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A chloroquinoline derivate presents effective *in vitro* and *in vivo* antileishmanial activity against *Leishmania* species that cause tegumentary and visceral leishmaniasis

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Abstract: The identification of new therapeutics to treat leishmaniasis is desirable, since available drugs are toxic and present high cost and/or poor availability. Therefore, the discovery of safer, more effective and selective pharmaceutical options is of utmost importance. Efforts towards the development of new candidates based on molecule analogs with known biological functions have been an interesting and cost-effective strategy. In this context, quinoline derivatives have proven to be effective biological activities against distinct diseases. In the present study, a new chloroquinoline derivate, AM1009, was *in vitro* tested against two *Leishmania* species that cause leishmaniasis. The present study analyzed the necessary inhibitory concentration to preclude 50% of the *Leishmania* promastigotes and axenic amastigotes (EC_{50} value), as well as the inhibitory concentrations to preclude 50% of the murine macrophages and human red blood cells (CC_{50} and RBC_{50} values, respectively). In addition, the treatment of infected macrophages and the inhibition of infection using pre-treated parasites were also investigated, as was the mechanism of action of the molecule in L. *amazonensis*. To investigate the *in vivo* therapeutic effect, BALB/c mice were infected with L. *amazonensis* and later treated with AM1009. Parasitological and immunological parameters were also evaluated. Clioquinol, a known antileishmanial quinoline derivate, and amphotericin B (AmpB), were used as molecule and drug controls, respectively. Results in both *in vitro* and *in vivo* experiments showed a better and more selective action of AM1009 to kill the *in vitro* parasites, as well as in treating infected mice, when compared to results obtained using clioquinol or AmpB. AM1009-treated animals presented significantly lower average lesion diameter and parasite burden in the infected tissue and organs evaluated in this study, as well as a more polarized antileishmanial Th1 immune response and low renal and hepatic toxicity. This result suggests that AM1009 should be considered a possible therapeutic target to be evaluated in future studies for treatment against leishmaniasis.

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1. Introduction

Leishmaniases are diseases caused by protozoan parasites of the *Leishmania* genus, which present an annual estimated incidence of 1.5 to 2.0 million new cases, which range between 1.0 and 1.5 million cases of tegumentary leishmaniasis (TL), coupled with approximately 0.5 million new cases of visceral leishmaniasis (VL). Epidemiological data have proven that this disease complex is endemic in 98 countries, mainly in subtropical and tropical regions in the world [1,2]. TL can lead to self-healing cutaneous lesions, even causing mutilation and morbidity in patients. The disease is usually caused by *Leishmania braziliensis*, *L. amazonensis*, *L. guyanensis*, *L. mexicana*, and L. *lainsoni* species in the Americas, whereas VL, which is considered a fatal disease if acute and left untreated, can be caused by L. *donovani* and L. *infantum* species [3,4].

Treatment against leishmaniasis has been based on the use of pentavalent antimonials, although other drugs, such as free and liposomal amphotericin B (AmpB), miltefosine, paramomycin, and pentamidine have been also used [5]. However, problems related to their toxicity, high cost, and/or development of resistant strains have limited the efficacy of these therapeutics [6,7]. The prolonged administration of these compounds can cause adverse effects, such as renal, hepatic, and cardiac toxicity. These can also show variable efficacy according to the parasite strain, immune status of the hosts, and the emergence of drug resistance, all of which have been observed in the subcontinent of India and other regions [8,9]. Consequently, since the search to identify new antileishmanial targets is hampered due to the high investment necessary to develop new products, as well as by the lack of a profitable drug market [10], alternative means through which to identify new antileishmanial agents, such as plant derivates or synthetic products, could help to solve this relevant problem and make it possible to identify new therapeutics to be applied against leishmaniasis [11,12].

The interest in natural and/or synthetic molecules has increased in recent decades, and new agents have been evaluated in experimental trials [13–16], although most have been tested in *in vitro* studies, and few *in vivo* treatment experiments have been developed using mammalian models [17–19]. Distinct molecule classes, such as flavonoids, terpenoids, quinolines, among others, have shown variable degrees of antileishmanial activity [20–23]. In this aspect, quinoline derivatives have been applied as an important drug class, showing potent antitumoral, antiprotozoal, antimicrobial, and anti-inflammatory activity, among others, and did not cause significant toxicity in the hosts [24–27].

Research groups have demonstrated the selective antileishmanial activity of the quinoline-based compounds against *Leishmania* models [28–30]. One of these molecules, clioquinol (5-chloro-7-iodoquinolin-8-ol), demonstrated high efficacy against L. *amazonensis* and L. *infantum* promastigotes and amastigotes, without causing toxicity in murine macrophages. It was also effective in the treatment of infected macrophages and in the inhibition of the infection of these cells using pre-treated parasites [31]. In another study, clioquinol was incorporated to a Poloxamer 407-based delivery system, and the formulation proved to be effective in the treatment of L. *amazonensis*-infected BALB/c mice [32].

In this context, in the present study, a new chloroquinoline derivate, AM1009 [N1-(7-chloroquinolin-4-yl)-N3-cyclohexylpropane-1,3-diamine], was *in vitro* tested against two *Leishmania* species that cause VL and TL around the world. The 50% *Leishmania* inhibitory concentration (EC_{50}) and the 50% macrophage inhibitory concentration (CC_{50}) in murine macrophages and in human red blood cells (RBC_{50}) were investigated. Additionally, the treatment of infected macrophages and the inhibition of infection using pre-treated parasites were also evaluated, as were the mechanism of action in L. *amazonensis* and the *in vivo* therapeutic efficacy in treating *Leishmania*-chronically infected BALB/c mice. As a molecule control, clioquinol was used in the *in vitro* and *in vivo* experiments, as was AmpB, which was used as a drug control. Our purpose is to identify new candidates to be tested in future studies for treatment against leishmaniasis, which can present higher activity and selectivity when compared to previously described antileishmanial agents.

2. Materials and methods

2.1. Synthesis of AM1009

To perform the synthesis of AM1009, 220 mg (0.668 mmol) N-(3-bromopropyl)-7-chloroquinolin-4-amine was added to a round bottom flask containing 464 mg (4.68 mmol) of cyclohexanamine, 462 mg (3.34 mmol) of potassium carbonate, and 1 mL of dimethylformamide PA. The flask was shaken for 20 h at 120 °C, and the reaction was completed by thin layer chromatography. After, the compound was mixed using distilled water and dichloromethane PA, and AM1009 was purified through a filtration using silica, which was eluted with 50% ethyl acetate and 50% methanol. The molecule was dried on the rotary evaporator, and characterized by ^{1}H ^{13}C NMR and mass spectrometry, presenting an estimated yield of 76.0%.

2.2. Mice and parasites

This study was approved by the Committee for the Ethical Handling of Research Animals (CEUA) from the Federal University of Minas Gerais (UFMG), logged under protocol number 085/2017. BALB/c mice (female, 8 weeks old) were purchased from the UFMG Institute of Biological Sciences, and were kept in pathogen-free conditions. *Leishmania amazonensis* (IFLA/BR/1967/PH-8) and L. *infantum* (MHOM/BR/1970/BH46) strains were used. Stationary promastigotes were grown in complete Schneider's medium (Sigma-Aldrich, USA), which consisted of a medium plus 20% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, USA) and 20 mM L-glutamine pH 7.4, at 24 °C. The soluble L. *amazonensis* antigenic extract (SLA) was prepared from stationary promastigote cultures as described [33]. To obtain the axenic amastigotes, 10^9 stationary promastigotes were washed three times in sterile phosphate buffer saline (PBS 1x) and incubated for 48 or 72 h (L. *amazonensis* and *L. infantum*, respectively) in 5 mL FBS at 37 °C. The parasites were then washed three times in cold PBS 1 ×, and their morphology was evaluated after staining by the Giemsa method in an optical microscope as described [19].

2.3. In vitro antileishmanial activity

The *in vitro* inhibition of *Leishmania* growth was evaluated by incubating L. *infantum* or L. *amazonensis* stationary promastigotes and axenic amastigotes (10^6 cells; each) in the presence of varied concentrations of AM1009 (0, 0.06, 0.12, 0.25, 0.49, 0.98, 1.97, 3.93, 7.87, 15.73, and 31.46 μM) or clioquinol (0, 0.07, 0.13, 0.26, 0.51, 1.03, 2.05, 4.09, 8.19, 16.37, and 32.73 μM), in 96-well culture plates (Nunc, Nunclon, Roskilde, Denmark) for 48 h at 24 °C. AmpB (0, 0.04, 0.07, 0.14, 0.27, 0.54, and 1.08 μM; Sigma-Aldrich, USA) was used as a drug control. Cell viability was assessed by the 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) method. The optical density (OD) values were read in a microplate spectrophotometer (Molecular Devices, Spectra Max Plus, San Jose, CA, USA) at 570 nm. The data were entered into Microsoft Excel (version 10.0) spreadsheets, and the 50% *Leishmania* inhibitory concentration (EC_{50}) evaluated for promastigotes and axenic amastigotes was calculated by applying a sigmoidal regression of the dose-response curve [34].

2.4. Cytotoxicity in murine macrophages and human red cells

The cytotoxicity was evaluated in murine macrophages (5×10^5 cells), which were incubated with varied concentrations of AM1009 (0, 0.06, 0.12, 0.25, 0.49, 0.98, 1.97, 3.93, 7.87, 15.73, and 31.46 μM) or

clioquinol (0, 0.07, 0.13, 0.26, 0.51, 1.03, 2.05, 4.09, 8.19, 16.37, and 32.73 μM) in RPMI 1640 medium and in 96-well plates (Nunc) for 48 h at 37 °C and 5% CO_2. AmpB (0, 0.08, 0.17, 0.34, 0.68, 1.35, 2.70, 5.40, and 10.80 μM) was used as a control. The macrophage viability was also assessed by the MTT method. The data were entered into Microsoft Excel (version 10.0) spreadsheets, and the 50% macrophage inhibitory concentration (CC_{50}) was calculated by applying a sigmoidal regression of the dose-response curve [34]. The cytotoxicity was also evaluated in human cells, when the 50% red blood cell inhibitory concentration (RBC_{50}) was determined by incubation of AM1009, clioquinol, or AmpB, in the same concentrations as described above, with a 5% red blood cell (human O^+ type) suspension, for 1 h at 37 °C. The material was centrifuged by 1000 ×*g* for 10 min, when the cell lyses was determined spectrophotometrically at 570 nm. Negative (saline) and positive (distilled water) controls were used, and the values were calculated by also applying a sigmoidal regression of the dose-response curve.

2.5. Treatment of infected macrophages and inhibition of the infection

To evaluate the efficacy of compounds in treatment-infected macrophages, cells (5×10^5) were plated on round glass coverslips within 24-well plates in RPMI 1640 medium, which was supplemented with 20% FBS and 20 mM L-glutamine, pH 7.4, and incubated for 24 h at 37 °C in 5% CO_2. The stationary promastigotes were the added to the wells, and cultures (10 parasites per macrophage) were incubated for 48 h at 37 °C in 5% CO_2. Free parasites were removed by extensive washing with RPMI 1640 medium, and infected macrophages were treated with AM1009 or clioquinol (0, 0.5, 1.0, 2.5, and 5.0 μM; each) for 48 h at 24 °C in 5% CO_2. AmpB (0, 0.11, 0.54, and 1.08 μM) was used as a control. To evaluate the inhibition of infection using pre-treated parasites, promastigotes (5×10^6 cells) were first incubated with AM1009, clioquinol, or AmpB, in the same concentrations as described above, for 4 h at 24 °C. The cells were then washed three times in RPMI 1640 medium, quantified, and added to infect murine macrophages (10 parasites per one macrophage), for 24 h at 37 °C in 5% CO_2. After fixation with 4% paraformaldehyde, cells were washed, stained with Giemsa and the evaluation of the infection, as well as the number of recovered amastigotes per infected cell, were determined by counting 200 cells in triplicate by using an optical microscope.

2.6. Mechanism of action in Leishmania amazonensi

2.6.1. Evaluation of mitochondrial membrane potential

Stationary promastigotes (10^7 cells) were cultured in the absence or presence of AM1009 (2.41 and 4.84 μM, corresponding to one and two times the EC_{50} values, respectively), for 24 h at 25 °C. The cells were then washed and incubated with JC-1 reagent (10 μg/mL; Sigma-Aldrich, USA) for 30 min and in the dark. After washing twice with HBSS, cells were added to a black 96-well plate, and the mitochondrial membrane potential (Δψm) was measured in a spectrofluorometer (FLx800, BioTek Instruments, Inc., Winooski, VT, USA) at 528 and 600 nm, using 485 nm as the excitation wavelength. FCCP (1.0 μM)-incubated promastigotes were used as a positive control. Δψm values were calculated by the ratio between the reading at 600 nm and 528 nm, as described [35].

2.6.2. Reactive oxygen species production

Stationary promastigotes (10^7 cells) were cultured in the absence or presence of AM1009 (2.41 and 4.84 μM) for 24 h at 25 °C. Parasites were then incubated with H_2DCFDA reagent (20 μM; Sigma-Aldrich, USA) for 30 min in the dark at room temperature. To evaluate the reactive oxygen species (ROS) production, the fluorescence intensity was measured in a spectrofluorometer (Varioskan® Flash, Thermo Scientific, USA) at 485 and 528 nm for excitation and emission, respectively. Miltefosine (22.1 μM)-treated promastigotes were used as a positive control [35].

2.6.3. Cell cycle analysis and autophagy vacuole formation

Initially, to evaluate the cell cycle, promastigotes (10^7 cells) were cultured in the absence or presence of AM1009 (2.41 and 4.84 μM) for 24 h at 25 °C. Parasites were then fixed with 70% ethanol for 1 h at 4 °C and incubated with ribonuclease A (200 μg/mL; Sigma-Aldrich, USA) for 1 h at 37 °C. Cells were stained with propidium iodide (7.0 μg/mL; Sigma-Aldrich, USA) for 20 min in the dark at room temperature. For each sample, 10,000 events were acquired in a FACsCanto II flow cytometer (Becton Dickinson, Rutherford, NJ, USA), which was equipped with DIVA software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA), as described [36]. To investigate the autophagy vacuole formation, treated parasites were incubated with monodansylcadaverin reagent (100 μM; Sigma-Aldrich, USA) for 1 h in the dark at 25 °C. Cells were washed twice with PBS 1×, and 200 μL of suspension were added into a black 96-well plate, at which time they were analyzed in a spectrofluorometer, by using 335 nm and 460 nm for excitation and emission, respectively [37].

2.7. In vivo antileishmanial activity

2.7.1. Infection and treatment schedule

To evaluate the *in vivo* therapeutic efficacy of AM1009, clioquinol, and AmpB in the treatment of BALB/c mice, animals ($n = 8$ per group) were subcutaneously infected in the base of the tail with L. *amazonensis* stationary promastigotes (10^6 cells). Fifty to 60 days after infection, mice were separated into groups, aiming to ensure a similar average lesion diameter between them. They then received subcutaneous injections near the site of infection, once a day and during 7 days, using saline (PBS 1× pH 7.4), AmpB (1 mg/kg body weight), clioquinol (5 mg/kg body weight), or AM1009 (5 mg/kg body weight). After the treatment, the lesion average diameter was measured using an electronic caliper (799–6/150 model, Starrett®, Brazil); 15 days later, they were euthanized, when parasitological and immunological evaluations were performed.

2.7.2. Parasite load in treated and infected animals

The infected tissue, spleen, liver, and draining lymph nodes (dLN) of treated and infected animals were collected, and the parasite load was evaluated by limiting dilution technique. For this, infected tissue and organs were macerated in a glass tissue grinder using sterile PBS 1×, tissue debris were removed by centrifugation at 150 ×*g*, and cells were concentrated by centrifugation at 2000 x*g*. The pellets were re-suspended in 1 mL of complete Schneider's medium. Log-fold serial dilutions were performed in Schneider's medium with a 10^{-1} to 10^{-12} dilution, and each sample was plated in triplicate and read 7 days after the beginning of the culture at 24 °C. Results were expressed as the negative log of the titer (*i.e.*, the dilution corresponding to the last positive well) adjusted per milligram of tissue or organ.

2.7.3. Investigating the immunological profile

Cell response was evaluated in spleen cells of treated and infected animals. For this, splenocytes (5×10^6 cells) were plated in duplicate in 24-well plates (Nunc) and incubated in complete DMEM (medium), which was composed by medium plus 20% FBS and 20 mM L-glutamine pH 7.4, or stimulated with L. *amazonensis* SLA (25.0 μg/mL), for 48 h at 37 °C, 5% CO_2. IFN-γ, IL-4, IL-10, IL-12p70, and GM-CSF levels were measured in the cell supernatant by capture ELISA (BD Pharmingen®, San Diego, CA, USA), according to the manufacturer's instructions. The nitrite production was also evaluated in the supernatant by the Griess method [37]. The humoral response was investigated by determining anti-parasite IgG1 and IgG2a antibody levels. For this, SLA was added in the ELISA plates (1.0 μg per well), sera samples were diluted at 1:100 in PBS-T (PBS 1× plus 0.05% Tween 20), and anti-mouse IgG1 and IgG2a horseradish-peroxidase conjugated antibodies (Sigma-Aldrich)

were employed both in a 1:10,000 dilution in PBS-T. Reactions were developed using H_2O_2, ortho-phenylenediamine and citrate-phosphate buffer, pH 5.0, for 30 min and in the dark, and were stopped by adding H_2SO_4 2 N. The OD values were measured in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada) at 492 nm.

2.7.4. Evaluating the organic toxicity

To verify if the compounds were toxic for treated and infected animals, commercial kits (Labtest Diagnostica®, Belo Horizonte) were tested using sera samples of these mice, at which time the hepatic (AST and ALT) and renal (urea and creatinine) functions were evaluated. Samples from non-treated and non-infected animals ($n = 4$) were employed as a control.

2.8. Statistical analysis

The EC_{50}, CC_{50}, and RBC_{50} values were entered into Microsoft Excel (version 10.0) spreadsheets and calculated by dose-response curves, which were plotted in GraphPad Prism 5.03. Results were analyzed by the one-way analysis of variance (ANOVA), followed by the Dunnett's post-test to comparison between the groups. Results were expressed as mean ± standard deviation. Three independent experiments, which presented similar results, were performed. Differences were considered significant with $P < .05$.

3. Results

3.1. Antileishmanial activity, cytotoxicity, and selectivity index

The *in vitro* antileishmanial activity of AM1009 was evaluated against L. *amazonensis* and L. *infantum* promastigotes and axenic amastigotes. As molecule and drug control, clioquinol and AmpB were used, respectively. The EC_{50} values for AM1009, clioquinol, and AmpB against L. *amazonensis* promastigotes were 2.41 ± 0.28, 7.90 ± 0.65, and 0.13 ± 0.04 μM, respectively, and of 1.03 ± 0.22, 2.27 ± 0.44, and 0.34 ± 0.11 μM against the axenic amastigotes, respectively. Regarding the L. *infantum* species, the EC_{50} values against the promastigotes were of 0.38 ± 0.13, 4.45 ± 0.98, and 0.09 ± 0.02 μM, respectively, and of 0.98 ± 0.15, 3.65 ± 0.25, and 0.18 ± 0.05 μM against the amastigote forms (Table 1). The cytotoxicity in murine macrophages showed CC_{50} values of 148.94 ± 10.12, 613.27 ± 20.53, and 0.85 ± 0.16 μM for AM1009, clioquinol, and AmpB, respectively; while the RBC_{50} values were 1739.69 ± 79.69, 1409.85 ± 64.35, and 13.93 ± 1.95 μM, respectively (Table 2). With such data, the SI values were calculated, and results were 61.80, 77.63, and 6.54 against L. *amazonensis* promastigotes, and of 144.6, 270.1, and 2.5 against the amastigotes, respectively. For the L. *infantum* species, the SI values were 392.0, 137.8, and 9.4 against the promastigotes, respectively, and of 152.0, 168.0, and 4.7 against the axenic amastigotes, respectively (Table 2).

Table 1
Antileishmanial activity against *Leishmania* promastigotes and axenic amastigotes. L. *amazonensis* and *L. infantum* stationary promastigotes and axenic amastigotes (10^6 cells; each) were incubated with AM1009 (0, 0.06, 0.12, 0.25, 0.49, 0.98, 1.97, 3.93, 7.87, 15.73, and 31.46 μM), clioquinol (0, 0.07, 0.13, 0.26, 0.51, 1.03, 2.05, 4.09, 8.19, 16.37, and 32.73 μM), or AmpB (0, 0.04, 0.07, 0.14, 0.27, 0.54, and 1.08 μM) for 48 h at 24 °C. The cell viability was analyzed by the MTT method. The 50% *Leishmania* inhibitory concentrations (EC_{50}) evaluated for each parasite stage were calculated by applying a sigmoidal regression of the dose-response curve. Results were expressed as mean ± standard deviation.

Compounds	Antileishmanial activity (EC_{50}; in μM)			
	Leishmania amazonensis		*Leishmania infantum*	
	Promastigotes	Axenic amastigotes	Promastigots	Axenic amastigotes
AM1009	2.41 ± 0.28	1.03 ± 0.22	0.38 ± 0.13	0.98 ± 0.15
Clioquinol	7.90 ± 0.65	2.27 ± 0.44	4.45 ± 0.98	3.65 ± 0.25
Amphotericin B	0.13 ± 0.04	0.34 ± 0.11	0.09 ± 0.02	0.18 ± 0.05

3.2. Treatment of infected macrophages and inhibition of infection

The treatment of infected macrophages was evaluated, and results showed that all therapeutics were able to reduce the percentage of infected macrophages and the number of recovered amastigotes after treatment. With the obtained values, reductions in the infectiveness degree and in the number of amastigotes by macrophage were calculated, and data showed that both AM1009 and clioquinol showed most significant reductions in the parasitism in the treated cells, when compared to the treatment using AmpB (Table 3). The inhibition of infection using pre-treated parasites also demonstrated that AM1009 and clioquinol significantly reduced the infection and parasitism in the mammalian cells, presenting similar values when both the percentage of infection and the number of recovered amastigotes were evaluated (Table 4).

3.3. Mechanism of action in L. amazonensis

The mechanism of action of AM1009 in *Leishmania* was evaluated using L. *amazonensis* species. The evaluation of ΔΨm illustrated that treatment with this molecule induced significant parasite membrane depolarization, in the order of 24.6%, when 2.41 μM of AM1009 was used. The positive control showed a ΔΨm reduction of 35.9% (Fig. 1). The ROS production was also evaluated as an indicator of oxidative stress, and the results showed that AM1009-treated parasites presented higher production of this molecule, when compared to the control (Fig. 2). AM1009-treated parasites also showed significant alterations in their cell cycle, due to the occurrence of cells in the sub-G0/G1 phase (Fig. 3), as well as by the formation of autophagic vacuoles, thus indicating cell apoptosis (Fig. 4).

3.4. In vivo therapeutic effect against L. amazonensis infection

To evaluate the *in vivo* therapeutic effect of compounds against *Leishmania* infection in a mammalian model, BALB/c mice were infected with L. *amazonensis* promastigotes. At 50 to 60 days post-infection, the mice were separated into groups, and received saline or were treated with AM1009, clioquinol, or AmpB. The therapeutic efficacy was then evaluated by measuring the average lesion diameter and parasite load in the animals' infected tissue, liver, spleen, and dLN. Results showed significant reductions in the average lesion diameter and in the parasite load in the treated mice, when compared to saline group (Fig. 5). Comparing the results between them, animals receiving AM1009 presented lower lesion diameter (Fig. 5A) and parasite load (Fig. 5B) in the infection site and evaluated organs, when compared to clioquinol and AmpB groups. In an attempt to investigate the immunological profile generated in the animals, Th1 and Th2-type cytokines were measured in the cell supernatant of stimulated splenocytes. Results showed that clioquinol or AM1009-treated mice presented a more polarized Th1 response, with specific production of IFN-γ, IL-12, and GM-CSF, which were associated with lower antileishmanial IL-4 and IL-10 production (Fig. 6). Treatment using AmpB also induced the production of IFN-γ, IL-12, and GM-CSF but only in lower levels when compared to values obtained in the other groups (Fig. 6A). In addition, AM1009-treated mice, as compared to the clioquinol group, showed higher IFN-γ and lower IL-4 and IL-10 production, although no significant difference has been found between them. In an attempt to evaluate the parasite-specific activation of macrophages in the treated groups, the nitrite presence in culture supernatants was assayed as an

Table 2
Evaluation of the cytotoxicity and selectivity index. Murine macrophages (5×10^5 cells) were incubated with AM1009 (0, 0.06, 0.12, 0.25, 0.49, 0.98, 1.97, 3.93, 7.87, 15.73, and 31.46 μM), clioquinol (0, 0.07, 0.13, 0.26, 0.51, 1.03, 2.05, 4.09, 8.19, 16.37, and 32.73 μM) or AmpB (0, 0.04, 0.07, 0.14, 0.27, 0.54, and 1.08 μM) for 48 h at 37 °C. The cell viability was analyzed by the MTT method. The cytotoxicity was also evaluated in human cells, at which time the 50% red blood cell inhibitory concentration was determined through the incubation of AM1009, clioquinol, or AmpB, in the same concentrations as described above, with a 5% red blood cell (human O^+ type) suspension, for 1 h at 37 °C. In both cases, the CC_{50} and RBC_{50} values were calculated by applying a sigmoidal regression of the dose-response curve. Results were expressed as a mean ± standard deviation.

Compounds	CC_{50} (μM)	RBC_{50} (μM)	Selectivity index			
			Leishmania amazonensis		*Leishmania infantum*	
			Promastigotes	Axenic amastigotes	Promastigotes	Axenic amastigotes
AM1009	148.94 ± 10.12	1739.69 ± 79.69	61.80	144.60	391.95	152.0
Clioquinol	613.27 ± 20.53	1409.85 ± 64.35	77.63	270.16	137.81	168.0
Amphotericin B	0.85 ± 0.16	13.93 ± 1.95	6.54	2.50	9.44	4.7

Table 3
Treatment of infected macrophages. Murine macrophages were infected with L. *amazonensis* promastigotes (10 parasites per one macrophage) and later treated with AM1009 or clioquinol (0, 0.5, 1.0, 2.5, and 5.0 μM; each) for 48 h at 24 °C in 5% CO_2. AmpB (0, 0.11, 0.54, and 1.08 μM) was used as the control. The reduction of infectivity and the percentage of amastigotes in treated and infected macrophages were determined after counting 200 cells in triplicate. Results were expressed as mean ± standard deviation.

Compounds	Concentration (μM)	Infectiveness reduction (%)		Number of recovered amastigotes in treated macrophages	
		L. amazonensis	*L. infantum*	*L. amazonensis*	*L. infantum*
AM1009	5.0	91.7 ± 5.5	89.9 ± 6.7	0.3 ± 0.1	0.2 ± 0
	2.5	72.9 ± 4.6	70.3 ± 5.2	0.7 ± 0.2	0.4 ± 0.2
	1.0	53.4 ± 3.8	52.4 ± 4.9	1.8 ± 0.3	1.4 ± 0.4
	0.5	28.9 ± 2.9	24.5 ± 3.3	2.9 ± 0.7	2.5 ± 1.0
	0	(−)	(−)	6.8 ± 1.6	4.7 ± 0.5
Clioquinol	5.0	80.9 ± 5.1	67.7 ± 4.0	2.0 ± 0.6	1.5 ± 0.6
	2.5	53.2 ± 4.7	40.8 ± 2.7	2.8 ± 0.9	1.9 ± 0.7
	1.0	28.7 ± 3.3	18.9 ± 3.4	3.5 ± 1.7	2.6 ± 1.0
	0.5	18.8 ± 2.5	10.4 ± 1.6	4.4 ± 1.1	3.5 ± 0.8
	0	(−)	(−)	6.8 ± 1.6	4.7 ± 0.5
Amphotericin B	1.08	89.1 ± 3.1	85.8 ± 2.5	1.7 ± 0.5	1.3 ± 0.4
	0.54	79.2 ± 4.2	71.8 ± 3.0	2.2 ± 0.6	2.0 ± 0.7
	0.11	65.2 ± 5.4	51.7 ± 2.7	3.7 ± 1.2	3.1 ± 1.2
	0	(−)	(−)	6.8 ± 1.6	4.7 ± 0.5

Table 4
Inhibition of infection of macrophages using pre-treated parasites. The inhibition of infection of macrophages using pre-treated parasites was evaluated by incubating cells (5×10^6) with AM1009 or clioquinol (0, 0.5, 1.0, 2.5, and 5.0 μM; each) for 1 h at 24 °C. AmpB (0, 0.11, 0.54, and 1.08 μM) was used as a control. Parasites were then washed, quantified, and incubated with macrophages (10 parasites per one macrophage) for 24 h at 37 °C in 5% CO_2. The percentage of infection using pre-treated parasites and the number of recovered amastigotes per infected macrophage were determined after counting 200 cells in triplicate. Results were expressed as mean ± standard deviation.

Compounds	Concentration (μM)	Percentage of infection using pre-treated parasites		Number of recovered amastigotes per infected macrophage	
		L. amazonensis	*L. infantum*	*L. amazonensis*	*L. infantum*
AM1009	5.0	10.2 ± 1.7	11.3 ± 2.0	0.5 ± 0.2	0.4 ± 0.2
	2.5	21.2 ± 3.5	24.3 ± 2.5	1.0 ± 0.4	0.9 ± 0.3
	1.0	48.5 ± 4.3	45.5 ± 5.0	2.3 ± 0.9	2.0 ± 0.6
	0.5	66.5 ± 5.7	58.8 ± 4.4	3.5 ± 0.9	3.1 ± 1.1
	0	85.5 ± 3.4	63.3 ± 3.2	6.5 ± 1.4	4.5 ± 0.3
Clioquinol	5.0	23.6 ± 3.3	26.2 ± 2.7	1.5 ± 0.8	1.3 ± 0.3
	2.5	51.5 ± 3.8	49.7 ± 6.0	2.5 ± 0.7	2.0 ± 0.6
	1.0	64.3 ± 4.4	52.6 ± 4.9	3.6 ± 0.7	2.7 ± 1.0
	0.5	73.5 ± 5.3	58.6 ± 3.4	4.1 ± 1.3	3.6 ± 0.7
	0	85.5 ± 3.4	63.3 ± 3.2	6.5 ± 1.4	4.5 ± 0.3
Amphotericin B	1.08	15.5 ± 1.6	17.7 ± 2.1	0.5 ± 0.1	0.5 ± 0.2
	0.54	29.8 ± 3.2	26.7 ± 1.8	1.5 ± 0.3	1.3 ± 0.2
	0.11	45.5 ± 4.2	41.2 ± 3.2	2.8 ± 0.5	2.3 ± 0.6
	0	85.5 ± 3.4	63.3 ± 3.2	6.5 ± 1.4	4.5 ± 0.3

indicator of NO production. Results showed that the nitrite production was higher in the AM1009-treated and infected animals, when compared to the other groups (Fig. 6B). To characterize the humoral response triggered by the treatments, we have analyzed the profile of parasite-specific IgG1 and IgG2a isotypes. Results showed that treated animals produced higher IgG2a isotype levels, as compared to the IgG1 production, consequently presenting a higher IgG2a/IgG1 ratio when compared to the values obtained in the saline group (Fig. 6C). AM1009-treated mice were those presenting higher antibody isotype ratios when compared to the other groups. The organic toxicity showed that AmpB-

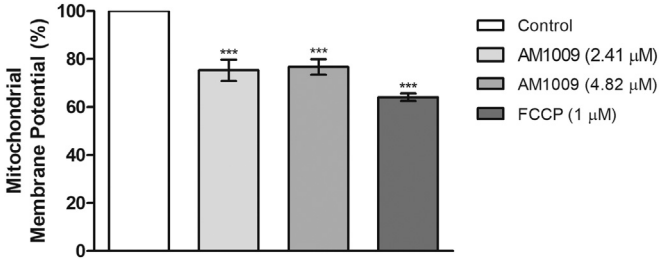

Fig. 1. Evaluation of the mitochondrial membrane potential in AM1009-treated parasites. *L. amazonensis* promastigotes (10^7 cells) were treated with 2.41 and 4.84 μM of AM1009 (corresponding to one and two times the EC_{50} values, respectively) for 24 h, when they were probed with JC-1 reagent. FCCP (1 μM)-treated promastigotes were used as a positive control. The analysis of labeling was performed in a fluorometer microplate reader. Data were expressed as mean ± standard deviation of the groups. (***) indicates statistically significant difference in relation to the control ($P < .001$).

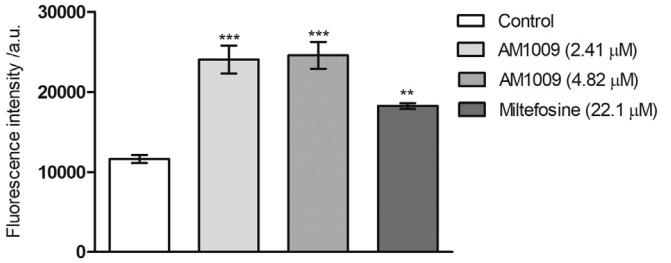

Fig. 2. Production of reactive oxygen species in AM1009-treated L. *amazonensis*. Promastigotes (10^7 cells) were untreated (negative control) or treated with AM1009 (2.41 and 4.84 μM, corresponding to one and two times the EC_{50} values, respectively) for 24 h, at which time they were probed with the H_2DCFDA reagent. Miltefosine (22.1 μM)-treated promastigotes were used as a positive control. The fluorescence intensity was evaluated in a fluorometer microplate reader. Data were expressed as mean ± standard deviation of the groups. (**) and (***) indicate statistically significant difference in relation to the control ($P < .01$ and $P < .001$, respectively).

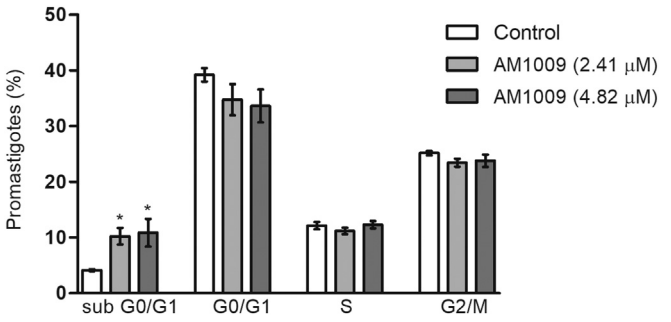

Fig. 3. Action of AM1009 on the L. *amazonensis* cell cycle. Parasites (10^7 cells) were incubated without (untreated cells) or with AM1009 (2.41 and 4.84 μM, corresponding to one and two times the EC_{50} values, respectively) for 24 h, at which time they were loaded with propidium iodide, and the DNA content was analyzed by flow cytometry. The promastigote percentage in each phase of the cell cycle was calculated and results are shown. Data were expressed as mean ± standard deviation of the groups. (*) indicates statistically significant difference in relation to the control ($P < .01$).

treated mice showed higher levels of renal and hepatic damage markers, when compared to the others (Fig. 7). Similar to that found in the immunological evaluation, AM1009-treated mice were those presenting lower levels of these enzymes, when compared to the other groups.

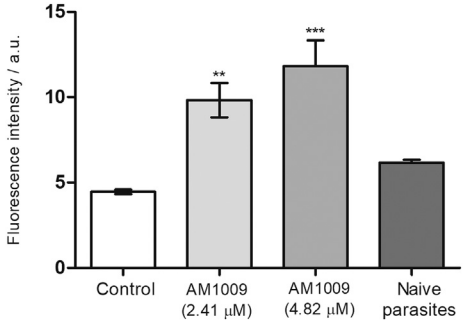

Fig. 4. Formation of autophagy vacuoles in AM1009-treated parasites. The formation of autophagy vacuoles in AM1009-treated *L. amazonensis* promastigotes was evaluated by fluorimetry, after incubation with AM1009 (2.41 and 4.84 μM, corresponding to one and two times the EC_{50} values, respectively) for 24 h. Non-treated promastigotes were used as a positive control. Results were expressed as mean ± standard deviation of the groups. (**) and (***) indicate statistically significant difference in relation to the control ($P < .01$ and $P < .001$, respectively).

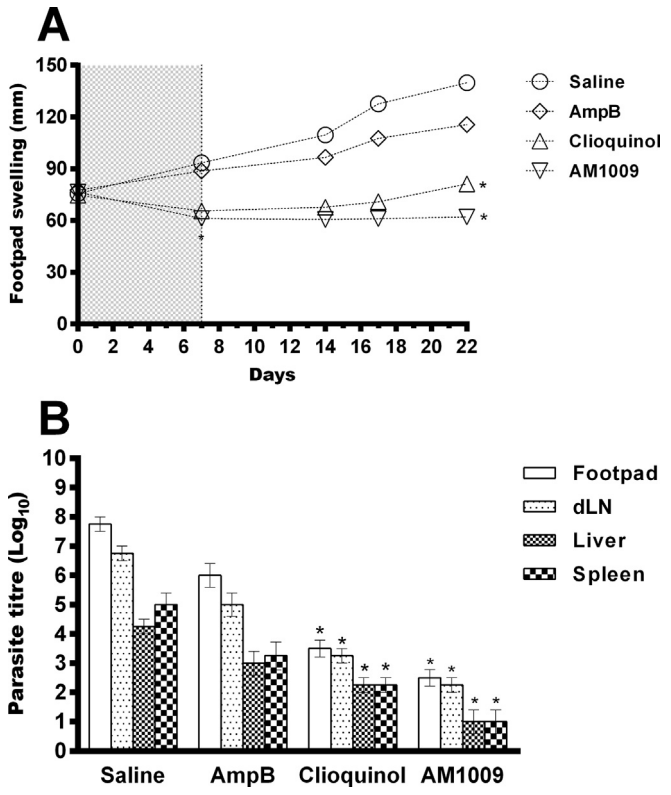

Fig. 5. *In vivo* therapeutic efficacy of the tested compounds. To evaluate the therapeutic action of AM1009, clioquinol, and AmpB in a mammalian model, BALB/c mice were infected with 10^7 *L. amazonensis* promastigotes. At 50 to 60 days post-infection, these mice were divided into groups ($n = 8$ per group) and received saline or were treated with AmpB, clioquinol, or AM1009. The lesion development was monitored weekly, and, 15 days after treatment, the parasite load was evaluated in the infected tissue, liver, spleen, and draining lymph nodes by applying the limiting dilution technique. Results showing the lesion's average diameter (A) and parasite load (B) are expressed as the mean ± standard deviation of the groups. (*) indicates a statistically significant difference in relation to the saline and AmpB groups ($P < .0001$).

4. Discussion

Advances in the biochemical research applied in *in vitro* and/or *in vivo* parasitological and immunological studies to kill *Leishmania* parasites have been developed in order to identify new and non-toxic

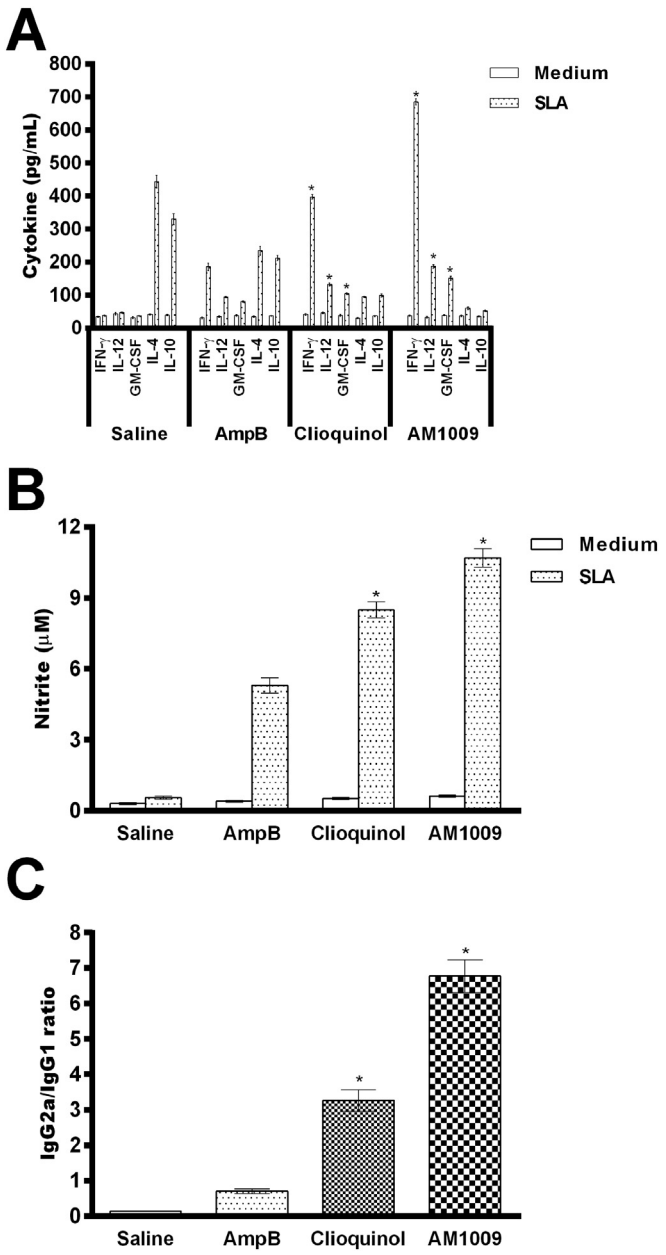

Fig. 6. Immune response developed in treated and infected animals. Splenocytes of treated and infected animals were collected 15 days after treatment, and cells were unstimulated (medium) or stimulated with L. *amazonensis* SLA (25.0 μg/mL) for 48 h at 37 °C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12p70, and GM-CSF levels (in A) were measured in the cell supernatant, using commercial kits. The nitrite production was also evaluated in the supernatants by Griess reaction (in B). In addition, sera samples were also collected of the animals, and the parasite-specific IgG1 and IgG2a isotype levels were evaluated by an indirect ELISA, at which time the ratio between the IgG2a/IgG1 values were calculated and are shown (in C). Bars represent the mean ± standard deviation of the groups. (*) indicate statistically significant difference in relation to the saline and AmpB groups ($P < .0001$).

and cost-effective antileishmanial products to control this neglected disease in the world [38]. However, current therapeutics present problems, such as side effects, including arthralgia, myalgia, fever, weakness, besides renal, hepatic and cardiac toxicity, together with high cost and/or poor availability. In addition, the parasite resistance against some drugs is increasing in several countries worldwide [39]. As a consequence, the search continues to find new antileishmanial targets, which should present a low toxicity and a lower cost but a high effectiveness in killing *Leishmania* parasites [40].

In the present study, a chloroquinoline derivative was tested for this biological purpose, and results proved to be highly effective in *in vitro* antileishmanial action against two important *Leishmania* species, L. *amazonensis* and *L. infantum*, in both parasite stages. Moreover, the molecule exhibited low toxicity in murine macrophages and in human red blood cells, showing satisfactory selectivity index values. The effect of AM1009 against intracellular amastigotes was observed in the treatment of infected macrophages, since this compound significantly diminished the infection percentage and the number of recovered amastigotes. In addition, the chloroquinoline derivate also demonstrated a potential to inhibit the macrophage infection, since the pre-treatment of parasites with the molecule induced a lower degree of infection in the murine cells, as well as a lower parasite load when the number of recovered amastigotes was evaluated. Another chloroquinoline derivative, clioquinol, was also used as a molecule control. Antileishmanial activity also combatted both parasite species, together with lower toxicity in murine macrophages and in human cells. However, results obtained with AM1009 were most significant in terms of efficacy of the treatment of infected macrophages and the inhibition of infection using pre-treated parasites.

Quinolines are heterocyclic compounds found in several plant families and that can be synthetized chemically. These are used in both folk and traditional medicines with a distinct biological activity, such as antimicrobicidal, anticancer, and antileishmanial agents [41]. In general, structural modifications in defined molecules are a cheaper and faster pathway to find new agents with biological activity [42]. In this aspect, quinoline derivatives have been produced and tested as antileishmanial agents, although the majority of the studies have performed *in vitro* experiments, while few are *in vivo* tested in infected hosts [32,40]. No less important, comparisons between the results obtained using such molecules in mammalian models must also be performed, especially when immunological and parasitological parameters are evaluated, in an attempt to select the best candidates for future studies in the treatment against disease.

Since the purpose of our study was to identify new antileishmanial targets to be applied in a future moment in clinical practice, *in vivo* experiments using a murine model were developed. For this, BALB/c mice were experimentally infected with L. *amazonensis* promastigotes and then treated with our molecule. Clioquinol and AmpB were used as controls. The efficacy of the products was evaluated by means of parasitological and immunological parameters, which were investigated 15 days after the treatment in the chronically-infected mice. Results showed that AM1009 was the most effective against L. *amazonensis* infection, since parasitological analyses demonstrated more significant reductions in the parasite load in infected tissue, spleen, liver, and dLN of the treated and infected animals, when compared to the others. Although clioquinol and AmpB have also showed reductions in the parasitism as compared to the saline group, values were higher in relation to those obtained using AM1009 in the infected animals. Similar results were found when some other natural or synthetic molecules, including those quinoline derivates, were tested in murine models against *Leishmania* infection [14,43].

The immune profile evaluated in the treated and infected animals showed the development of polarized Th1 response in AM1009-treated animals, which was based on high levels of IFN-γ, IL-12, GM-CSF, and nitrite, as well as by the presence of the parasite-specific IgG2a isotype antibody. The nitrite production by activated macrophages is known to play a major role in the elimination of parasites, such as *Leishmania* [44]. Data showed here suggest the macrophage activation in AM1009-treated animals, based on the higher antileishmanial nitrite levels that were found, thus relating to the lower parasitism found in infected tissue and organs of these animals. Clioquinol or AmpB-treated and infected mice also showed an antileishmanial Th1 response, although their spleen cells have produced lower IFN-γ and nitrite levels, as well as higher IL-4 and IL-10 levels, when compared to the AM1009-treated

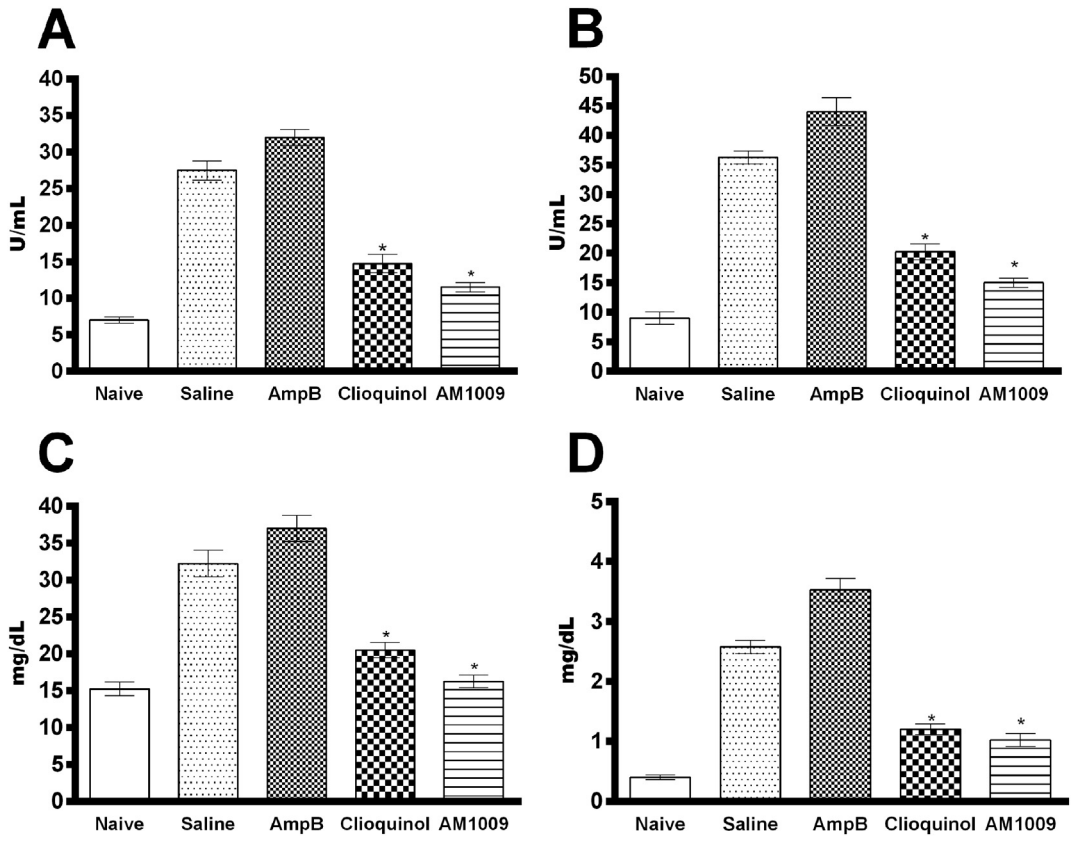

Fig. 7. Evaluation of *in vivo* toxicity in treated and infected mice. To evaluate the organic toxicity induced by the therapeutics, alanine aminotransferase (A), aspartate aminotransferase (B), urea (C), and creatinine (D) levels were measured in the sera samples collected from treated and infected animals (n = 8 per group), at 15 days post-treatment. Samples from naive (untreated and uninfected) animals ($n = 4$) were used as the control. Bars indicate the mean ± standard deviation of the groups. (*) indicates statistically significant difference in relation to the saline and AmpB groups ($P < .0001$).

group. On the other hand, saline group mice mounted a polarized Th2 response, which was characterized by high levels of IL-4, IL-10, and anti-*Leishmania* IgG1 antibody, corroborating with the susceptibility profile found when this mouse lineage is infected with L. *amazonensis* [45,46]. As a limitation of the study, the therapeutic efficacy in different periods of time after the treatment was not evaluated, and different dose schedules and the incorporation to delivery systems were not performed. Nevertheless, data presented here allow one to infer about an *in vitro* and *in vivo* effective antileishmanial action of AM1009, as well as by lower toxicity found in two distinct types of mammalian cells. Thus, this molecule can be considered to be an alternative antileishmanial derivate for studies against this disease.

The toxicity induced in treated and infected BALB/c mice was evaluated, and the results demonstrated that AM1009 or clioquinol caused no significant toxicity in the animals, since renal and hepatic markers were near the values found for the non-treated and non-infected animals. By contrast, AmpB-treated mice presented high levels of AST, ALT, urea, and creatinine, thus reflecting the toxicity of this drug when used in mammalians, such as that reported in other studies [47,48]. Although AmpB is considered to be an immunomodulatory agent, as it is able to induce the production of IL-1, TNF-α, and nitric oxide, it is also considered to be potentially nephrotoxic, the acute toxicity of which can cause fever, chills, nausea, vomiting, diarrhea, and headaches in patients [49,50].

The mechanism of action of AM1009 was evaluated in L. *amazonensis*. Results showed that the molecule induced ΔΨm depolarization, stimulated ROS production, and promoted alterations in the *Leishmania* cell cycle by increasing the sub-G0/G1 phase cell population. The ROS production is considered relevant for organisms, since it regulates the signaling pathways and cell proliferation; however, when present in high levels, it can cause oxidative stress and cell death. This production can also induce deleterious effects in the cell mitochondrial membrane, leading to ΔΨm depolarization and cell death [51,52]. Alterations in the sub-G0/G1 phase cell cycle indicate parasite stress, with the presence of a cell subpopulation presenting low DNA content, which is compatible with cell degradation [53]. In conclusion, these findings suggest that mitochondria were the target organelles in AM1009-treated parasites. Other quinoline derivates have also demonstrated a similar mechanism of action in *Leishmania*, such as 4-hydrazinoquinoline [54], QuinDer1 [55], and Flau-A [56].

Efforts towards the development of new antileishmanial candidates have been performed. This action has led to the discovery of new products with higher therapeutic efficacy, reduced toxicity, and broader utility. Here, a new chloroquinolin derivate presented a more selective action against two important *Leishmania* species. In addition, it was not toxic to mammalian cells derived from mice and humans and was effective in the treatment of infection caused in a mammalian host. Although other quinoline derivates are also under production and are being tested as antileishmanial targets, new options containing more effective and selective molecules are desirable, since the therapeutic arsenal available to treat against leishmaniasis is scarce, and the pharmaceutical industry is not interested in developing new products against this neglected disease. As a result, this study presents AM1009 as a new candidate to be tested in future studies for treatment against leishmaniasis in mammalian hosts.

Declaration of Competing Interest

The authors confirm that they have no conflicts of interest in relation to this work.

Acknowledgments

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