Activity of antimalarial drugs in vitro and in a murine model of cutaneous leishmaniasis

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The currently used treatments for leishmaniasis, a neglected parasitic disease, are associated with several side effects, high cost and resistance of the Leishmania parasites. Here we evaluated in vitro and in vivo the antileishmanial activity of five antimalarial drugs against Leishmania amazonensis. Mefloquine was effective against promastigotes in axenic cultures and showed an IC50 (concentration giving half-maximal inhibition) value of 8.4 ± 0.7 µM. In addition, mefloquine, chloroquine and hydroxychloroquine were active against intracellular amastigotes in macrophage-infected cultures, presenting IC50 values of 1.56 ± 0.19 µM, 0.78 ± 0.08 µM and 0.67 ± 0.12 µM, respectively. The ultrastructural analysis of chloroquine- or mefloquine-treated amastigotes showed an accumulation of multivesicular bodies in the cytoplasm of the parasite, suggesting endocytic pathway impairment, in addition to the formation of myelin-like figures and enlargement of the Golgi cisternae. CBA mice were infected with L. amazonensis in the ear dermis, and treated by oral and/or topical routes with chloroquine and mefloquine. Treatment of L. amazonensis-infected mice with chloroquine by the oral route reduced lesion size, which was associated with a decrease in the number of parasites in the ear, as well as the parasite burden in the draining lymph nodes. In contrast, mefloquine administration by both routes decreased the lesion size in infected mice without causing a reduction in parasite burden. Our results revealed a promising antileishmanial effect of chloroquine and suggest its use in cutaneous leishmaniasis treatment.

INTRODUCTION

Cutaneous leishmaniasis is a complex disease caused by protozoan parasites belonging to the genus Leishmania and transmitted by the bite of the female sand fly vector. Several Leishmania species are responsible for the wide spectrum of clinical manifestations of cutaneous leishmaniasis, including the mild cutaneous form (localized cutaneous leishmaniasis), multiple non-ulcerative nodules (diffuse cutaneous

leishmaniasis) and the disfiguring mucosal form (mucocutaneous leishmaniasis) (Kaye & Scott, 2011; Reithinger et al., 2007; Murray et al., 2005). Currently, the disease is present in 88 countries located in tropical and subtropical regions of the world, affecting about 12 million people, and presents significant social burdens due to the resulting deformities and disfiguring scars (Desjeux, 2001).

First-line drugs for the treatment of cutaneous leishmaniasis are the pentavalent antimonials. The two main antimonials in clinical use are N-methyl-D-glucamine and sodium stibogluconate (Frézard et al., 2009). Although the pentavalent antimonials represent the first-line treatment against all forms of leishmaniasis, this treatment can be
complicated by decreased sensitivity or resistance in some *Leishmania* species, variations in pharmacokinetics and drug–host immune response interaction (Croft *et al.*, 2006), as well as difficulties in parenteral route administration. In addition, antimonials present several side effects, including pain at the injection site, myalgia, anorexia, cardiotoxicity, and hepatic and renal insufficiency, which require long-term treatment and increase the cost of therapy. Second-line drugs like amphotericin B, pentamidine and paramomycin can be used for leishmaniasis treatment, but present similar limitations to those observed for antimonial treatment. The lipid formulation of amphotericin B and miltefosine have shown efficacy in the treatment of leishmaniasis. However, the high cost and side effects limit their usefulness (Oliveira *et al.*, 2011; WHO, 2010).

Thus, it is pivotal to develop a safer and more cost effective therapy against leishmaniasis. In this context, drug repositioning is a strategy to identify and develop new uses of existing drugs, reducing expenditure and research time (Ashburn & Thor, 2004). Antimalarial drugs may represent a promising source of new molecules with antileishmanial activity. Some of these drugs have pharmacokinetic profiles that are important for the treatment of intracellular pathogens which multiply within acidified vacuoles. Moreover, these drugs are cheap, well tolerated and have oral bioavailability (Rolain *et al.*, 2007; Bermudez *et al.*, 1999). Previous studies have showed the effect of some antimalarial drugs, like artemisinin and artsunate, against *Leishmania major* and *Leishmania donovani in vitro* and in murine disease models caused by infection with these parasite species (Yang & Liew, 1993; Sen *et al.*, 2010). Moreover, the use of mefloquine and chloroquine showed great efficacy in the treatment of human cutaneous leishmaniasis, in Ecuador (Landires *et al.*, 1995) and in Pakistan (Noor *et al.*, 2005; Khan *et al.*, 2007), respectively. The present study evaluated the activity of five antimalarial drugs (artesunate, chloroquine, hydroxychloroquine, mefloquine and primaquine), *in vitro* and in a murine model of cutaneous leishmaniasis caused by *Leishmania amazonensis*.

**METHODS**

**Drugs.** The antileishmanial activity of five antimalarial drugs was analysed in this study. Artesunate, chloroquine, mefloquine and primaquine were obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Galena Química Farmacêutica. All antimalarial drugs were obtained from Galena Quimica Farmaceutica. All antimalarial drugs were obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. Hydroxychloroquine was obtained from Farmanguinhos, Fundación Oswaldo Cruz, Brazil. Animals were housed in temperature-controlled rooms (22–25 °C) under a 12 : 12 h light–dark cycle, and provided with rodent diet and water *ad libitum*. Animals were handled according to the NIH (USA National Institutes of Health) guidelines for animal experimentation. Ketamine (100 mg kg⁻¹) and xylazine (10 mg kg⁻¹) was used as anaesthetics. All procedures described here had prior approval from the local animal ethics committee.

**Parasites.** *L. amazonensis* (MHOM/BR88/BA-125) were isolated from popliteal lymph nodes of infected C57BL/6 mice after cultivation in bificas Novy–Nicolle–MacNeal (NNN) medium containing Schneider’s insect medium (Sigma Aldrich) supplemented with 10 % fetal bovine serum (Gibco Laboratories) and 50 μg gentamicin ml⁻¹ (Hipolabor) at 24 °C. The promastigotes obtained were transferred to Schneider’s insect medium and cultivated until stationary growth phase was reached.

**Axenic culture of *L. amazonensis* promastigotes and treatment with antimalarial drugs.** *L. amazonensis* promastigotes, in stationary growth phase, were plated in 96-well plates at a density of 2 x 10⁶ parasites ml⁻¹ in 200 μl Schneider’s insect medium, either in the absence or presence of the test drugs at concentrations ranging from 0.6 to 50 μM. The parasite number was evaluated using a Neubauer chamber, every 24 h, for 72 h. The percentage of parasite growth inhibition in relation to the control was used to calculate the concentration of antimalarial drugs necessary to reduce the parasite growth by 50 % (IC₅₀, concentration giving half-maximal inhibition), after 72 h of culture. The IC₅₀ was assessed using concentration logarithm values followed by a non-linear regression curve fit.

**Cytotoxicity assays.** For the antimalarial drugs, the lethal concentration killing 50 % of cells (LC₅₀) was determined in cultures of peritoneal macrophages obtained from CBA mice 4 days after the injection of 3 % thiglycolate medium (Sigma Aldrich). Peritoneal exudate macrophages (5 x 10⁶ cells per well in 200 μl) were cultured in NNN plates in the presence of antimalarial drugs at concentrations ranging from 3.7 to 300 μM, for 48 h at 37 °C in 5% CO₂. Cells were incubated with 20 μl alamarBlue per well (Invitrogen) for 24 h. Colorimetric absorbance readings were performed at 570 and 600 nm, and the LC₅₀ value was calculated in relation to untreated cultures using concentration logarithm values, followed by a non-linear regression curve fit. Amphotericin B was used at concentrations ranging from 0.62 to 50 μM and gentian violet at concentrations ranging from 1.23 to 100 μM.

**In vitro macrophage infection and treatment with antimalarial drugs.** Peritoneal exudate macrophages were obtained as described above, and infected with *L. amazonensis*, as previously described by Gomes *et al.* (2003). Briefly, 2 x 10⁵ cells ml⁻¹ were plated in 24-well plates containing 13 mm-diameter glass coverslips. Peritoneal macrophage cultures were washed with sterile saline (NaCl 154 mM) to remove non-adherent cells and infected with *L. amazonensis* promastigotes in stationary growth phase at a ratio of 10 : 1 for 6 h at 37 °C in 5% CO₂. After washing to remove non-internalized parasites, cultures were treated with the drugs at a maximum concentration of 5 μM at 24 and 48 h. In another set of experiments, macrophages were infected for 6 h and left untreated for 48 h to allow the differentiation and replication of parasites into amastigotes before exposure to the drugs (Chaves *et al.*, 2009). The macrophage cultures were then treated with the selected drugs, based on the previous *in vitro* infection, at concentrations ranging from 0.06 to 5 μM for 48 h, to determine their IC₅₀ values. At the end of each treatment, the cells were fixed with ethanol and stained with conventional haematoxylin and eosin (H&E). The selective index (SI) was defined as the ratio between LC₅₀ and IC₅₀ (Bézivin *et al.*, 2003). To determine the viability of the remaining parasites, cultures of infected macrophages
were washed with sterile saline after drug treatment and incubated with Schneider’s insect medium at 24 °C. Intracellular survival and viability of *L. amazonensis* was measured by counting motile promastigotes after 96 h of culture.

**Transmission electron microscopy.** Peritoneal macrophages obtained from CBA mice were infected with *L. amazonensis*, as described above, and treated, after 48 h to allow amastigote differentiation, with chloroquine and mefloquine for an additional 48 h. Infected macrophages were fixed in a solution of 2.5 % glutaraldehyde, 2 % formaldehyde and 2.5 mM CaCl₂ in 0.1 M sodium cacodylate buffer pH 7.2, followed by post-fixation in 1 % osmium tetroxide and 0.8 % potassium ferricyanide in the same buffer and acetone gradient dehydration. Finally the material was embedded in Poly/Bed resin; ultrathin sections were stained with uranyl acetate and lead citrate and observed under a JEM 1320 transmission electron microscope (JEOL).

**In vivo infection with *L. amazonensis***. Female CBA mice, 4 to 6-weeks-old, were infected in the right ear dermis with 5 × 10³ *L. amazonensis* promastigotes in stationary growth phase in 10 μl of saline (NaCl 154 mM). After 1 week of infection, mice were treated daily with chloroquine or mefloquine by oral or topical routes, or with a combined administration by both routes, for 4 weeks. The ointment was formulated in commercially available cold cream and the topical treatment was performed by spreading the ointment in the infected ear. The ear swelling was monitored weekly, using a digital caliper (Mitutoyo) and was determined as the difference in thickness between the infected and contra-lateral uninfected ear. The control groups received saline and the base ointment formulation.

**Parasite quantification.** The parasite number in the draining lymph nodes was estimated by limiting dilution assay, as described previously by Guimaraes *et al.* (2006), with some modifications. Briefly, the right auricular lymph nodes were removed and used to prepare a cell suspension in ice-cold saline solution. The cells were plated in Schneider’s insect medium at a density of 5 × 10⁶ cells per well in a 24-well cell culture plate. The cell suspension then underwent 10-fold serial dilutions and was distributed in 96-well culture plates (8 replicates per dilution). After 7 to 10 days of incubation at 24 °C, the wells were examined with an inverted microscope to evaluate the presence or absence of the promastigote form. Results were expressed as the log of the highest cell suspension dilution for wells positive for *Leishmania* promastigotes.

**Histopathological and immunohistochemical evaluation.** Infected ears of CBA mice were removed after 5 weeks of infection and fixed in 10 % formaldehyde. After 12–24 h of fixation, tissue slices were embedded in paraffin. Sections at 3–5 μm thick were stained with conventional H&E, and analysed by light microscopy. To demonstrate the presence of parasites at the site of infection, we performed an immunostaining for *L. amazonensis* in the 3–5 μm thick sections obtained from formalin-fixed and paraffin-embedded tissue, according to Guimaraes *et al.* (2006). Briefly, the sections were incubated with a rabbit polyclonal antibody against *Leishmania*. The primary antibody was detected using a biotinylated goat anti-rabbit antibody (American Qualex) and streptavidin-conjugated peroxidase was used for secondary antibody detection. The reaction was revealed using 3, 3’-diaminobenzidine (DAB) (Sigma Aldrich) in PBS. Tissue sections were then counterstained with Harris’s haematoxylin and examined under light microscopy.

**Statistical analyses.** According to normality analysis (Kolmogorov–Smirnov), mean differences between groups were analysed using either one-way ANOVA or two-way ANOVA, when one variable was evaluated or two independents variables were evaluated at the same time, followed by Bonferroni’s multiple comparison test. An unpaired Student’s *t*-test was used to compare the means between only two groups. For non-parametric data, the Mann–Whitney test was used. All analyses were performed using GraphPad Prism version 5.01 (GraphPad Software). Data were presented as the mean ± SEM and statistical difference was considered significant at *P* < 0.05.

## RESULTS

**Activity of antimalarial drugs against axenic promastigotes of *L. amazonensis***

The activity of the antimalarial drugs was first evaluated against axenic promastigotes of *L. amazonensis*, in order to determine the IC₅₀ values of these molecules. Artemisinate, chloroquine, hydroxychloroquine, mefloquine and primaquine were tested at five different concentrations, ranging from 0.6 to 50 μM, over 72 h. Chloroquine and hydroxychloroquine did not significantly affect the parasitic growth even at 50 μM (Fig. 1a, b). At this concentration, artesunate and primaquine significantly affected the parasite growth, but the inhibition was less than 50 % (Fig. 1c, d). Mefloquine at 50 μM, as well as amphotericin B (tested at 1 μM), inhibited completely the growth of *L. amazonensis* compared to untreated controls (Fig. 1e, f). After 72 h, mefloquine inhibited promastigote growth with an IC₅₀ of 8.4 ± 0.7 μM. However, this molecule was less potent than amphotericin B, which presented an IC₅₀ of 0.13 ± 0.01 μM (Table 1).

**Effects of antimalarial drugs in in vitro macrophage infection with *L. amazonensis***

To test the effects of antimalarial drugs in *L. amazonensis* infection, we first determined the cytotoxicity to mammalian cells for the five compounds. Peritoneal macrophages were incubated with artesunate, chloroquine, hydroxychloroquine, mefloquine and primaquine at five different concentrations. All molecules tested presented a lower cytotoxicity than gentian violet, a standard cytotoxic drug. Artesunate had the lowest cytotoxicity of the five antimalarials tested, while mefloquine was the most toxic to macrophages (Table 1).

The activity of the antileishmanial drugs was evaluated next in cultures of *L. amazonensis*-infected peritoneal macrophages. Chloroquine and mefloquine (5 μM) significantly reduced the percentage of infected cells and the number of parasites 24 h after treatment (*P* < 0.0001, Fig. 2a, b). Chloroquine reduced the parasitism by 86.8 %, while mefloquine showed antileishmanial activity similar to that of amphotericin B (95 % reduction of parasite numbers) after 48 h of treatment. At this treatment time point, hydroxychloroquine also significantly reduced the percentage of infected cells and, similar to artesunate and primaquine, reduced the parasitism (*P* < 0.0001, Fig. 2c, d). In cultures incubated with the drugs for 96 h, we did not find a statistically significant difference between the activity of chloroquine and amphotericin B, whereas...
hydroxychloroquine inhibited the parasitism by 68.7% (Fig. S1 available in JMM Online). Based on these results, chloroquine, hydroxychloroquine and mefloquine were selected for the following experiments.

To validate the activity of the selected drugs against intracellular parasites, *L. amazonensis*-infected macrophages were cultured for 48 h for complete amastigote differentiation and parasite replication, followed by treatment with the antimalarial drugs for an additional 48 h. Chloroquine, hydroxychloroquine and mefloquine at 5 μM reduced the number of infected macrophages by 53, 43 and 58%, respectively (Fig. 3a). In addition, chloroquine and mefloquine decreased the parasite burden by 75% and 77%, respectively, while hydroxychloroquine was less effective, reducing 59% of the amastigotes when compared to untreated controls (Fig. 3b). Amphotericin B

![Graphs showing growth kinetics of parasites and drugs](image)

**Fig. 1.** Effects of antimalarial drugs on the growth of *L. amazonensis* axenic promastigotes. The parasites (2×10^6 cells ml⁻¹) were cultivated in Schneider’s medium with antimalarial drugs at a maximum concentration of 50 μM (a–e) or amphotericin B at a maximum concentration of 1 μM (f). The growth kinetics were assessed by counting cells in a Neubauer chamber every 24 h. Results shown are the means ± SEM of six replicates, and represent one experiment of three performed. *, *P < 0.0001 compared to the control group (by unpaired Student’s *t*-test).

The IC₅₀, LC₅₀ and SI values of antimalarial drugs are summarized in Table 1.

**Table 1.** IC₅₀, LC₅₀ and SI values of antimalarial drugs

<table>
<thead>
<tr>
<th>Antimalarial drug</th>
<th>IC₅₀ (μM)</th>
<th>LC₅₀ (μM)</th>
<th>SI</th>
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<tr>
<td></td>
<td><em>L. amazonensis</em></td>
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<tr>
<td>Promastigotes</td>
<td>Amastigotes</td>
<td></td>
<td></td>
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<tr>
<td>Artesunate</td>
<td>&gt;50*</td>
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<td>Chloroquine</td>
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<td>Hydroxychloroquine</td>
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<td>0.67 ± 0.12†</td>
<td>140.60 ± 10.34</td>
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<td>Mefloquine</td>
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<td>1.56 ± 0.19†</td>
<td>11.95 ± 0.93</td>
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<tr>
<td>Primaquine</td>
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<td>68.59 ± 4.18</td>
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<tr>
<td>Gentian violet</td>
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<td>ND</td>
<td>4.31 ± 0.22</td>
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<tr>
<td>Amphotericin B</td>
<td>0.13 ± 0.01</td>
<td>0.09 ± 0.02</td>
<td>47.98 ± 7.65</td>
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*Treatment at 50 μM inhibited less than 50% of parasite growth.
†*P < 0.05 compared to amphotericin B (unpaired Student’s *t*-test).
almost completely reduced both the number of infected macrophages, as well as the parasite burden (Fig. 3a, b). The viability of the macrophages was confirmed by alamarBlue metabolism after 48 h of treatment (data not shown). Chloroquine significantly reduced the parasite burden at concentrations as low as 0.06 &mu;m (P<0.001), while the lowest active concentration of hydroxychloroquine and mefloquine was 0.56 &mu;m (P<0.001, Fig. 3c). Chloroquine and hydroxychloroquine had IC_{50} values of 0.78 ± 0.08 &mu;m and 0.67 ± 0.12 &mu;m, respectively, while mefloquine had an IC_{50} of 1.56 ± 0.19 &mu;m. Amphotericin B, however, was more potent than the antimalarial drugs analysed and presented an IC_{50} of 0.09 ± 0.02 &mu;m (Table 1).

Next we evaluated the viability of the parasites after drug treatment, by using an assay to differentiate intracellular amastigotes into promastigotes. After drug treatment, infected macrophages were cultured in Schneider’s insect medium at 24 °C for 96 h. As expected, there were significantly less promastigotes in wells from infected macrophages treated with chloroquine and mefloquine when compared to the control group (P<0.0001; Fig. 3d). However, after withdrawal, the treatment parasites were able to differentiate and proliferate as promastigotes.

Ultrastructural analysis of amastigotes treated with chloroquine and mefloquine

An ultrastructural analysis of differentiated intracellular amastigotes treated with chloroquine or mefloquine was performed. As shown in Fig. 4a, the untreated amastigotes had organelles with normal morphology and an organized cytoplasm. Treatment of *L. amazonensis*-infected cells with chloroquine caused an accumulation of multivesicular bodies in the parasite cytoplasm, a complete disorganization of parasite structure and disruption of the plasma membrane (Fig. 4b, c). Moreover, mefloquine was able to induce the formation of myelin-like figures and enlargement of Golgi cisternae, as well as the accumulation of multivesicular bodies (Fig. 4d, e, f).

Treatment of *L. amazonensis*-infected CBA mice with chloroquine and mefloquine

CBA mice were infected in the right ear dermis with 5 × 10^5 promastigotes of *L. amazonensis* and treated daily, after 1 week of infection, with chloroquine or mefloquine through oral and/or topical routes. Four weeks after treatment, the group treated with chloroquine by the oral route had significantly smaller lesions than those of animals treated...
by the topical route, as well as vehicle-treated mice ($P<0.01$, Fig. 5a). The parasite burden in the draining lymph nodes of mice orally treated with chloroquine with or without topical treatment was lower than that of the control group ($P=0.01$, Fig. 5b). Oral chloroquine treatment reduced the number of inflammatory cells, the

![Graphs showing percentage of infected macrophages and percentage of parasites](image)

**Fig. 3.** Effects of antimalarial drugs against amastigotes from *L. amazonensis*. Macrophages from peritoneal exudate were collected from CBA mice and infected with *L. amazonensis* promastigotes in stationary growth phase. Cells were incubated for 48 h without treatment for amastigote differentiation and parasite replication. After this period, the macrophages were treated with selected antimalarial drugs at 5 µM or lower concentrations for 48 h. Amphotericin B was used as a positive control at 5 µM. (a) The percentage of infected cells was evaluated by counting 100 cells. The control group was considered as 100 % (86.17 ± 1.24 %). (b) Parasite number was evaluated by counting 100 cells. The control group was considered as 100 % (624.67 ± 65.18 amastigotes per 100 macrophages). (c) Concentration–response curve. The number of amastigotes was evaluated as described above for each concentration after treatment of the infected macrophages. The control group was considered 100 % (817.83 ± 52.31 amastigotes per 100 macrophages). *, $P<0.0001$ repeated measures (ANOVA followed by Bonferroni’s multiple comparison test). Results shown are the means ± SEM of the percentages related to the control of three experiments performed in triplicate. (d) Parasite viability was evaluated by transformation of amastigotes in motile promastigotes after medium change. *, $P<0.0001$ (one-way ANOVA followed by Bonferroni’s multiple comparison test). Results shown are the means ± SEM of six replicates, and represent one of two experiments performed.

![Transmission electron microscopy images](image)

**Fig. 4.** Transmission electron microscopy images of amastigotes from *L. amazonensis* after treatment with chloroquine or mefloquine at 5 µM for 48 h. (a) Untreated intracellular parasite displaying organelles with normal morphology and an organized cytoplasm. (b, c) Parasites treated with chloroquine presenting multivesicular bodies in the cytoplasm (arrow and inset), complete disorganization of cellular structure and disruption of plasma membrane (star). (d, e, f) Parasites treated with mefloquine showing myelin-like figures (white arrow), accumulation of multivesicular bodies in the cytoplasm (black arrow) and enlargement of the Golgi cisternae (star and inset), respectively. k, kinetoplast; m, mitochondrion; n, nucleus.
presence of vacuolated macrophages and intracellular parasite number in the infected ear, while vehicle-treated animals had an extensive collection of vacuolated and heavily parasitized macrophages at the lesion site, as demonstrated by histopathological analysis (Fig. 6a, b) and immunostaining (Fig. 6c, d). Moreover, animals treated with chloroquine by the oral route also presented a mixed infiltrate composed of polymorphonuclear cells and lymphocytes.

Regardless of the route of administration, mefloquine-treated mice also presented significantly smaller lesions than vehicle-treated mice ($P < 0.01$, Fig. 5c). However, the parasite burden in the draining lymph nodes (Fig. 5d), as well as the parasitism in the infected ear (data not shown), did not differ from that of the control group.

**DISCUSSION**

In this work we tested the drug repositioning strategy to evaluate the antileishmanial activity of five drugs currently used in the treatment of *Plasmodium* spp. infection. Five antimalarial drugs were tested against *L. amazonensis*, a parasite species that causes localized and diffuse cutaneous leishmaniasis in the New World (Kaye & Scott, 2011).

According to the present study, artesunate was not capable of reducing promastigote growth in axenic cultures, and presented a reduced activity against the intracellular form of the parasite. In a previous report, the antileishmanial activity of artesunate was demonstrated against *L. major* in an experimental model of cutaneous leishmaniasis (Yang & Liew, 1993). The divergence between these results may be related to the susceptibility of the different species of parasite to the compound tested as a consequence of the different genotypes between *Leishmania* species (Smith *et al.*, 2007).

Primaquine also showed reduced activity against promastigotes and intracellular *L. amazonensis*. A published work showed moderate activity of primaquine against intracellular *L. donovani* in an *in vitro* infection model, unless loaded in nanocapsules (Heurtault *et al.*, 2001).

Chloroquine and its derivative compound, hydroxychloroquine, were significantly active against *L. amazonensis* amastigotes. However, mefloquine presented significant activity against both promastigotes and amastigotes. It is
Chloroquine treatment by the oral route showed a promising pharmacological effect in *L. amazonensis*-infected animals. In addition to reducing the lesion size that is related to intense parasite multiplication in the infected ear, this treatment decreased the parasitism in the draining lymph nodes of CBA mice. To date, no literature evidence has demonstrated a mouse strain completely resistant to *L. amazonensis* infection. BALB/c and C57BL/6 mice present high susceptibility to infection, while C3H, DBA, CBA and C57BL/6 have partial resistance to infection with *L. amazonensis* (Pereira & Alves, 2008). According to Awasthi et al. (2004), the BALB/c model is not suitable for testing new compounds since the effective dose would be much higher to cure *Leishmania* infection in this model because of the high susceptibility of this mouse strain and fast progression of the disease. However, CBA mice have slow lesion development following persistent parasitism. Based on that, CBA mice are a useful experimental model of cutaneous leishmaniasis caused by *L. amazonensis* to evaluate the *in vivo* activity of a new drug candidate (Lemos de Souza et al., 2000; de Oliveira Cardoso et al., 2010). The anti-inflammatory features of chloroquine, such as decreased tumour necrosis factor alpha production (Jang et al., 2006), may be important to reduce tissue damage in cutaneous leishmaniasis lesions (de Oliveira Cardoso et al., 2010; Faria et al., 2005). Moreover, previous reports have suggested a large spectrum of action of chloroquine in infectious diseases (Rolain et al., 2007), including the efficacy of its oral and intraleisional treatments in human cutaneous leishmaniasis (Khan et al., 2007; Noor et al., 2005).

In our study, treatment with mefloquine was not associated with a reduction of parasite burden in the draining lymph nodes, despite the reduction of the lesion size. Mefloquine also has a large range of action against other parasite infections besides malaria, such as alveolar echinococcosis (Küster et al., 2011) and human schistosomiasis (Keiser et al., 2011; Ingram et al., 2012). However, the pharmacological effect of mefloquine in *Leishmania* infection remains controversial. According to Galvão et al. (2000) and Laguna-Torres et al. (1999), oral treatment with mefloquine had a limited therapeutic effect in C57BL/6 mice infected with *L. amazonensis*, as well as in the treatment of human cutaneous leishmaniasis caused by *Leishmania braziliensis*. However, Landires et al. (1995) reported a promising therapeutic effect of mefloquine in human cutaneous leishmaniasis caused by *Leishmania panamensis*. The positive results reported by Landires et al. (1995) during infection by *L. panamensis* may be due to the combination of the host immune response with drug action.
to the species of parasite involved in the infection, which is known to be associated with more positive post-treatment (Laguna-Torres et al., 1999). Finally, the genetic and biological diversity of the parasites involved in such studies may contribute to observed differences (Smith et al., 2007).

Based on the results presented here, chloroquine showed a promising pharmacological effect for the treatment of cutaneous leishmaniasis. In addition, chloroquine has low toxicity and cost, and exhibits oral route bioavailability, making its use easier in endemic areas and improving patient compliance with the treatment. Chloroquine may also be used in combined therapy with conventional first or second-line antileishmanial drugs, thereby reducing the dose, side effects and time of treatment. The investigation of the mechanism involved in the effect observed in this work will improve the understanding of the chloroquine action in *Leishmania* parasites. This study may help the development of new therapies of leishmaniasis based on the use of chloroquine alone or as combined therapy.

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**REFERENCES**


