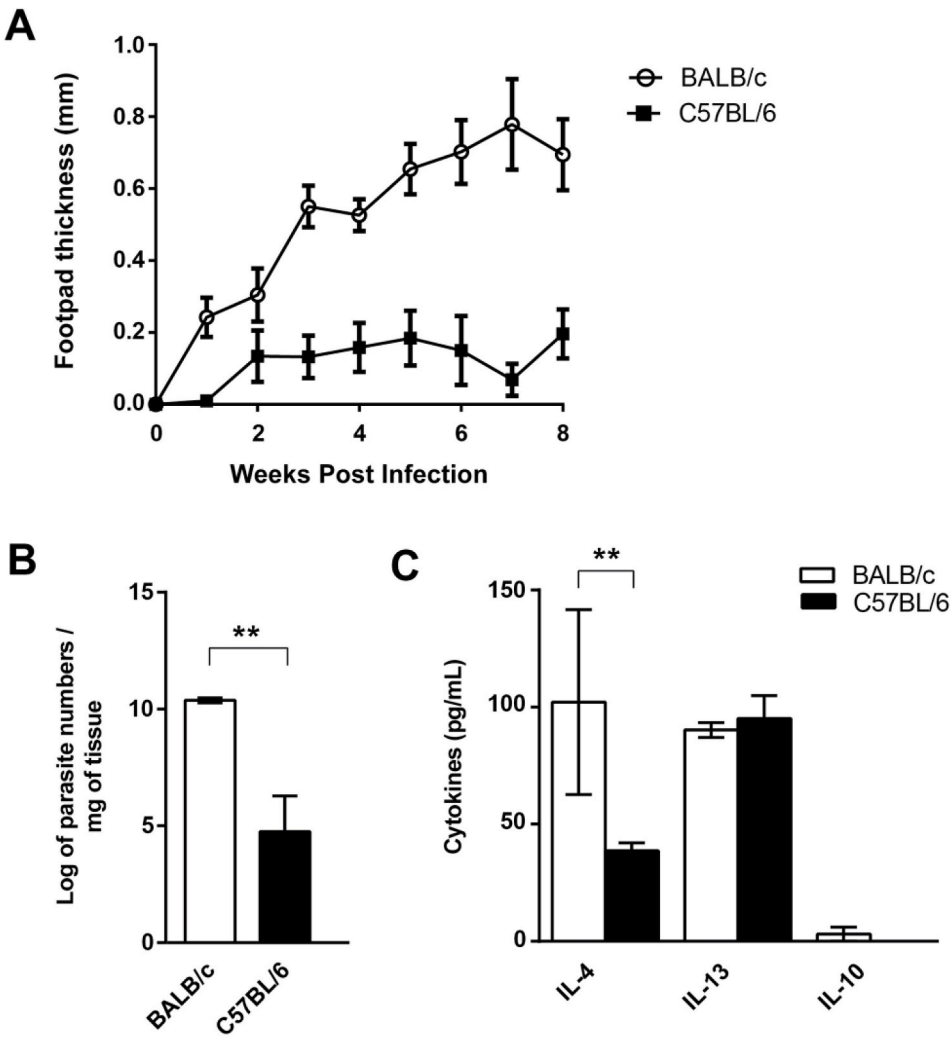

Fig. 1. Characterization of *L. panamensis* infection in murine models. (A) Course of *L. panamensis* infection in BALB/c and C57BL/6 mice. Animals were inoculated with stationary phase promastigotes (10^5). Lesion development was evaluated weekly, measuring increase in the thickness of the inoculated footpad. **(B)** Parasite load determined by limiting dilution at 8 weeks post infection. Results are represented as mean ± standard error of the mean (SEM) and are representative of at least three independent experiments ($n = 5$ per group). **(C)** Cytokine production by popliteal lymph node cells stimulated with soluble *L. panamensis* antigen (SLA), obtained from BALB/c and C57BL/6 mice at week 8 post infection ($n = 5$ per group). Results are represented as mean ± SEM from samples assayed in triplicates and are representative of two independent experiments. **, $P < 0.01$.

made in BALB/c mice, in order to achieve better infectivity in this mouse strain.

We used an inoculum of 10^5 stationary phase promastigotes, since preliminary tests with this parasite dose resulted in a more gradual and sustained course of infection when compared to 10^6 (Fig. S1). Infection in the hind footpad showed localized inflammation at the inoculation site in both mouse strains, although it was stronger in BALB/c mice than in C57BL/6 (Fig. 1A). At the end of the experiments, animals were sacrificed and the parasite load was assessed with a limiting dilution assay. Presence of parasites at the site of inoculation was observed in both strains (Fig. 1B), although C57BL/6 mice showed 45% less parasitic load than BALB/c mice, on average. These results are consistent with previous studies describing the higher susceptibility of the BALB/c strain to infection with *L. panamensis* promastigotes, regardless of the inoculation site (Castilho et al., 2010; Guevara-Mendoza et al., 1997; Neal and Hale, 1983; Rojas et al., 1993). An important source of variability in this scenario are the differences in tissue tropism among *Leishmania* species, which result in differences in lesion development related to inoculation sites in animal models (Osorio et al., 2003). Doses of infection also determine the inflammation rates and the appearance of lesions. While relatively high doses (~10^7) tend to induce a more acute course of infection (Neal and Hale, 1983; Rojas et al., 1993), lower doses (~10^4) have been used for the development of a chronic model of infection (Castilho et al., 2010). In our experimental setup, we found that a dose of 10^5 induced a course of infection that was more appropriate for testing antileishmanial molecules.

In order to evaluate the immune response of both strains to infection, cytokine production was measured in response to incubation with soluble *L. panamensis* antigens (SLA). Draining lymph nodes were isolated from infected animals and levels of cytokines were determined in the supernatant of the cells stimulated with SLA. At week 8 after infection, BALB/c mice showed a significantly higher production of IL-4, when compared to C57BL/6 (Fig. 1C). At week 12 post infection the levels of this cytokine produced by BALB/c were similar to those observed for C57BL/6 at week 8 (Fig. S2). The levels of IL-10 at week 8 were quite low for both, BALB/c and C57BL/6 (Fig. 1 C). Although we observed high levels in the production of IL-13 from popliteal lymph node cells in both animal strains, this appears to be not relevant in the development of the footpad inflammation and parasite load for C57BL/6 (Fig. 1).

These results are consistent with other studies that showed high levels of IL-4 in BALB/c mice infected with different *Leishmania* species (Launois et al., 1997; Rosas et al., 2005). It has been shown that this cytokine promotes the differentiation of Th2 cells and has the capacity to interfere with macrophage activation through the inhibition of nitric oxide production (Hurdayal and Brombacher, 2014; Jones et al., 2000; Scott and Kaufmann, 1991). Sustained production of IL-4 in BALB/c mice during the course of *L. major* infection has been associated with susceptibility of this mice strain (Himmelrich et al., 2000). Although C57BL/6 mice also produced IL-4 early in the infection with *L. major*, the levels of this cytokine decline with time (Morris et al., 1992). Susceptibility of BALB/c mice to *L. panamensis* infection might be associated with IL-4 and IL-13 (Castilho et al., 2010). Surprisingly, popliteal lymph

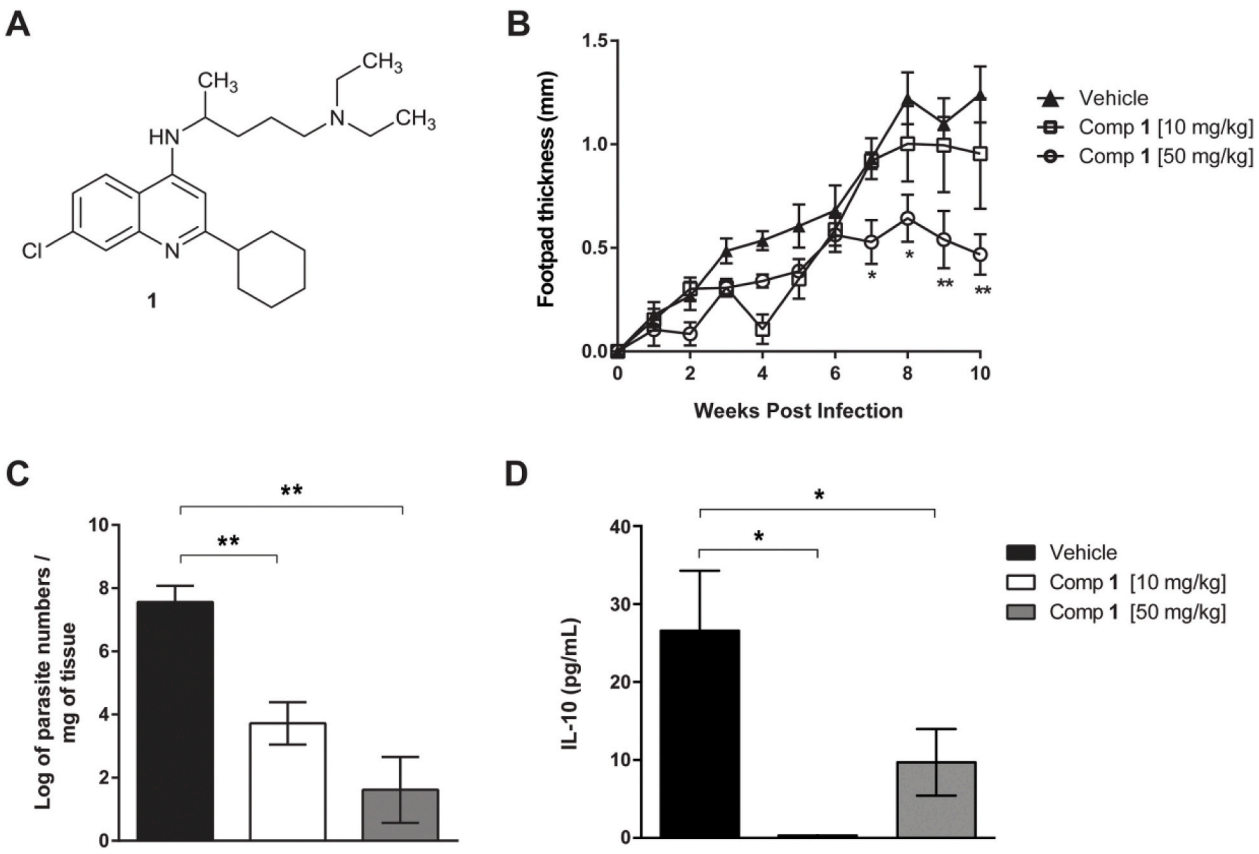

Fig. 2. Compound 1 reduces inflammation and parasitic load in BALB/c infected with *L. panamensis*. (A) Schematic representation of compound **1**. **(B)** Course of the infection in the presence or absence of 10 mg/kg (open squares) or 50 mg/kg (open circles) of compound **1**. Lesion development was evaluated weekly measuring the increase of thickness of the inoculated footpad. **(C)** Parasitic load at 10 weeks post *L. panamensis* infection in mice treated or not with compound **1** at doses of 10 and 50 mg/kg **(D)** IL-10 production by SLA-stimulated popliteal lymph node cells at week 10 post infection, treated with or without 10 or 50 mg/kg of compound **1**. Results are represented as mean ± SEM and are representative of two independent experiments ($n = 5$ per group). *, $P < 0.05$; **, $P < 0.01$ compared to vehicle treatment.

node cells from C57BL/6 mice produced high levels of IL-13, similar to those produced for BALB/c mice cells (Fig. 1C). This cytokine is also associated with a Th2 response and could have a regulatory role in the progression of *Leishmania* infection (Wynn, 2003). A combined action of IL-4 and IL-13 seems to reinforce the susceptibility to cutaneous leishmaniasis (Hurdayal and Brombacher, 2017, 2014). These results strengthen our data showing that BALB/c mice are more susceptible to the *L. panamensis* challenge than C57BL/6 mice. However, these cytokines can function independently to generate a susceptible phenotype (Hurdayal and Brombacher, 2017). Although it has been previously reported that IL-13 determines susceptibility to *L. panamensis* infection in BALB/c mice (Castilho et al., 2010), further studies are necessary to characterize the role of this cytokine in the infection of resistant mice strains, such as C57BL/6, to *L. panamensis*. Previous reports of cytokine production in murine models, in response to *L. panamensis* infection, have shown mixed Th1/Th2 cytokines responses, similar to those observed in humans (Castilho et al., 2010; Díaz et al., 2010; Ehrlich et al., 2014). This mixed response in patients infected with *L. (Viannia)* species is characterized by the presence of IL-10, IL-13, IFN-γ, TNF and IL-4 (Bosque et al., 2000). Curiously, although production of IFN-γ has been previously reported in C57BL/6 mice (Belkaid et al., 2002), we did not find detectable levels of this cytokine either in C57BL/6 or in BALB/c mice.

We used BALB/c mice to evaluate the effect of compound **1** *in vivo*, since we observed a higher susceptibility to infection in this strain. This compound, synthesized according to previously reported procedures (Larionov et al., 2014), is a chloroquine derivative from the quinoline family (Fig. 2A). In order to evaluate the activity of the compound *in vivo*, animals were infected in the hind footpad and monitored weekly to record the progress of the infection through the development of localized inflammation at the inoculation site. By week 6 after inoculation, we observed that the infection was apparently established in all of the animals evaluated, with an inflammation-associated thickness increase of over 0.5 mm (Fig. 2B). At this time point, we began to administer the compound at a dose of 10 mg/kg or 50 mg/kg. The higher dose of compound significantly reduced the footpad thickness from week 7-post infection and the effect was maintained until the end of the evaluation period (Fig. 2B). This effect was associated with a 78% decrease in the parasitic load (Fig. 2C). Significant reduction in parasite number was observed in the animals treated with the lower dose of compound; however, this reduction had no effect on footpad inflammation (Fig. 2B and C). Chloroquine, instead, did not show any effect, neither on the inflammation of the hind footpad nor on the parasite load (Fig. S3). Similar results after treatment with chloroquine alone have been reported in murine models of *L. major* and *L. amazonensis* (Wijnant et al., 2017).

In order to evaluate the specific immune response in the animals treated with compound **1**, cells obtained from the lymph node of infected BALB/c mice, treated or not with compound **1**, were stimulated *in vitro* with SLA. Despite low levels of IL-10 in controls, animals treated with compound **1** showed a significant reduction in the production of this cytokine (Fig. 2D). Previous results demonstrated that this compound is associated with a reduction of this cytokine produced by macrophages infected *in vitro* with *L. panamensis* (Herrera et al., 2016). We did not observe significant differences in the levels of IL-13 or IL-4, but the latter tended to be reduced in all the experiments (Fig. S4).

IL-10 is a potent anti-inflammatory cytokine produced by cells of the immune system, including Th2 cells, dendritic cells, macrophages and neutrophils (Saraiva and O'Garra, 2010). Production of this cytokine by infected macrophages appears to be essential for the survival and persistence of the *Leishmania* parasites in their host, due to the inhibition of the protective activities in the infected cells (Geiger et al., 2016; Kane and Mosser, 2001; Verma et al., 2016). A decrease in the parasite burden associated with low levels of IL-10 has been shown in BALB/c mice infected with *L. amazonensis* (Mendonça et al., 2019). Moreover, the IL-10 secreted by T-cells influences the susceptibility of BALB/c mice to *L. major* infection (Schwarz et al., 2013). IL-10 detected in the skin lesions of *L.* (*Viannia*) species-infected humans has been associated with a susceptible phenotype and unresponsiveness to treatment (Bourreau et al., 2009, 2001; Salhi et al., 2008). The blockage of IL-10 has been suggested as a potential treatment for cutaneous leishmaniasis (Castellano et al., 2015). Our results suggest that the effect of compound **1** on the control to infection might be partially related to IL-10 regulation. Although no effect on inflammation or parasite load was observed in animals treated with chloroquine, we did find a reduction in the production of IL-10 induced by the treatment with this drug (Fig. S3). Further studies are needed to better characterize the effects of compound **1** and chloroquine on cytokine secretion in *L. panamensis* infection.

The chloroquine scaffold has shown promise in the development of new antiparasitic drug candidates. Here, we demonstrated the antileishmanial activity of a chloroquine derivative in an *in vivo* model of *L. panamensis* infection. Researchers have synthesized and evaluated chloroquine analogues as an alternative to overcome emerging resistance to antimalarials and as an alternative in the search of active compounds against *Leishmania* spp. (Hanif et al., 2016). Chloroquine and its derivatives have been previously shown to be active against several *Leishmania* species, including *L. amazonensis* (Rocha et al., 2013), *L. donovani* (Mwololo et al., 2015) and *L. braziliensis* (Soares et al., 2017). Authors have shown that some of these compounds appear to be more active against amastigotes and can accumulate in relatively high concentrations within acidic vacuoles in the infected host cells (Rocha et al., 2013). Compound **1** appears to modulate the production of IL-10 by macrophages (Herrera et al., 2016). Our results suggest then that this compound may have potential as a new antileishmanial agent and as a source scaffold in subsequent lead optimization experiments.

Author contributions

Conceptualization, P.L.F. and R.L.; methodology, L.H., J.A., K.D., C. M.R. and R.R.; formal analysis, L.H., A.L. and C.M.R.; resources, D.E.S., H.T.D. and O.V.L.; data curation, L.H.; writing—original draft preparation, L.H., A.L. and P.L.F.; writing—review and editing, A.L., C.M.R., O. V.L., R.L. and P.L.F.; supervision, P.L.F. and R.L.; project administration, P.L.F.; funding acquisition, P.L.F. and L.H. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2020.08.002.

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