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 IC_{50} (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 IC_{50} 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

Abbreviations: H&E, haematoxylin and eosin; IC_{50}, concentration giving half-maximal inhibition; LC_{50}, lethal concentration killing 50 % of cells; SI, selective index.

A supplementary figure is available with the online version of this paper.

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 leishmaniases. 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 artesunate, 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 diluted in saline (NaCl 154 mM), with the exception of the artesunate, which was diluted in 100 % DMSO (Sigma Aldrich). When used in culture medium, the maximum concentration of DMSO was 0.2 % at the highest concentration of artesunate. The same amount of solvent was used in the control group. Amphotericin (Gibco Laboratories) was used as a positive control. Gentian violet (Synth) was used as a cytotoxicity control.

Mice. Male or female, 4 to 8-week-old C57BL/6 and CBA mice were obtained from the Animal Facilities of the Gonçalo Moniz Research Center-FIOCRUZ (Fundação 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 bifasic 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 2×10^6 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 % (IC_{50}, concentration giving half-maximal inhibition), after 72 h of culture. The IC_{50} 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 (LC_{50}) was determined in cultures of peritoneal macrophages obtained from CBA mice 4 days after the injection of 3 % thioglycolate medium (Sigma Aldrich). Peritoneal exudate macrophages (5×10^4 cells per well in 200 μl) were cultured in 96-well plates in the presence of antimalarial drugs at concentrations ranging from 3.7 to 300 μM, for 48 h at 37 °C in 5 % CO_2. 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 LC_{50} 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, 2×10^5 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% CO_2. 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 IC_{50} 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 LC_{50} and IC_{50} (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 $CaCl_2$ 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 5×10^5 *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 Guimarães *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 5×10^5 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 Guimarães *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 $\pm$ 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 IC_{50} values of these molecules. Artesunate, 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 IC_{50} of 8.4 ± 0.7 µM. However, this molecule was less potent than amphotericin B, which presented an IC_{50} 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

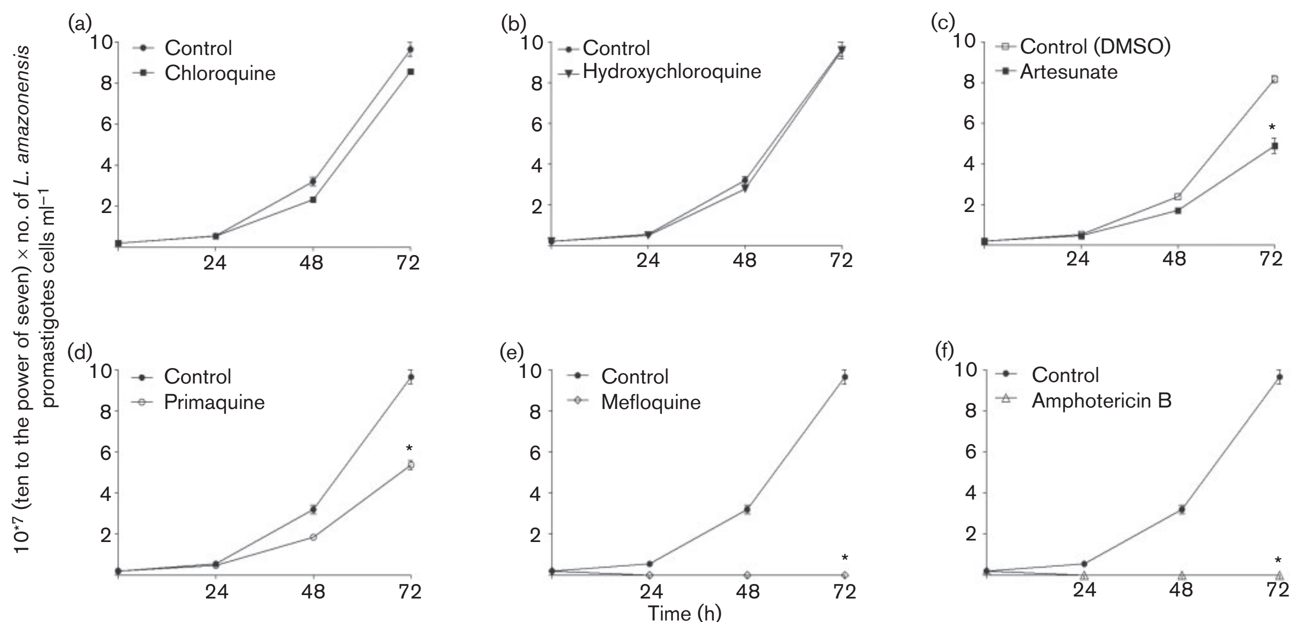

Fig. 1. Effects of antimalarial drugs on the growth of *L. amazonensis* axenic promastigotes. The parasites (2×10^6 cells ml^{-1}) were cultivated in Schneider's medium with antimalarial drugs at a maximum concentration of 50 μM (a–e) or amphotericin B at a maximum concentration of 1 μM (f). The growth 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).

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

Table 1. IC_{50}, LC_{50} and SI values of antimalarial drugs

Values represent the mean ± SEM from two independent experiments performed in triplicate. ND, Not determined.

Antimalarial drug	IC_{50} (μM)		LC_{50} (μM)	SI
	L. amazonensis		Macrophages	
	Promastigotes	Amastigotes		
Artesunate	>50*	ND	142.12 ± 483	ND
Chloroquine	>50*	0.78 ± 0.08†	108.12 ± 10.61	138.61
Hydroxychloroquine	>50*	0.67 ± 0.12†	140.60 ± 10.34	209.85
Mefloquine	8.4 ± 0.7†	1.56 ± 0.19†	11.95 ± 0.93	7.66
Primaquine	>50*	ND	68.59 ± 4.18	ND
Gentian violet	ND	ND	4.31 ± 0.22	ND
Amphotericin B	0.13 ± 0.01	0.09 ± 0.02	47.98 ± 7.65	533.11

*Treatment at 50 μM inhibited less than 50 % of parasite growth.
†$P<0.05$ compared to amphotericin B (unpaired Student's *t*-test).

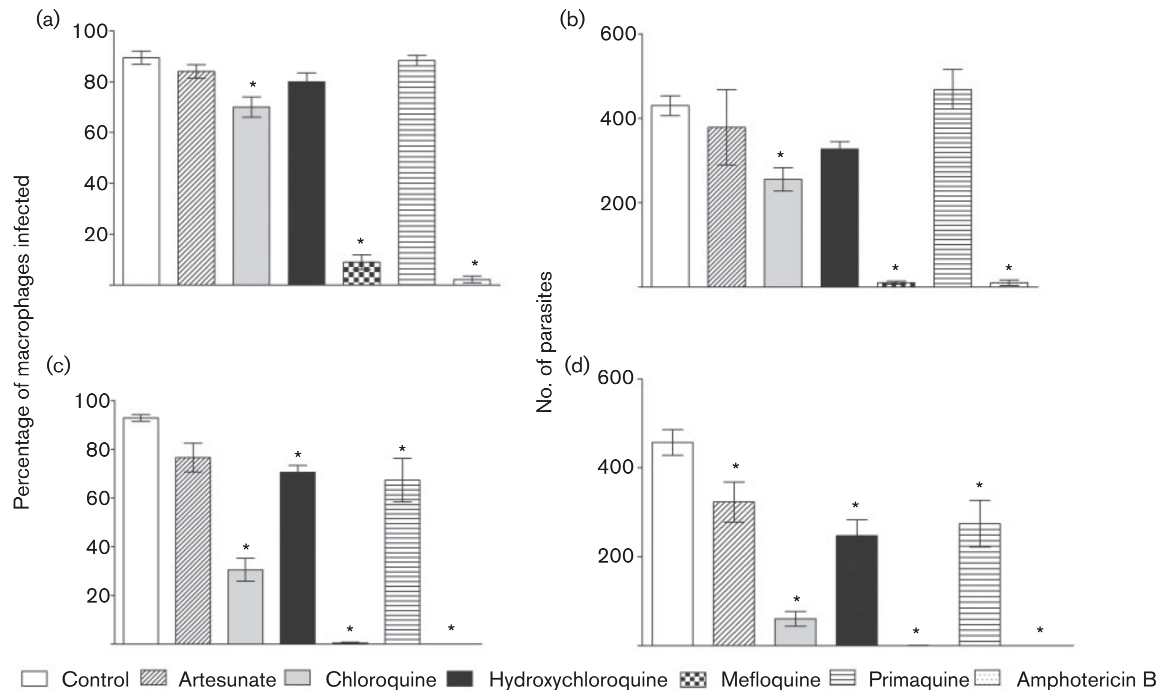

Fig. 2. Screening of antimalarial drugs against intracellular *Leishmania* parasites. Macrophages from peritoneal exudate were collected from CBA mice and infected with *L. amazonensis* promastigotes in the stationary phase of growth. The macrophages were treated with antimalarial drugs or amphotericin B at 5 μM, and the percentage of infected cells and the number of parasites were measured 24 (a, b) and 48 h (c, d) after treatment by counting 100 macrophages. Results shown are means ± SEM of triplicates, and represent one experiment of three performed. *, $P<0.0001$ compared to the control group (by one-way ANOVA followed by Bonferroni's multiple comparison 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 μM ($P<0.001$), while the lowest active concentration of hydroxychloroquine and mefloquine was 0.56 μM ($P<0.001$, Fig. 3c). Chloroquine and hydroxychloroquine had IC_{50} values of 0.78 ± 0.08 μM and 0.67 ± 0.12 μM, respectively, while mefloquine had an IC_{50} of 1.56 ± 0.19 μM. Amphotericin B, however, was more potent than the antimalarial drugs analysed and presented an IC_{50} of 0.09 ± 0.02 μM (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

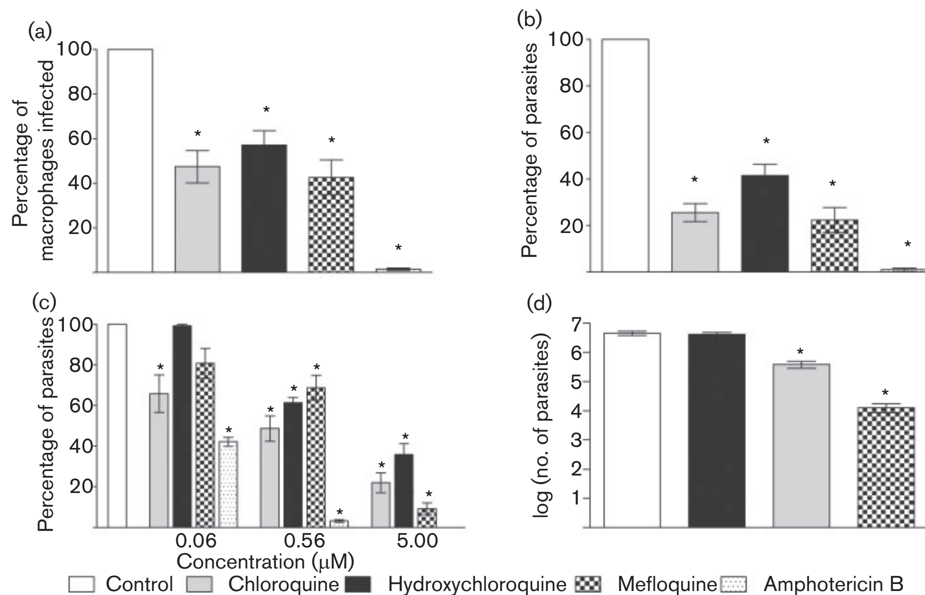

Fig. 3. Effects of antimalarial drugs against amastigotes from *L. amazonensis*. Macrophages from peritoneal exudate were collected from CBA mice and infected with *L. amazonensis* promastigotes in stationary growth phase. Cells were incubated for 48 h without treatment for amastigote differentiation 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.

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

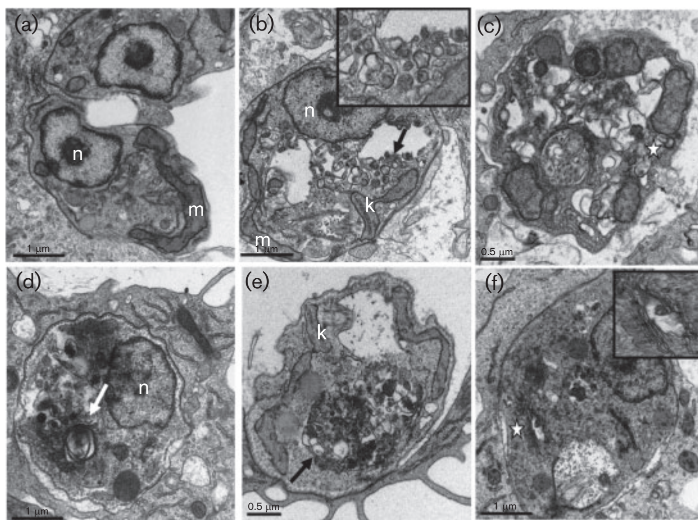

Fig. 4. Transmission electron microscopy images of amastigotes from *L. amazonensis* after treatment with chloroquine or mefloquine at 5 μM for 48 h. (a) Untreated intracellular parasite displaying organelles with normal morphology and an organized cytoplasm. (b, c) Parasites treated with chloroquine 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.

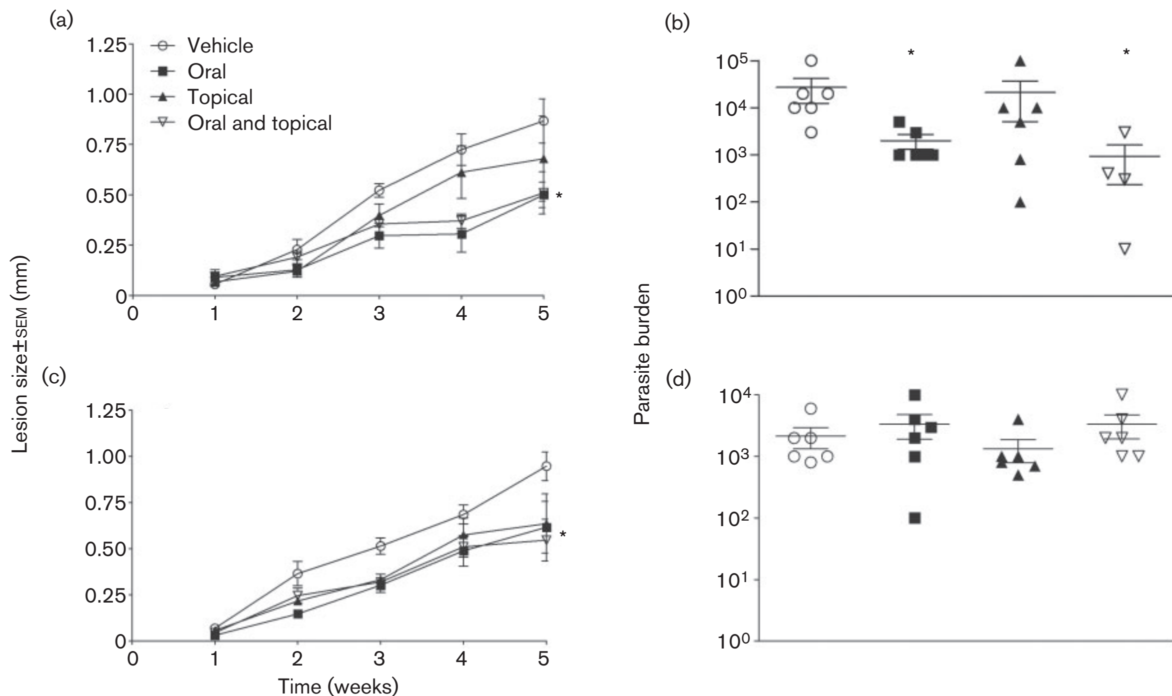

Fig. 5. Pharmacological effect of antimalarial drugs in an experimental model of cutaneous leishmaniasis. CBA mice were infected with 5×10^5 *L. amazonensis* promastigotes and lesion development was monitored for 5 weeks, as well as the number of parasites at the end of the experiment. (a, c) Lesion size expressed as the mean ± SEM of a representative experiment from two performed ($n=6$ per group) after treatment with chloroquine (a) or mefloquine (c) by oral (50 mg chloroquine kg^{-1} or 20 mg mefloquine kg^{-1}), topical (1 % for both drugs) and both routes of administration. *, $P<0.01$ (two-way ANOVA followed by Bonferroni's multiple comparison test). (b, d) Parasite burdens in the draining lymph nodes after treatment with chloroquine (b) or mefloquine (d) as above were measured after 5 weeks post-infection by limiting dilution assay. Data are expressed as the mean ± SEM of a representative experiment from two performed. *, $P=0.01$ (Mann–Whitney test).

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

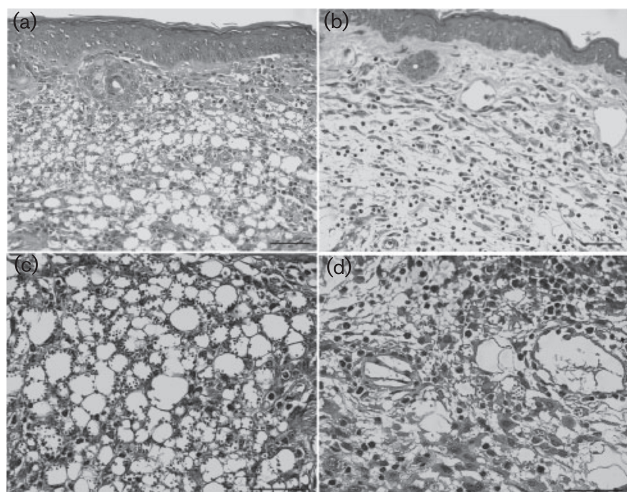

Fig. 6. Histology of the infected ears of CBA mice after oral treatment with chloroquine. Vehicle-treated mice presented an extensive collection of vacuolated and heavily parasitized macrophages, as demonstrated by histological analysis (a) and immunoperoxide staining for *L. amazonensis* amastigotes (c). Oral route chloroquine-treated animals displayed a reduced number of vacuolated macrophages in the infected ear (b), as well as a reduced number of parasites (d). (a, b) H&E stained cells, magnification ×40. (c, d) Immunohistochemical stained cells using a polyclonal anti-*Leishmania* antibody, magnification ×60.

noteworthy that these quinoline molecules were more active against intracellular parasites than promastigotes. This may be explained by the fact that these molecules are able to accumulate in high concentrations within acidic compartments like the parasitophorous vacuoles (O'Neill *et al.*, 1998). In addition, the modification of the physicochemical properties of the acidic compartments, such as the increase in pH caused by chloroquine, may decrease the acquisition of iron ions and other nutrients (Taylor & Kelly, 2010), consequently inhibiting amastigote proliferation (Huynh *et al.*, 2006). In this context, the pharmacological activity of chloroquine against intracellular microorganisms, such as bacteria and fungi, by affecting nutrient acquisition has been reported (Byrd & Horwitz, 1991; Fortier *et al.*, 1995).

The ultrastructural analysis of amastigotes treated with chloroquine and mefloquine suggests an impairment of the endocytic pathway, as shown by the accumulation of multivesicular bodies and alterations in the Golgi complex. The endocytic pathway of amastigotes is related to the absorption of macromolecules through the flagellar pocket (Borges *et al.*, 1998). Moreover, the acidic profile of endocytic vesicles may be important for chloroquine and mefloquine molecules accumulating due to their lysosomotropic characteristic (Ghedin *et al.*, 2001). The fact that chloroquine is able to inhibit nitric oxide (NO) production by macrophages and its NO-dependent antimicrobial functions (Hrabák *et al.*, 1998), as well as the release of tumour necrosis factor alpha by monocytic cells (Jang *et al.*, 2006), reinforces the hypothesis that the mechanism of drug action is based on the impairment of macromolecule absorption rather than a mechanism based on cell activation. Further studies are necessary to evaluate if the impairment of macromolecule absorption occurs in macrophages infected with *L. amazonensis* after chloroquine or mefloquine treatment.

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/10 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 intralesional 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 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 leishmaniases based on the use of chloroquine alone or as combined therapy.

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