Antileishmanial activity of ezetimibe: inhibition of sterol biosynthesis, in vitro synergy with azoles and efficacious in experimental cutaneous leishmaniasis

Running title: Antileishmanial activity of ezetimibe

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ABSTRACT

Leishmaniasis affects mainly low-income populations in tropical regions. Radical innovation in drug discovery is time- and money-consuming, causing severe restrictions to launch new chemical entities to treat neglected diseases. Drug repositioning is an attractive strategy to attend a specific demand more easily. In this project, we have evaluated the antileishmanial activity of 30 drugs currently in clinical usage for different morbidities. Ezetimibe, clinically used to reduce intestinal cholesterol absorption in dyslipidemic patients, killed promastigotes of *L. amazonensis*, with an IC$_{50}$ of 30 μM. Morphological analysis revealed that ezetimibe caused the parasites to become rounded with multiple nuclei and flagellae. Analysis by GC/MS showed that promastigotes treated with ezetimibe had a lower amount of C14-demethylated sterols and accumulated cholesterol and lanosterol. Then, we evaluated the combination of ezetimibe with well-known antileishmanial azoles. The Fractional Inhibitory Concentration Index (FICI) indicated synergy when ezetimibe is associated with ketoconazole or miconazole. Ezetimibe activity was confirmed against intracellular amastigotes, with an IC$_{50}$ of 20 μM, and reduced the IC$_{90}$ of ketoconazole and miconazole from 11.3 and 11.5 μM to 4.14 and 8.25 μM, respectively. Following, ezetimibe confirmed its activity in vivo, decreasing the lesion development and parasite load in murine cutaneous leishmaniasis. We concluded that ezetimibe has promising antileishmanial activity and should be considered in association with azoles in further preclinical and clinical studies.

Keywords: Sterol biosynthesis, *Leishmania amazonensis*, azoles, ezetimibe, drug repositioning.
Leishmaniasis is a non-contagious infectious disease caused by parasites of the genus *Leishmania* and is usually transmitted by sandflies belonging to the genera *Phlebotomus* and *Lutzomyia*. According to the species of *Leishmania* involved, the infection can affect the skin and mucous membranes (cutaneous leishmaniasis - CL) or the internal organs (visceral leishmaniasis - VL) (1, 2). Official estimates point an annual incidence of approximately 300,000 VL cases and 1.0 million CL cases worldwide (3). The basis of chemotherapy of leishmaniasis has its origin in the work of Vianna (1912), whom successfully treated a patient with emetic tartar (trivalent antimonial), a formulation broadly used at that time to treat infectious diseases (4). From this empirical approach, pentavalent antimonials were developed in the 1940’s and have saved thousands of lives for several decades. Other drugs have been repositioned to treat leishmaniasis, such as amphotericin B, pentamidine and more recently, miltefosine. Unfortunately, the therapeutic arsenal for leishmaniasis has become quite limited and outdated. Antimonials have many toxic effects including cardiac, hepatic, pancreatic and renal toxicity and should be used with caution and with clinical and laboratory monitoring in patients with heart disease and liver disease (5). Miltefosine, the only oral drug licensed, was the most important advance for the treatment of leishmaniasis in recent years. Currently, it is the treatment of choice of program elimination of visceral leishmaniasis in India and recently was approved by FDA for all forms of this disease, but cases of resistance have been reported with increased failure of treatment (6-9). This scenario is a reflex of the difficulty of launching new chemical entities as drugs for this disease. Radical innovation in drug discovery is a time- and money-consuming process. Drug repositioning is an approach very used in the past and that can be useful nowadays to address specific demands in areas of public health, such as neglected and orphan diseases (10-12). This strategy offers the advantage of working with compounds that have already been proved to be safe and to have a favorable pharmacokinetic profile in humans. Thereby, we selected a small set of drugs currently in clinical usage for several morbidities for testing against *Leishmania amazonensis*, the causal agent of cutaneous diffuse leishmaniasis.
Ezetimibe, the most active in the initial screening, was then evaluated about its mode of action and its effectiveness in association with well-known leishmanicidal azoles in vitro and in vivo.

MATERIALS AND METHODS

Drugs. Ezetimibe was extracted from sixty Ezetrol® 10 mg tablets (Merck Sharp & Dohme Corp., Kenilworth, NJ, USA), using dichloromethane in the proportion of 1:0.3 (wt/v). The mixture was incubated for 30 min under agitation and filtrated afterwards. The solvent was evaporated, yielding 200 mg. The extracted product was dissolved in deuterated chloroform and analyzed in a 400 MHz 1H-NMR (Fig. S1). All other drugs were provided by the Sigma–Aldrich Corp. (St Louis, MO, USA). The drugs were dissolved in a 10 mM stock of dimethylsulfoxide (DMSO) or PBS and stored at -20°C.

Maintenance and cultivation of parasites. Leishmania amazonensis promastigotes (strain MHOM/BR/77/LTB 0016) were maintained at 26°C in RPMI medium (Sigma-Aldrich Corp., St. Louis, MO, US) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, 100 U/ml penicillin and 5 mg/ml hemine. Subcultures were performed twice a week until the tenth passage. Afterwards, old cultures were discarded and fresh parasites were obtained from infected BALB/c mice.

Promastigote morphology. L. amazonensis promastigotes (1.0 x 10^6/mL) were grown at 10, 20 and 40 μM ezetimibe. After 72 hours, the parasites were washed twice with PBS, fixed, and stained using the Instant Prov hematological dye system (Newprov, Curitiba, Brazil).

Antipromastigote activity. Assays were performed with promastigotes of L. amazonensis at 26°C in RPMI medium without phenol red (Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented as described above. The tests were performed in 96-well plates, with an initial inoculum of 1.0 x 10^6 cells/ml and compound concentrations ranging to 24 μM for ketoconazole and miconazole, and to 100 μM for all other drugs. In association experiments, ezetimibe concentrations were 2.5, 5 and 10 μM. Plates were incubated at 26°C for 72 hours. After this period, parasite growth was evaluated by adding 10% tetrazolium salt (MTT) (5 mg/ml) per well. The plates were incubated at 26°C for a
further 1 hour, and formazan crystals were dissolved by adding 80 μl DMSO each well. The reaction was analyzed in a spectrophotometer at a 570 nm wavelength. IC\textsubscript{50} values were obtained by non-linear regression using Graphpad Prism 6 software (GraphPad Software, Inc., La Jolla, USA).

The fractional inhibitory concentration (FICI) for the analysis of synergy was calculated as follows:

\[
\text{FICI} = \frac{\text{IC}_{50} \text{ of drug A in combination}}{\text{IC}_{50} \text{ of drug A alone}} + \frac{\text{IC}_{50} \text{ of drug B in combination}}{\text{IC}_{50} \text{ of drug B alone}}.
\]

The interpretation of FICI, according to published guidelines are: synergy (FICI ≤ 0.5), antagonism (FICI > 4.0) and ‘no interaction’ (FICI > 0.5–4.0) (13).

**Toxicity to macrophages.**

Mouse peritoneal macrophages in 96-well plates were treated with ezetimibe, ketoconazole and miconazole alone or in combination for 72 hours at 37°C. After this period, the cell viability was evaluated by adding 10% tetrazolium salt (MTT) (5 mg/ml) to each well. The plates were incubated at 37°C for another four hours, and the resulting formazan crystals were dissolved by adding 80 μl DMSO to each well. The plates were then read at 570 nm. The results were expressed as the percentage of viable cells compared to the untreated control.

**Antiamastigote activity.** Peritoneal macrophages from BALB/c mice were infected with promastigotes of *L. amazonensis* (stationary growth phase) at a 3:1 parasite-to-macrophage ratio and incubated for 4 h in Lab-Tek chambers (Nunc, Roskilde, Denmark) and kept at 37°C. After 4 hours, the chambers were washed, and the cultures were treated with theazole derivatives alone or combined with ezetimibe in supplemented RPMI medium for 72 hours. For initial screening, the concentration of drugs was fixed in 25 μM and a threshold of at least 50% of inhibition was established. A drug-activity curve was done with ezetimibe, the only drug that reached the threshold, with concentration ranging to 40 μM. In the association assays, the concentration of ezetimibe was fixed at 20 μM, while the concentration of ketoconazole and miconazole ranged to 16 and 32 μM, respectively. After incubation, the slides were stained, and the infection rate was determined by counting under a light microscope. The infection rate was calculated using the formula: % infected macrophages x number of amastigotes / number of total macrophages.
Extraction of lipids. Lipids from promastigotes of *L. amazonensis* were extracted using the method of Bligh and Dyer (1959) (14). Briefly, samples were pelleted, and a solution of methanol, chloroform and water (2:1:0.5 v/v) was added. After stirring the mixture for 1 hour, the samples were centrifuged for 20 min at 3,000 rpm and the supernatant, containing the lipids was separated from the precipitate. The precipitate was subjected to a second extraction under the same conditions. The supernatants were combined, and chloroform/water (1:1 v/v) was added. After 40 seconds of stirring, the material was centrifuged (3,000 rpm/30 min) again. The lower layer (organic) containing the lipids was then separated with the aid of a glass syringe and transferred to a 1.5 ml tube resistant to organic solvents (Axygen Scientific, Inc., Union City, CA, USA). The solvent was evaporated under N₂ flux, and the lipids were analyzed by gas chromatography-mass spectrometry (GC/MS), as described below.

Analysis of the sterol profile by gas chromatography coupled with mass spectrometry (GC/MS). Promastigotes of *L. amazonensis* were cultured with 10, 20 or 40 μM ezetimibe or in culture medium alone. After 72 hours, 1x10⁸ parasites of each culture were washed 3 times in cold PBS (pH 7.5), and the sterols were extracted as described above. The samples were injected into a GCMS - QP2010 Ultra Machine (Shimadzu Scientific Instruments, Tokyo, Japan). After injection, the column temperature was maintained at 50°C for 1 minute and then increased to 270°C at a rate of 10°C/min and finally to 300°C at a rate of 1°C/min. The flow of the carrier gas (He) was kept constant at 1.1 ml/min. The temperatures of the injector and detector were 250°C and 280°C, respectively (15).

Ethics Statement

Studies in *L. amazonensis*-infected BALB/c mice were performed in accordance with protocols approved by the Ethics Committee for Animal Use of the Instituto Oswaldo Cruz (L026/2015).

In vivo assay. To assess the in vivo activity of the combination of ezetimibe and ketoconazole, BALB/c mice (9 animals per group) were infected on the right ear with 2x10⁶ promastigotes of *L. amazonensis* in stationary phase. The treatment started ten days after infection. The animals were
treated with ezetimibe (10 mg/kg/day), ketoconazole (100 mg/kg/day), miltefosine (20 mg/kg/day) and the combination of ezetimibe + ketoconazole (10 mg/kg/day + 100 mg/kg/day) by oral route.

The animals were treated 5 days per week in a total of 20 doses. Negative controls were also similarly treated with PBS. The ear measurement was recorded once a week. After the treatment period, the animals were euthanized for determination of parasite load and toxicological analysis.

Serum levels of urea (URE), albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CREA), total bilirubin (BIL), creatine kinase (CK) and cholesterol (CHO) were measured in a Vitros 250 equipment (Ortho Clinical - Johnson & Johnson) using dry chemistry methodology.

RESULTS

Antileishmanial screening. The complete list of drugs and the IC₅₀ for promastigotes and intracellular amastigotes of *L. amazonensis* can be found in the Table 1. Among all tested compounds, only glimepiride, domperidone and ezetimibe showed activity against promastigotes, with IC₅₀ of 54, 51 and 30 μM, respectively. For intracellular amastigotes, the clinically relevant stage of the parasite, only ezetimibe showed IC₅₀ lower than the arbitrated threshold of 25 μM, reaching to 20 μM. Ezetimibe also drew attention by provoking striking morphological changes on the promastigotes of *L. amazonensis*. We observed that, in a concentration dependent manner, the parasites became rounded and displayed multiple nuclei and flagellae, mainly at 40 μM (Fig. 1).

Ezetimibe interferes with sterol biosynthesis in *L. amazonensis*. The morphological alterations seen in ezetimibe-treated promastigotes induced us to investigate whether this drug interferes with sterol biosynthesis of the parasite. Table 2 shows an analysis of the relative amount of each sterol indicated by GC/MS. Promastigotes treated with miconazole, an inhibitor of C14-demethylase, showed a decrease in the ergostane-derived sterols ergosta-5,7,24-trien-3β-ol (dehydroepisterol) (3) and ergosta-7,24-dien-3β-ol (episterol) (4). Miconazole also induced the accumulation of exogenous cholesterol collected from culture medium and an increase in the methylated sterols
14α-methylergosta-8,24(28)-dien-3β-ol (2) and 4α-14α-dimethylergosta-8,24(28)-dien-3β-ol (obtusifoliol) (5). Treatment with ezetimibe also decreased dehydroepisterol and episterol (C14-demethylated sterols) and promoted the accumulation of cholesterol (1) and lanosterol (C14-methylated sterol) (7). We also observed the accumulation of an unknown sterol (6).

**Ezetimibe potentiates the effect of azoles.** Considering the significative alteration in sterol pattern of the parasites after ezetimibe treatment, the antileishmanial activity of this drug was also evaluated in combination with azoles (ketoconazole and miconazole) in promastigotes and amastigotes of *L. amazonensis*. To graphically evaluate the interaction of ezetimibe with azoles in promastigotes, the IC$_{50}$ of the drugs alone and in combination was plotted as isobolograms, and to estimate the type of interaction (synergy, antagonism or neutral), the fractional inhibitory concentration index (FICI) was calculated. As expected, miconazole and ketoconazole were active against the promastigotes, with IC$_{50}$ of 2.5±0.1 and 2.7±0.1 μM, respectively. The IC$_{50}$ of the azoles decreased when in combination with ezetimibe, generating fully concave isobolograms (Fig. 2). The calculation of FICI resulted in values of 0.4 for both combinations, confirming the synergism. Due to the experimental difficulty of performing a “checkerboard” approach to draw isobolograms for antiamastigote activity, we fixed the concentration of ezetimibe at the IC$_{50}$ (20 μM) (Fig. 3A) and varied the concentration of the azoles to calculate the IC$_{90}$ (concentration that inhibits 90% of the parasites) of the combination. When associated with ezetimibe, the IC$_{90}$ of ketoconazole and miconazole were reduced from 11.3±0.2 μM and 11.5±0.1 μM to 4.14±0.3 μM and 8.25±0.2 μM, respectively (Figs. 3B and C). Furthermore, ezetimibe, miconazole, ketoconazole alone or combined showed no toxic effects to macrophages (Fig. 4A-E). Figure 5 shows representative micrographs of infected macrophages treated with ezetimibe alone and combined with ketoconazole and miconazole that have normal morphology and a reduced parasite load (Fig. 5).

For in vivo assays, BALB/c mice were infected with *L. amazonensis* and treated with ezetimibe (10 mg/kg/day) and ketoconazole (100 mg/kg/day) alone or in combination by oral route. As can be seen in Figure 6A, ketoconazole and ezetimibe were able to individually control the
lesion development. When both drugs were combined, the lesion growth was more effectively controlled. All treatments reduced the parasite load significantly (Fig. 6A – insert). Nonetheless, no significant differences in serum levels of urea, albumin, ALT, AST, creatinine, bilirubin and creatine kinase were observed in treated and untreated animals, demonstrating that the treatment was not toxic to the mammalian host. Furthermore, no significant change in cholesterol level in serum was observed (Figs. 6B-I).

DISCUSSION

Thirty drugs in clinical usage were included in this study. Instead of focusing in antimicrobial agents, we selected compounds belonging to a wide spectrum of pharmacological classes (Table 1). There is no previous data in literature about antileishmanial activity of glimepiride and ezetimibe but, interestingly, domperidone has been used in clinical trials in naturally infected dogs, aiming veterinary use (16, 17). However, in those studies, no intrinsic antileishmanial activity was assigned to domperidone. The therapeutic effect of this drug in dogs has been attributed to its immunomodulatory activity (17).

Among the active drugs, ezetimibe drew attention due to its property of causing dramatic morphological alterations in the promastigotes (Fig. 1). These morphological changes are consistent with those previously described as consequence of drug interference in sterol metabolism of trypanosomatids (18-20). In fact, we observed that ezetimibe causes alterations in the sterol composition of the parasites compatible with inhibition of C14-demethylase (21-23). Curiously, ezetimibe is a potent inhibitor of the intestinal cholesterol absorption mediated by the Niemann–Pick C1-like 1 protein (NPC1L1) and it is clinically used to reduce cholesterolemia (24).

Nevertheless, no inhibition in the human sterol biosynthesis pathway has been described for ezetimibe. However, the sterol pathway of the trypanosomatids has diverged sufficiently to allow selective pharmacological inhibition. Thereby, besides its effect on transport of cholesterol in humans, it is possible that ezetimibe also could interfere with sterol biosynthesis in Leishmania.
It is assumed that the sterol biosynthetic pathway is essential for *Leishmania*, because inhibition of diverse enzymes of this pathway such as, HMG-CoA reductase, squalene epoxidase, C-14 demethylase and C-24 methyltransferase, results in parasite death (25-30). Drug combination in the treatment of infectious diseases is an interesting approach. Drugs acting in the same pathway can be associated to improve their activity (31). Thus, we evaluated the activity of ezetimibe in combination with ketoconazole or miconazole. This association showed synergistic effect on promastigotes (Fig. 2) and increased the activity of these azoles on intracellular amastigotes of *L. amazonensis* (Figs. 3B and C).

The next step was to evaluate the leishmanicidal activity of ezetimibe alone or combined with ketoconazole *in vivo* (Figs. 6A and B). Ezetimibe and ketoconazole alone reduced the lesion growth and parasite load equally in murine model of cutaneous leishmaniasis. The combination was more efficacious in controlling the lesion development, but no difference was observed in parasite burden. Ketoconazole was chosen to be evaluated in vivo because it showed better result on intracellular amastigotes, when in combination with ezetimibe (Fig. 3C). Moreover, clinical trials have demonstrated that ketoconazole could be advantageous for patients with New World species of *Leishmania* (32). Oral ketoconazole combined with intra-lesional injections of meglumine antimoniate (Glucantime) has been shown to be more effective than Glucantime alone in the treatment of localized cutaneous leishmaniasis (33). Furthermore, ketoconazole (100 mg/kg/day) reduced splenic parasite load in murine model of visceral leishmaniasis caused by *L. infantum*, and in the lower dose of 50 mg/kg/day, potentiated the effect of meglumine antimoniate (34).

In summary, our screening of FDA approved drugs pointed out ezetimibe as a promising antileishmanial agent. It has antileishmanial activity in vitro and in vivo, supposedly by its effect on parasite sterol biosynthesis, and acts synergistically with azoles. These data suggest that ezetimibe could be useful, in association or not with azoles, in further treatments for leishmaniasis.
ACKNOWLEDGMENTS The authors thank the Program for Technological Development in Tools for Health-PDTIS, FIOCRUZ for evaluating the serum toxicological markers.

FOOTNOTE PAGE

FUNDING: This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Programa Estratégico de Apoio à Pesquisa em Saúde (PAPES/FIOCRUZ; grant 407680/2012-8 to E. C. T. S.).

CONFLICT OF INTEREST

All authors: No reported conflict of interest.

REFERENCES


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<th>Promastigotes IC₅₀ (μM)</th>
<th>Intracellular amastigotes IC₅₀ (μM)</th>
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<th>Chemical Class</th>
<th>Mode of Action</th>
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<td>Promotes sodium, chloride, and water excretion by inhibiting sodium reabsorption in the distal tubules of the kidneys.</td>
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<td>Inhibits RNA-dependent protein synthesis in many types of aerobic, anaerobic, gram-positive, and gram-negative bacteria.</td>
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<td>Non-selective competitive inhibition of u-adrenoceptors in vascular smooth muscle.</td>
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<td>Aminosalicylic acid</td>
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<td>Hydrochlorothiazide</td>
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<td>&gt;25</td>
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<td>Antiulcer agent, gastric acid secretion inhibitor</td>
<td>Aminomethyl-substituted fumar derivative</td>
<td>Competitive blockade of histaminergic H₂ receptors.</td>
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<td>Antichoimnergic</td>
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Table 2: Sterol composition of L. amazonensis promastigotes under effect of ezetimibe.

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<th>Compound</th>
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<td>(1) Cholesterol</td>
<td>386</td>
<td>1.58</td>
<td>31.15</td>
<td>16.91</td>
<td>19.30</td>
<td>43.28</td>
<td></td>
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<tr>
<td>(2) 14α-methyllyergosta-8,24(28)-dien-3β-ol</td>
<td>412</td>
<td>-</td>
<td>41.18</td>
<td>4.44</td>
<td>6.21</td>
<td>2.43</td>
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<tr>
<td>(3) Ergosta-5,7,24-trien-3β-ol (dehydroepisterol)</td>
<td>396</td>
<td>93.32</td>
<td>-</td>
<td>62.18</td>
<td>23.70</td>
<td>4.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4) Ergosta-7,24-dien-3β-ol (Episterol)</td>
<td>398</td>
<td>5.10</td>
<td>-</td>
<td>12.20</td>
<td>9.83</td>
<td>1.72</td>
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<tr>
<td>(5) 4β,14α-dimethyllyergosta-8,24(28)-dien-3β-ol, (obtusifoliol)</td>
<td>426</td>
<td>-</td>
<td>33.60</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>(6) Unknown</td>
<td>424</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.71</td>
<td>8.75</td>
<td></td>
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<tr>
<td>(7) Lanosterol</td>
<td>426</td>
<td>-</td>
<td>-</td>
<td>3.34</td>
<td>37.88</td>
<td>39.24</td>
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</table>

MW - Molecular Weight; Relative Amount (%) - Relative to total lipid content from each sample.
Legends to Figures

Fig. 1. Micrographs of promastigotes of *L. amazonensis* treated with ezetimibe. *L. amazonensis* promastigotes were grown at 10, 20 and 40 μM of ezetimibe to 72 hours. A control, B ezetimibe 10 μM, C ezetimibe 20 μM, D ezetimibe 40 μM. The slides were stained using Instant Prov hematological dye system.

Fig. 2. Isobolographic analysis of the antileishmanial activity of association of ezetimibe and azoles. *L. amazonensis* promastigotes were incubated with ezetimibe and the indicated azoles in different concentrations for 72 hours at 26°C. Each plotted point in the isobolograms is the IC₅₀ of the drugs alone or in combination. The straight lines connecting the individual IC₅₀s represent the theoretical line of additivity for each combination. The experiments were performed in triplicate, n = 3. The graphics are representative of a single experiment, with the standard deviation value. The graphics and IC₅₀ values were produced with GraphPad Prism 4 software.

Fig. 3. Antiamastigote activity of ezetimibe combined with azoles. Peritoneal macrophages infected with *L. amazonensis* treated with ezetimibe and the indicated azoles were incubated for 72 hours at 37°C. After incubation, the cells were fixed and stained. The infection rate was calculated as indicated in the methodology. The experiments were performed in triplicate, n = 3. The graphics are representative of a single experiment, with the standard deviation value. * p < 0.05, ** p < 0.01, *** p < 0.001.

Fig. 4. Toxicity to macrophages. Uninfected macrophages were incubated with ezetimibe, miconazole, ketoconazole alone or in combination for 72 hours at 37 °C. After this period, the cells were incubated with MTT for 1 hour at 37 °C. The absorbance was read in a spectrophotometer at 570 nm. (A) ezetimibe, (B), ketoconazole (C), miconazole (D) + Eze20 ketoconazole (E) Eze20 + miconazole. The experiments were performed in triplicate, n = 3. *** p <0.001 compared to the control.
Fig. 5. Micrographs of *L. amazonensis*-infected macrophages treated with ezetimibe and azoles. Peritoneal macrophages infected with *L. amazonensis* treated with ezetimibe and the indicated azoles were incubated for 72 hours at 37°C. After incubation, the cells were fixed and stained. (A) control, (B) ezetimibe 20 μM, (C) ketoconazole 8 μM, (D) ketoconazole 8 μM + ezetimibe 20 μM, (E) miconazole 4 μM, (F) miconazole 4μM + ezetimibe 20 μM.

Fig. 6. In vivo activity of ezetimibe in combination with ketoconazole. BALB/c mice (9/group) were infected on the right ear with 2 x 10⁶ promastigotes of *L. amazonensis* stationary phase and orally treated with ezetimibe (10 mg/kg/day) (Eze10), ketoconazole (100mg/ kg/day) (Keto100), miltefosine (20 mg/kg/day) and ezetimibe + ketoconazole (10 mg/kg/day + 100 mg/kg/ day) (Eze10 + keto100). The treatment started ten days after infection. The animals were treated 5 days per week, in a total of 20 doses. The euthanasia was proceeded after 35 days of infection. Negative controls were also similarly treated with PBS. A - Lesion growth and parasite load. B-I - Biochemical evaluation of toxicity of the treatment, according to the indicated parameters. Urea (URE), albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CREA), total bilirubin (BIL), creatine kinase (CK) and cholesterol (CHO). 2way ANOVA, * p <0.05, ** p <0.01 *** p <0.001 compared to the control group. # # p <0.01 compared to the Eze10 group. π p <0.05, π π p <0.01 π π π p <0.001 compared to the Keto100 group.