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In vitro antileishmanial activity of ravuconazole, a triazole antifungal drug, as a potential treatment for leishmaniasis

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Objectives: Leishmaniasis, one of the most significant neglected diseases around the world, is caused by protozoan parasites of the *Leishmania* genus. Nowadays, the available aetiological treatments for leishmaniasis have variable effectiveness and several problems such as serious side effects, toxicity, high cost and an increasing number of resistance cases. Thus, there is an urgent need for safe, oral and cost-effective drugs for leishmaniases. Previously, our group has shown the effect of the ergosterol biosynthesis inhibitors on *Leishmania amazonensis*. Herein, we showed the effect of ravuconazole against *L. amazonensis*; ravuconazole is a second-generation triazole antifungal drug that has good bioavailability after oral administration and a long terminal half-life in humans, a broad activity spectrum, high effectiveness in treatment of mycosis and negligible side effects.

Methods: Several methodologies were used: cell culture, fluorescence and electron microscopy, high-resolution capillary GC coupled with MS, fluorimetry and flow cytometry.

Results: Our results showed that ravuconazole was able to inhibit the proliferation of *L. amazonensis* promastigotes and intracellular amastigotes *in vitro*, with single-digit to sub-micromolar IC_{50} values, causing several alterations in the morphology, ultrastructure, cell viability and physiology of the parasites. The mitochondrion was significantly affected by the treatment, resulting in a collapse of the mitochondrial transmembrane potential that consequently led to inhibition of ATP production, combined with an increase in reactive oxygen species and mitochondrial superoxide production; by transmission electron microscopy, the organelle displayed a completely altered ultrastructure. The treatment changed the lipid profile, showing a profound depletion of the 14-desmethyl endogenous sterol pool.

Conclusions: These results suggest that ravuconazole could be an alternative option for the treatment of leishmaniasis.

Introduction

Leishmaniasis is a neglected disease, distributed throughout the world, caused by protozoan parasites of the *Leishmania* genus. It is prevalent in 98 countries, with a total of 350 million people at risk and 12 million cases of infection every year.[1] The leishmaniases have been divided into three main clinical manifestations: cutaneous, mucocutaneous and visceral.[2,3] Brazil is one of the most significant countries in its epidemiology.[1]

For several years, pentavalent antimonials have been used as first-line treatment,[4] with the exception of India,[5] where the number of resistance cases has increased significantly. The second-line treatment is based on the use of amphotericin B or pentamidine, which are toxic and expensive.[6] Moreover, they have several limitations, such as serious adverse effects, high cost and cases of resistance.[7] In 2002, miltefosine was registered as the first oral treatment for visceral leishmaniasis in India;[5] however, nowadays, liposomal amphotericin B is the first-line treatment in Asia, Africa and Europe, as recommended by the WHO.[7,8] Although miltefosine displays good efficacy, it is teratogenic, hepatotoxic and nephrotoxic.[9] Thus, there is an urgent need to identify

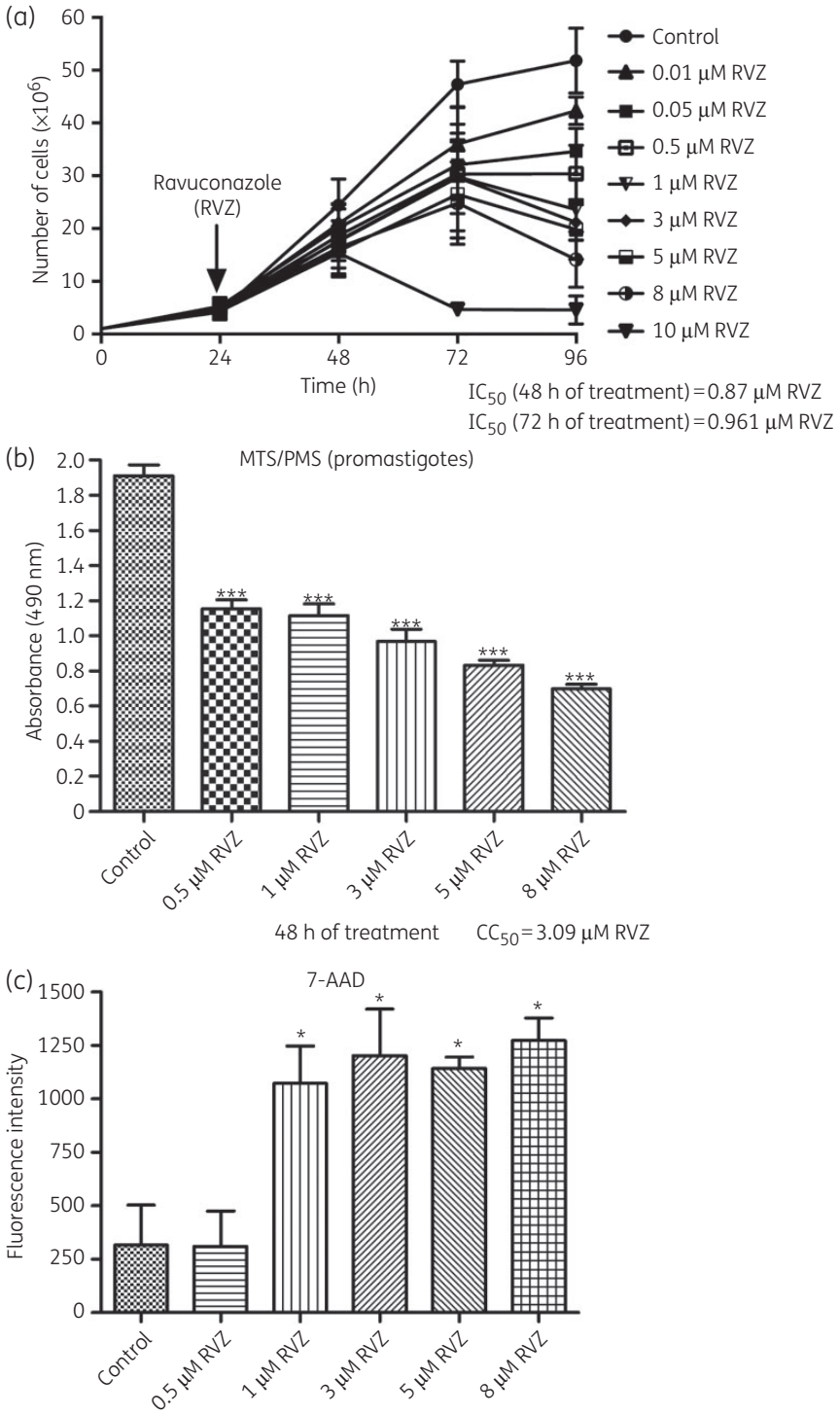


Figure 1. Evaluation of antiproliferative and cytotoxicity effects of ravuconazole on *L. amazonensis* promastigotes. (a) Parasites were treated with ravuconazole for 72 h to evaluate parasite growth. Ravuconazole at different concentrations was added after 24 h of growth (arrow). The IC_{50} after 48 h of treatment was 0.87 μM ravuconazole. (b) Cell viability and cytotoxicity were evaluated against promastigotes using the MTS/PMS reaction after 48 h of treatment. The CC_{50} value was 3.09 μM ravuconazole. (c) Evaluation of plasma membrane integrity of *L. amazonensis* promastigotes with 7-AAD after 48 h of treatment with ravuconazole. There was a significant increase in the fluorescence intensity at concentrations higher than 1 μM ravuconazole. This increase represents a decrease in viability of the treated cells. Bars represent standard deviation. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

Table 1. CC_{50}, CC_{90}, IC_{50} and IC_{90} for *L. amazonensis* and macrophages after treatment with different concentrations of ravuconazole determined by different methodologies such as MTS/PMS and cellular density by counting the cells in a Neubauer chamber

	Evaluation by MTS/PMS after 48 h of treatment	Evaluation by MTS/PMS after 72 h of treatment	IC_{50}/IC_{90} after 48 h of treatment	IC_{50}/IC_{90} after 72 h of treatment
Promastigotes	$CC_{50} = 3.09\ \mu M$ $CC_{90} > 8\ \mu M$	$CC_{50} = 3.99\ \mu M$ $CC_{90} > 8\ \mu M$	$IC_{50} = 0.87\ \mu M$ $IC_{90} = 9.74\ \mu M$	$IC_{50} = 0.961\ \mu M$ $IC_{90} = 9.87\ \mu M$
Peritoneal macrophages	$CC_{50} > 70\ \mu M$ $CC_{90} > 70\ \mu M$	$CC_{50} = 47.68\ \mu M$ $CC_{90} > 70\ \mu M$	—	—
Intracellular amastigotes	—	—	$IC_{50} = 5.11\ \mu M$ $IC_{90} = 9.31\ \mu M$	$IC_{50} = 1.65\ \mu M$ $IC_{90} = 8.41\ \mu M$

All of the methodologies used for these results are described in the Materials and methods section.

new therapeutic alternatives, since the current treatments are unsatisfactory.

Members of the Trypanosomatidae family such as *Leishmania* have a strict requirement for ergosterol and other 24-alkyl sterols that are essential for the maintenance of cell membrane structure and function and are absent in mammalian cells.[10] Several studies have shown that the sterol biosynthesis pathway might be an important druggable target in *Leishmania* parasites.[10–12] Azoles are a well-known class of antifungal agents that inhibit the conversion of lanosterol to zymosterol by the cytochrome P450-containing monooxygenase lanosterol C14α-demethylase.[10,13] Some azoles have been described to be active against different *Leishmania* spp., such as ketoconazole, fluconazole, itraconazole and posaconazole.[8] Furthermore, our group showed the potent effect of itraconazole and posaconazole against *Leishmania amazonensis* alone or in combination with E5700, a known squalene synthase inhibitor, another essential enzyme in the sterol biosynthesis pathway.[14,15]

Ravuconazole (Eisai Co. Ltd, Japan) is a second-generation triazole antifungal drug that has potent activity against *Trypanosoma cruzi in vitro*, but its curative activity in murine and canine models of acute Chagas' disease is limited by the relatively short terminal half-life of the drug in these animal models.[16–18] Ravuconazole has a much longer half-life in humans (>120 h), offering the opportunity to assess the drug as chemotherapy for human Chagas' disease.[17,19] The drug has good bioavailability after oral administration, particularly in its prodrug formulation (E1224, fosravuconazole[19]), a broad activity spectrum, high effectiveness in the treatment of deep-seated mycosis and a very good safety profile. Its absorption, distribution, metabolism and excretion (ADME) profile is similar to that of posaconazole owing to its lipophilicity, which is an important feature for membrane permeability and large volumes of distribution.[20,21]

In this work, we showed the potent effect of ravuconazole *in vitro* against *L. amazonensis* promastigotes and intracellular amastigotes, inhibiting growth and inducing several alterations in morphology, ultrastructure, sterol composition and physiology of the parasite.

Materials and methods

Leishmania cultures

The MHOM/BR/75/Josefa strain of *L. amazonensis* (provided by the *Leishmania* Collection of the Instituto Oswaldo Cruz) was used in this study and it has been maintained by inoculation into the base of BALB/c mice tails. Amastigotes were obtained from mice and transformed into promastigotes that were axenically cultured in Warren's medium[22] supplemented with 10% FBS at 25°C.

Drug

Ravuconazole, [R-(R*,R*)]-4-[2-[2-(2,4-difluorophenyl)-2-hydroxy-1-methyl-3-(1*H*-1,2,4-triazol-1-yl)propyl]-4-thiazolyl]benzonitrile, was provided by Eisai Co. Ltd, dissolved in DMSO as a 10 mM stock solution and stored at −20°C. New dilutions were prepared in culture medium during the experiments, but the DMSO concentration did not exceed 0.1%.

In vitro antiproliferative effects of ravuconazole

Promastigote cultures were initiated at a cell density of 1.0×10^6 cells/mL. After 24 h of growth, ravuconazole was added at different concentrations from the stock solution. Cell densities were determined every day in a Neubauer chamber during 96 h of growth. The effects of ravuconazole on *L. amazonensis* intracellular amastigotes were also evaluated following experimental protocols previously published.[14,15] The IC_{50} was calculated for promastigotes and intracellular amastigotes by fitting the values to a non-linear curve analysis. The regression analyses were performed with SigmaPlot 10 software.

Cell viability assays in murine macrophages and *L. amazonensis* promastigotes

The CellTiter 96® AQueous MTS Assay (Promega) was used to evaluate the cytotoxicity effects of ravuconazole in mammal cells and the cell viability of the *L. amazonensis* promastigotes. MTS/PMS is a colorimetric method used to evaluate the number of viable cells in proliferation, where MTS is 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium and PMS is phenazine methosulphate.[23] Murine macrophages[15] were treated daily with different concentrations of ravuconazole for 72 h. Promastigotes of *L. amazonensis* were cultivated at a cell density of 1.0×10^6 cells/mL and, after 24 h of growth, ravuconazole was added at different concentrations. The cell viability was measured after 48 and 72 h of treatment in 96-well plates in triplicate. Either macrophages or promastigotes were evaluated by MTS/PMS assay reaction according to the

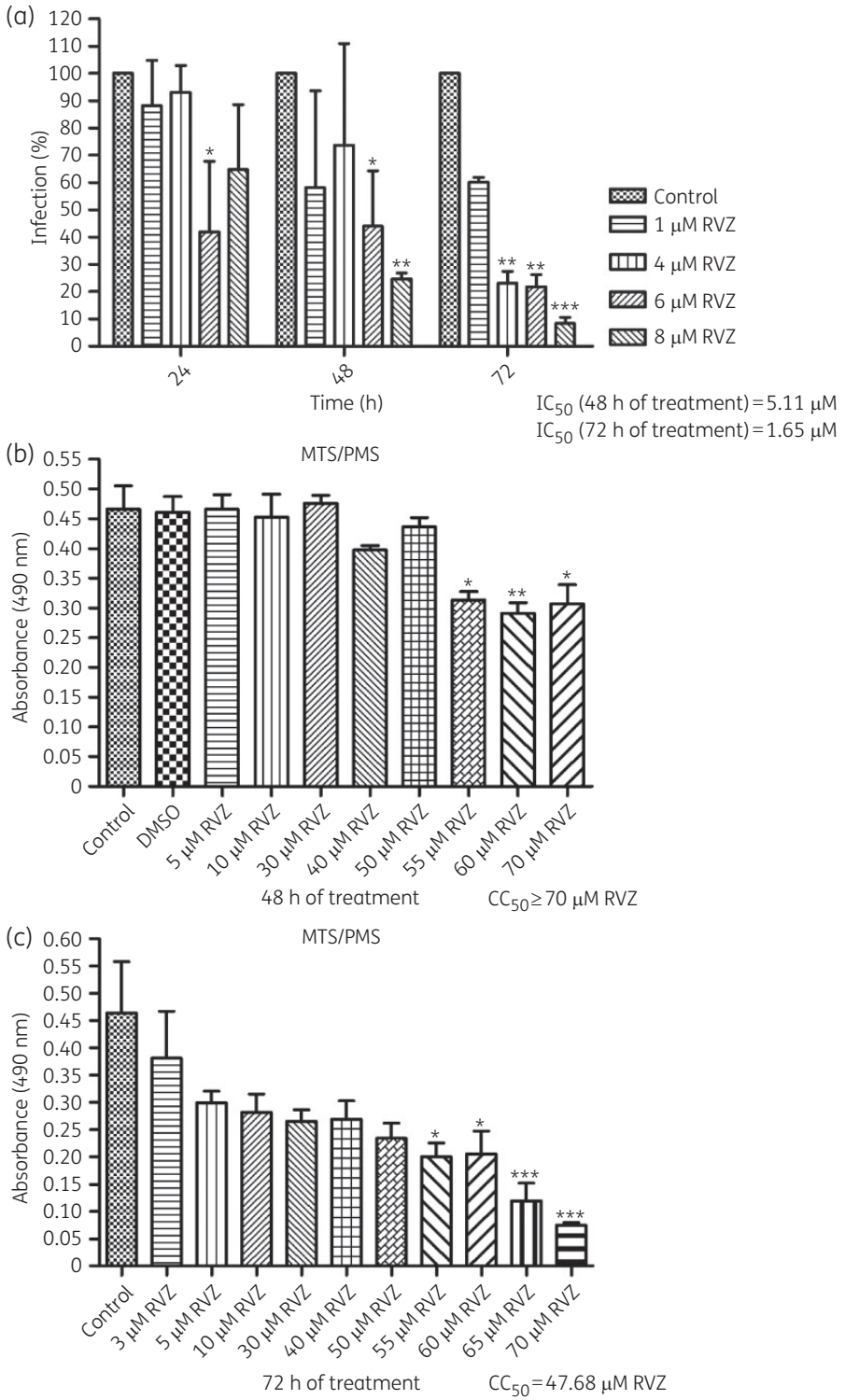


Figure 2. Evaluation of antiproliferative and cytotoxicity effects of ravuconazole on *L. amazonensis* intracellular amastigotes and murine macrophages. (a) Parasites were treated with ravuconazole for 72 h to evaluate parasite growth. After 72 h of treatment, the effect was concentration-dependent with an IC_{50} of 1.65 μM ravuconazole. (b and c) The MTS/PMS reaction was used to evaluate the cytotoxicity of ravuconazole against murine macrophages. The CC_{50} values were ≥70 and 47.68 μM for 48 and 72 h of treatment, respectively. Bars represent standard deviation. *$P<0.05$, **$P<0.01$ and ***$P<0.001$. RVZ, ravuconazole.

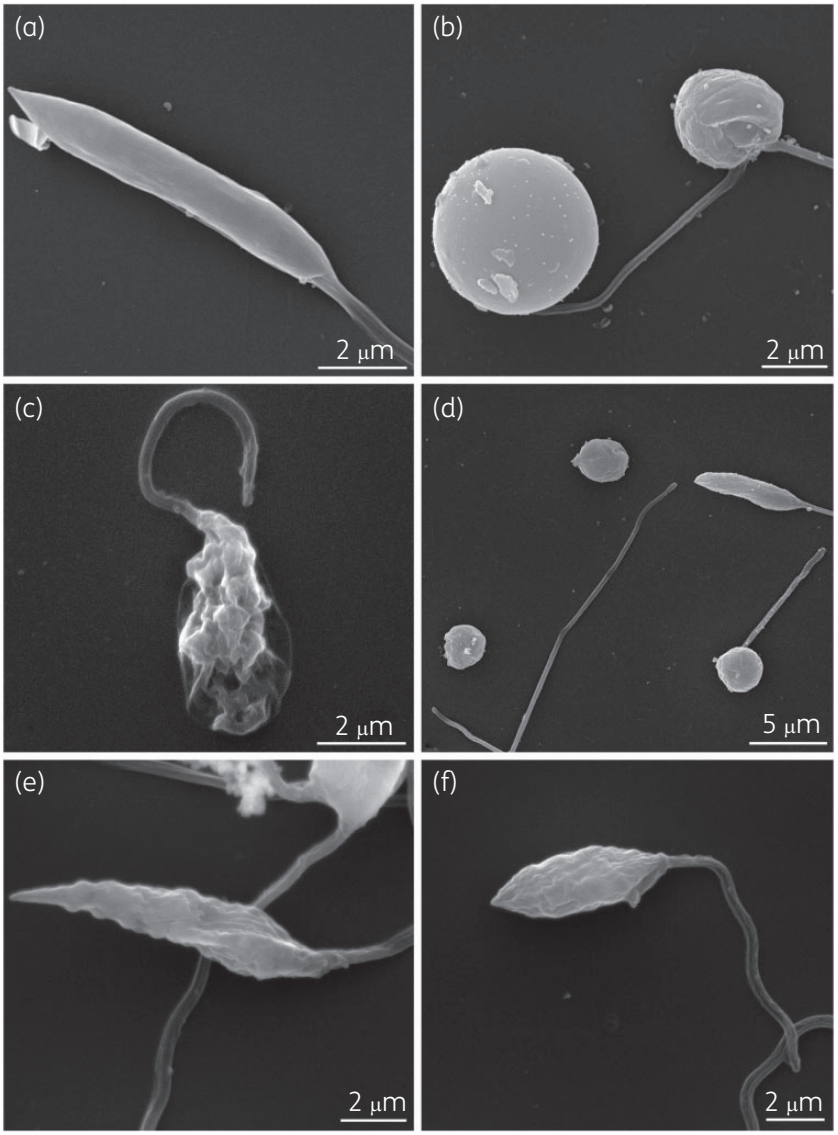


Figure 3. Scanning electron microscopy of *L. amazonensis* promastigotes treated with ravuconazole for 48 h. (a) Control. (b) 0.5 μM ravuconazole. (c) 1 μM ravuconazole. (d, e and f) 3 μM ravuconazole. The images show a dramatic alteration in the shape of promastigotes after treatment. The promastigotes appeared rounded and usually the cell surface was crumpled.

protocol published previously.[23] The CC_{50} for viable macrophages and promastigotes was determined by non-linear regression using SigmaPlot software.

Electron microscopy

Control and treated promastigotes and intracellular amastigotes were fixed for at least 2 h in 2.5% glutaraldehyde (Sigma) in 0.1 M cacodylate buffer (pH 7.2) and post-fixed in a solution containing 1% OsO_4, 1.25% potassium ferrocyanide, 5 mM $CaCl_2$ and 0.1 M cacodylate buffer (pH 7.2) for 30 min. Experimental protocols for scanning and transmission electron microscopy were published previously.[15] Ultrathin sections were observed under a Zeiss 900 electron microscope.

Immunofluorescence assays

Promastigotes of *L. amazonensis*, untreated and treated with different concentrations of ravuconazole, were washed twice with PBS (pH 7.2), fixed with 4% freshly prepared formaldehyde for 30 min and placed onto poly-L-lysine-coated coverslips to adhere. Thereafter, experimental protocols previously published by our group were used here for α-tubulin labelling.[24] Samples were observed using a Leica TCS SPE confocal microscope.

Estimation of mitochondrial transmembrane electric potential ($\Delta\psi_m$)

$\Delta\psi_m$ of the untreated and treated promastigotes was analysed using the JC-1 fluorochrome (Molecular Probes), a lipophilic

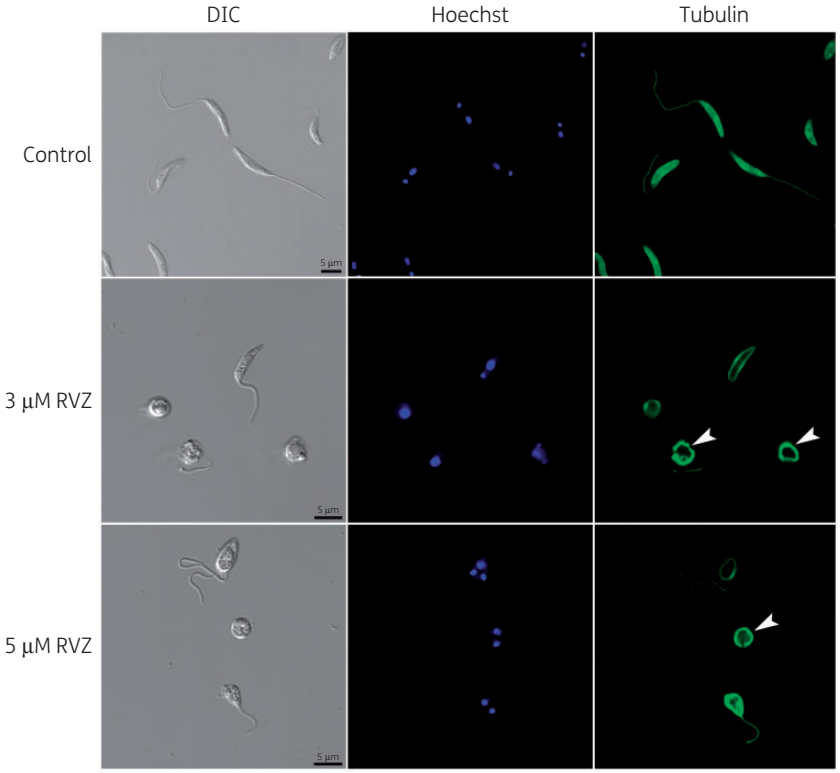


Figure 4. DIC microscopy and immunofluorescence with anti-α-tubulin antibody of *L. amazonensis* control promastigotes and those treated with ravuconazole. Images show the formation of large tubulin clusters (white arrowheads) in treated parasites, which are absent in control parasites. DIC images confirmed the potent alterations of the shape of promastigotes induced by ravuconazole (3 and 5 μM). These alterations should be related to the remodelling of the tubulin cytoskeleton. RVZ, ravuconazole. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

and cationic mitochondrial vital dye that accumulates in the mitochondria in response to $\Delta\psi_m$.[24,25] Parasites and experimental conditions were prepared as previously described.[14,15,25] The experiments were repeated at least three times in triplicate and Figure 7(a) shows the mean and standard deviation of one representative experiment.

Quantification of intracellular ATP

Intracellular ATP levels were measured using a bioluminescence assay based on the reaction catalysed by luciferase, in which its substrate D-luciferin is metabolized in the presence of ATP resulting in light that is measured at the maximum emission wavelength of 560 nm. Firstly, a standard curve of ATP concentrations was obtained, varying the ATP concentration from 1 to 100 nM; samples were analysed in a luminometer measuring the luminescence. After that, 1×10^7 parasites (untreated and treated with ravuconazole) were harvested, washed in PBS (pH 7.2), resuspended in 1 mL of boiling water using a vortex mixer and then incubated at 4°C. The amount of ATP in the supernatant was measured using the ATP Determination Kit in a luminometer (PerkinElmer) at the maximum emission wavelength of 560 nm. Experiments were repeated at least three times in triplicate and Figure 7(b) shows the mean and standard deviation of one representative experiment.

Measurements of reactive oxygen species and mitochondrial superoxide

Reactive oxygen species (ROS) and mitochondrial superoxide were measured using two different techniques: (i) incubation with H_2DCFDA (Molecular Probes), a non-fluorescent cell-permeant 2′,7′-dichlorodihydrofluorescein diacetate that can be converted to a fluorescent 2′,7′-dichlorodihydrofluorescein by intracellular esterases and oxidation in the presence of high amounts of ROS; and (ii) incubation with MitoSOX Red indicator (Molecular Probes), which is a fluorogenic dye for highly selective detection of mitochondrial superoxide in live cells. In the mitochondrion, MitoSOX Red is oxidized exclusively by superoxide with emission fluorescence at 580 nm (red channel). For the analyses, 3×10^7 cells were prepared according to previous work.[15] Experiments were repeated at least three times in triplicate and Figure 7(c–d) shows the mean and standard deviation of one representative experiment.

Evaluation of plasma membrane integrity

Plasma membrane integrity was evaluated using the fluorescent intercalator 7-aminoactinomycin D (7-AAD), which is excluded from live cells and undergoes a spectral shift upon association with DNA. Control and treated promastigotes were washed in PBS (pH 7.2) and resuspended in a volume of 500 μL. After that, 2 μg/mL 7-AAD (Molecular Probes) was added and samples

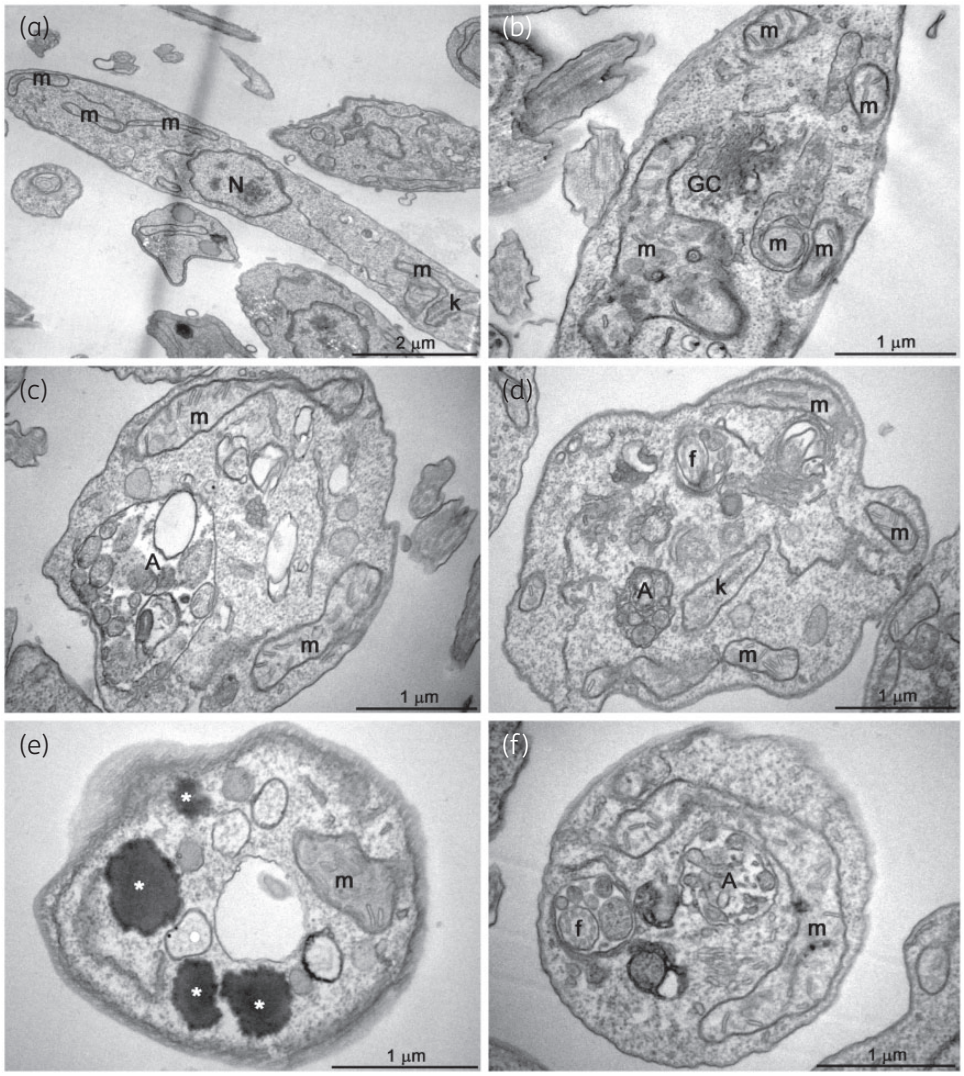


Figure 5. Ultrathin sections of *L. amazonensis* control promastigotes (a) and those treated with ravuconazole for 48 h [(b) 0.5 μM ravuconazole, (c and d) 3 μM ravuconazole and (e and f) 5 μM ravuconazole]. Several alterations were observed, such as the presence of several lipid bodies (e), intense disorganization and mitochondrial swelling (b–f) and the presence of autophagosome-like structures (c, d and f), sometimes in close association with important organelles, such as the mitochondrion (f). f, flagellum; N, nucleus; m, mitochondrion; k, kinetoplast; asterisks, lipid bodies; A, autophagosome-like structure; GC, Golgi complex.

were incubated for 15 min at 25°C, protected from light. For this analysis, a positive control with 0.1% Triton X-100 (Sigma) was used. Samples were then analysed by flow cytometry (BD Accuri C6) using an argon-ion laser for excitation at 488 nm and the maximum emission was measured at 647 nm. Experiments were repeated at least three times in triplicate and Figure 1(c) shows the mean and standard deviation of one representative experiment.

Neutral lipid accumulation

Presence of lipid bodies was evaluated by incubating parasites with Nile red, a fluorescence marker for neutral lipid. For that, 1.0×10^7 promastigotes were harvested, washed in PBS (pH 7.2) and incubated with 10 μg/mL Nile red (Sigma) for 20 min. After that, cells were washed twice, resuspended in 200 μL of PBS and then added to a black 96-well plate. The experiments were performed in triplicate and samples were read in a microplate reader and spectrofluorimeter SpectraMax M2/M2e at 485 and 538 nm wavelengths for excitation and emission, respectively. After readings, control and treated parasites were fixed with 4% nascent formaldehyde in 0.1 M phosphate buffer (pH 7.2) and observed using a Leica DMI 6000 microscope.

Extraction, separation of neutral lipids and free sterol analysis

For the analysis of the effects of ravuconazole on the free sterol composition of the promastigotes, total lipids were extracted from control and drug-treated *L. amazonensis* promastigotes, as

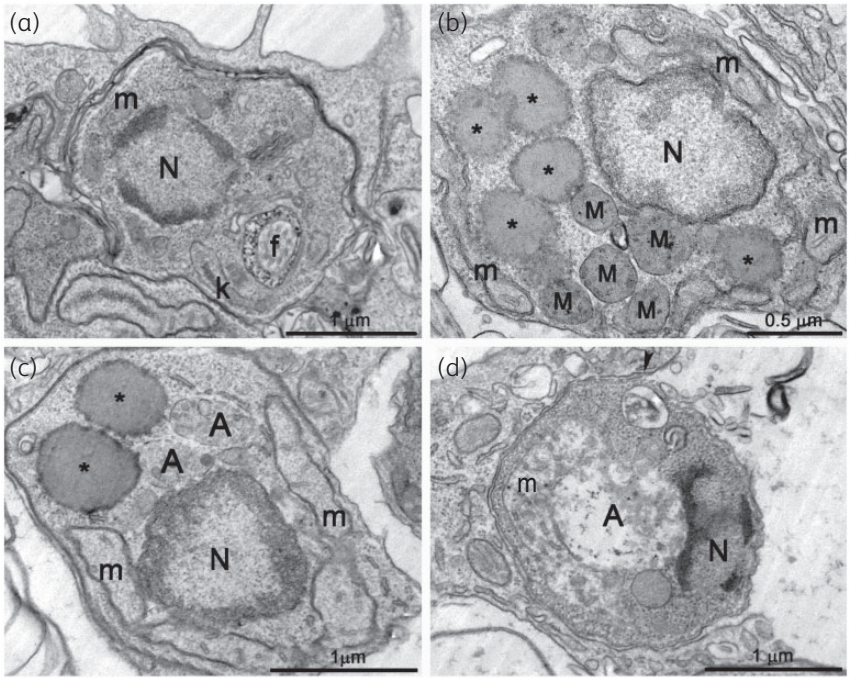


Figure 6. Ultrathin sections of *L. amazonensis* intracellular control amastigotes (a) and those treated with 6 μM ravuconazole (b–d) for 48 h. Different alterations were observed, such as the presence of several lipid bodies (b and c; asterisks), mitochondrial swelling (b–d) and loss of the matrix content (d) and the presence of structures similar to autophagosomes close to mitochondria (d) and the nucleus (c and d). The arrowhead (d) indicates a possible region of rupture of the amastigote induced by the treatment. N, nucleus; m, mitochondrion; k, kinetoplast; asterisks, lipid bodies; f, flagellum; A, autophagosome; M, megasome.

described previously.[26,27] Neutral lipids were analysed by MS and mass spectra were obtained by electron ionization (EI) at 70 eV according to the protocol published previously.[26,27] The assignment of structures was based on relative chromatographic behaviours as well as the characteristic fragmentation patterns in MS and by comparison of the mass spectra with those available in the National Institute of Standards and Technology (NIST) Research Library located at the NIST Mass Spectrometry Data Center.

Ethics

All animal models used were approved by the Ethics Committee for Animal Experimentation of the Health Sciences Centre, Federal University of Rio de Janeiro (Protocols IBCCF096/097/106), according to the Brazilian Federal Law (11.794/2008, Decreto n° 6.899/2009). Animals received humane care in compliance with the 'Principles of Laboratory Animal Care' formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences, USA.

Statistical analyses

Statistical significance of differences among the groups was assessed using the one-way analysis of variance (ANOVA) test followed by Bonferroni's multiple comparison test in GraphPad Prism 4 Software. Graphics were made using the mean of three independent experiments, where bars represent the standard deviation between them. Results were considered statistically significant when $P < 0.05$.

Results

Ravuconazole induced growth inhibition of L. amazonensis with low cytotoxicity to mammal cells

Figure 1 shows the antiproliferative effect of ravuconazole on the proliferation of *L. amazonensis* promastigotes and intracellular amastigotes. Ravuconazole had significant activity against promastigotes resulting in a concentration-dependent effect on growth inhibition and an IC_{50} value of 0.87 μM after 48 h of treatment (Figure 1a and Table 1). In addition, analysis of cell viability using the MTS/PMS assay and 7-AAD indicated that ravuconazole is lytic for promastigotes at concentrations above 0.5 μM (Figure 1b and c). As is characteristic of the effects of ergosterol biosynthesis inhibitors (EBIs) on *Leishmania* spp. and other susceptible organisms,[14–16] a delayed lytic effect was observed; at 48 h of exposure to $\geq$0.5 μM of the drug, complete growth arrest and loss of cell viability ensued, which is associated with the depletion of essential C-24 alkyl sterols (Table 2).

Ravuconazole was also tested against *L. amazonensis* intracellular amastigotes, presenting IC_{50} values of 5.11 and 1.65 μM after 48 and 72 h of treatment, respectively (Figure 2a). We also evaluated the cytotoxic effects of treatment with ravuconazole on murine macrophages using the MTS/PMS assay and CC_{50} values of $\geq$70 and 47.68 μM were observed for 48 and 72 h of treatment, respectively (Figure 2b and c). Thus, the selectivity index obtained was 28.9 after 72 h of treatment. IC_{90} values for both promastigotes and intracellular amastigotes were around 8–10 μM (Table 1).

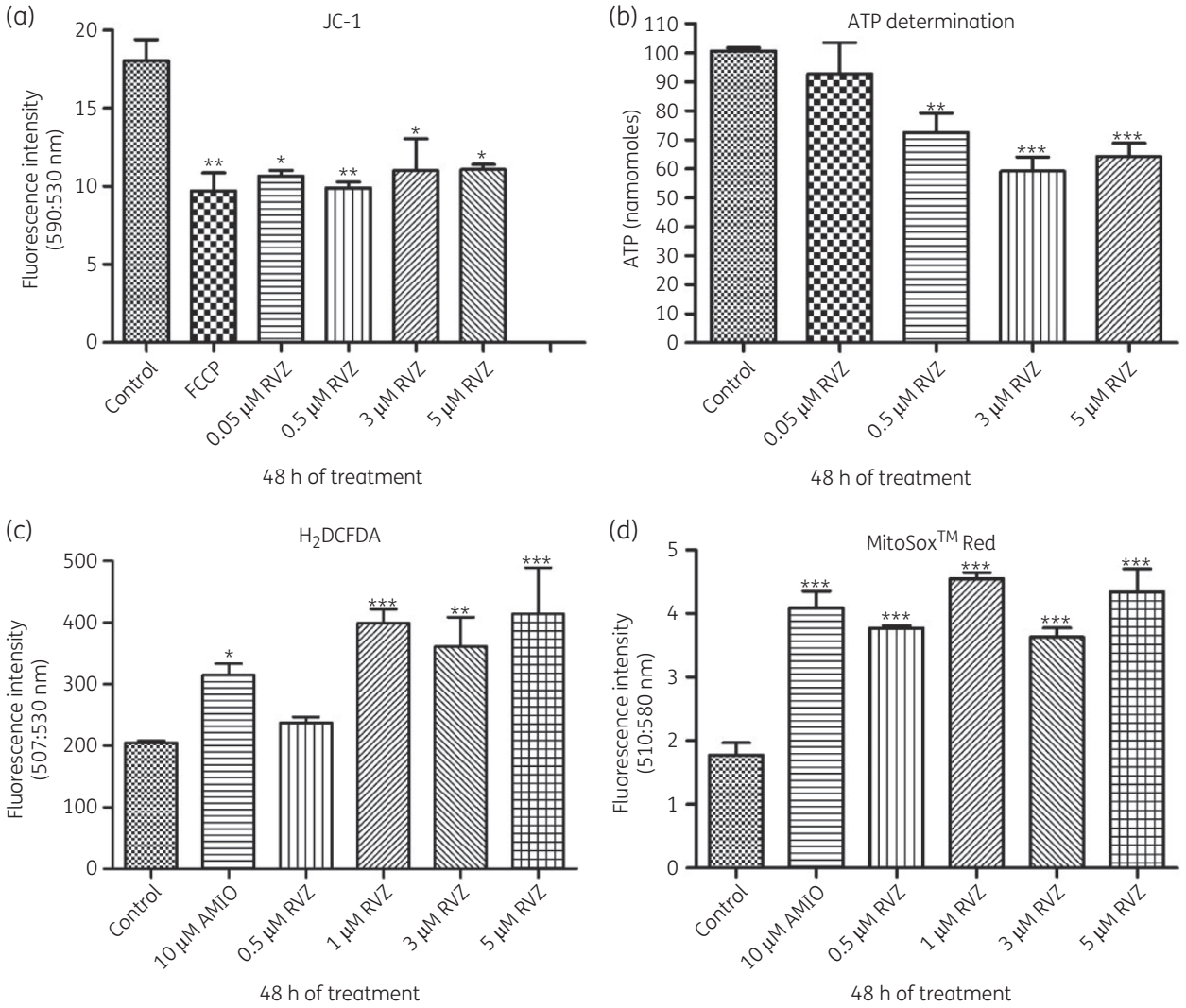


Figure 7. Evaluation of the mitochondrial physiology and function of *L. amazonensis* control promastigotes and those treated with ravuconazole for 48 h. (a) Measurement of $\Delta\psi_m$ using the JC-1 marker. (b) Measurement of ATP. (c) Determination of intracellular ROS by incubating the cells with H_2DCFDA. (d) Measurement of mitochondrial superoxide production by the use of MitoSox™ Red. (a) The $\Delta\psi_m$ was evaluated during 26 min; after this time, 2 μM FCCP was added to abolish $\Delta\psi_m$. In addition, 2 μM FCCP was also used as a positive control. The decrease in the $\Delta\psi_m$ value indicates a collapse in the mitochondrial transmembrane potential. (b) Ravuconazole also induced a significant reduction in the intracellular ATP levels at concentrations $\geq$0.5 μM. (c and d) We also measured the oxidative stress by incubating the cells with H_2DCFDA for total ROS production and MitoSox™ Red for mitochondrial superoxide. Results suggest a significant increase at concentrations higher than 1 μM for ROS production, and at all concentrations tested for mitochondrial superoxide. For these analyses, amiodarone was used as a positive control. The experiments were performed three times, each time in triplicate, and the figures shown are representative of these experiments. Bars represent standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. AMIO, amiodarone; RVZ, ravuconazole.

Ravuconazole altered the morphology of the promastigotes followed by changes in the cytoskeleton

Scanning electron microscopy revealed profound alterations in the morphology of promastigotes after treatment with ravuconazole for 48 h (Figure 3b–f). Promastigotes treated with 0.5 μM ravuconazole appeared rounded and swollen (Figure 3b and d). Interestingly, at different concentrations the cell surface of promastigotes became crumpled (Figure 3c, e and f). Alterations in the shape of promastigotes were also observed by differential interference contrast (DIC) microscopy when promastigotes were treated with 3 and 5 μM ravuconazole (Figure 4). Changes in the tubulin labelling for subpellicular microtubules were observed after treatment; fluorescence images revealed the formation of large tubulin clusters (white arrowheads) in treated parasites (Figure 4). Incubation with Hoechst to label the nucleus and kinetoplast showed that ravuconazole did not interfere with the cell cycle, a result that was corroborated by flow cytometry analysis of propidium iodide labelling (data not shown).

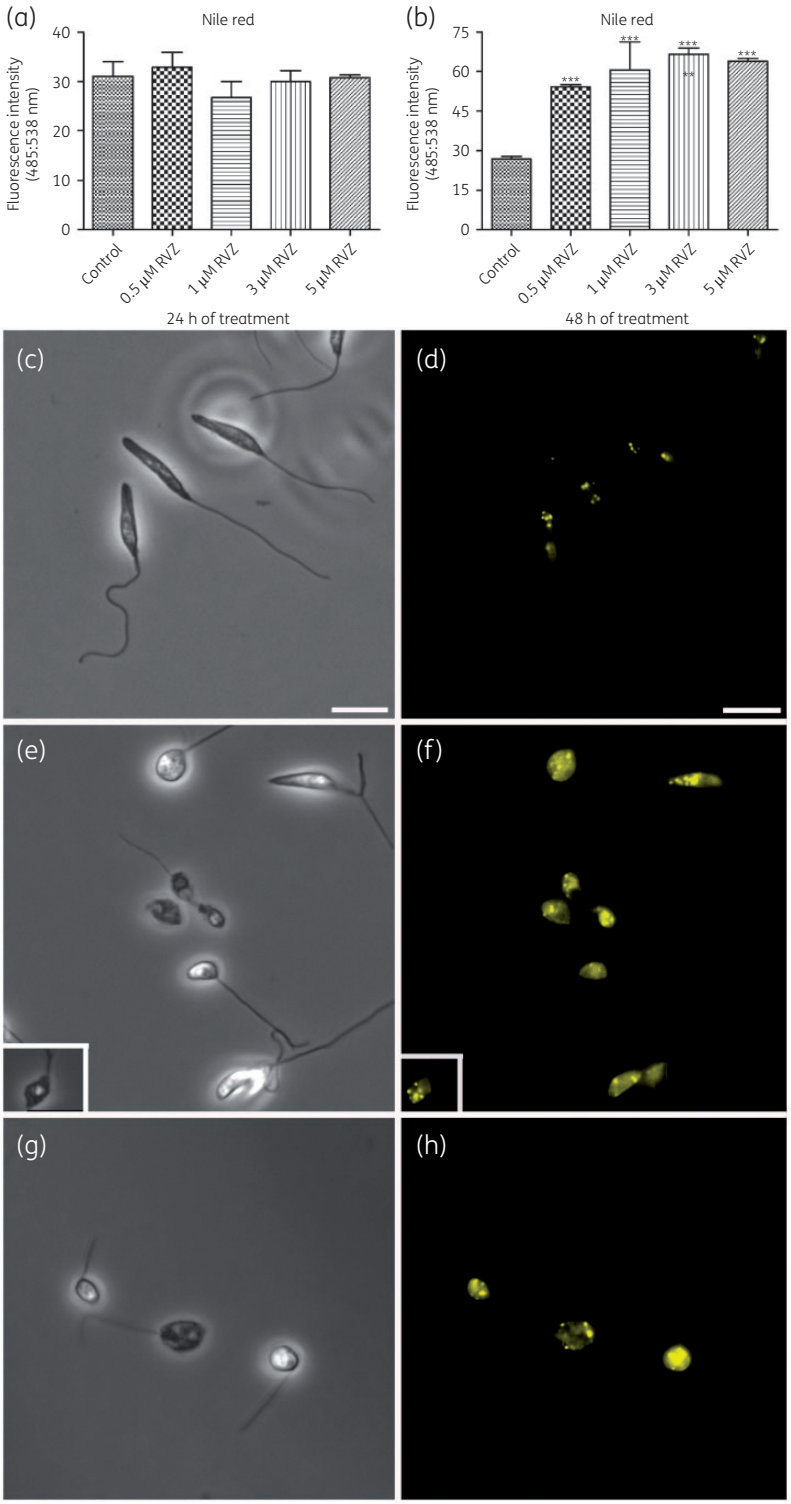
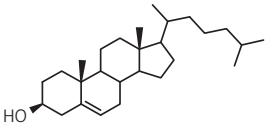
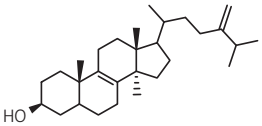
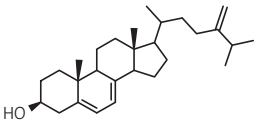
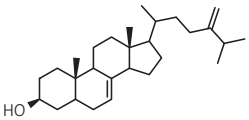
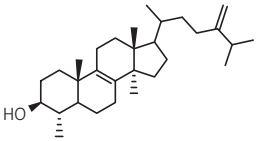
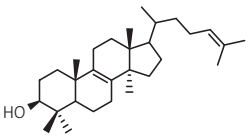


Figure 8. Analysis of Nile red accumulation and the presence of lipid bodies in *L. amazonensis* promastigotes after treatment with ravuconazole. (a and b) Fluorimetric analyses indicate that there is a significant increase in Nile red accumulation after treatment with concentrations of ravuconazole $\geq$0.5 μM for 48 h (b), different from the results observed with 24 h of treatment, in which the treated parasites presented the same values as control promastigotes (a). Fluorescence intensity is expressed as arbitrary units. The experiments were performed three times, each time in triplicate, and the figures shown are representative of these experiments. ***$P < 0.001$. (c–h) The presence of lipid bodies, randomly distributed throughout the cytoplasm, was confirmed by fluorescence microscopy. Contrast phase images also showed the significant alteration in the shape of the cell body induced by ravuconazole (e and g). (c and d) Control promastigotes. (e and f) 3 μM ravuconazole. (g and h) 5 μM ravuconazole. Bars (a and b) indicate standard deviation; scale bars, 10 μm for fluorescence images (c–h). RVZ, ravuconazole. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Table 2. Free sterols present in *L. amazonensis* in the absence or presence of ravuconazole

Compound	Structure	Retention time (min)	Percentage of compound among free sterols in promastigotes exposed to ravuconazole at				
			control	0.5 μM	1.0 μM	5.0 μM	8 μM
Cholesterol		27.2	10.8	9.0	7.1	7.6	6.4
14α-Methyl-ergosta-8, 24(24′)-dien-3β-ol		30.7	—	62.8	57.5	38.7	30.1
5-Dehydroepisterol		31.2	76.4	8.8	6.3	6.5	6.2
Episterol		31.8	12.8	—	—	—	—
Obtusifoliol		32.3	—	19.4	29.1	47.2	50.6
Lanosterol		33.6	—	—	—	—	6.7

Ravuconazole altered mitochondrial ultrastructure and function

Transmission electron microscopy was used to study the ultrastructure of treated *Leishmania* to identify the main organelles affected by ravuconazole. Figure 5(a) shows a control *L. amazonensis* promastigote presenting a normal ultrastructure for organelles such as the mitochondrion and nucleus. The mitochondrion was the organelle most altered by the drug treatment (Figure 5b–f), appearing swollen and completely disorganized. Some images also suggested a close association between autophagosomes and mitochondria (Figure 5c, d and f). Similar alterations were observed in intracellular amastigotes (Figure 6), in which the mitochondrion appeared swollen (Figure 6c). In addition, Figure 6(d) indicates loss of the mitochondrial matrix content and the presence of a giant autophagosome near the mitochondrion.

Since the mitochondrion ultrastructure was significantly altered in treated cells, we decided to analyse mitochondrial function using four criteria: $\Delta\psi_m$ using JC-1 fluorochrome; measurement of ATP levels; production of ROS; and production of superoxide radicals. Promastigotes were treated with ravuconazole and then analysed to evaluate $\Delta\psi_m$ in parallel with measurements of ATP production. The pre-treatment with ravuconazole for 48 h led to a marked reduction of $\Delta\psi_m$, similar to that observed with Carbonyl cyanide 4-(trifluoro-methoxyl)phenylhydrazone (FCCP), a classical protonophore that abolishes $\Delta\psi_m$ (Figure 7a). The decrease in $\Delta\psi_m$ was associated with a reduction in intracellular ATP levels after treatment with 0.5, 3 and 5 μM ravuconazole (Figure 7b). We also analysed the effect of ravuconazole on ROS and mitochondrial superoxide production in treated promastigotes; we used amiodarone as a positive control, owing to its known inhibitory activity on oxidative phosphorylation via dissipation of $\Delta\psi_m$ and increase in ROS and superoxide production.[15,25] Treatment with ravuconazole induced an increase in ROS; the effect was most evident with 1, 3 and 5 μM (Figure 7c). Furthermore, all treatments also induced a significant increase in mitochondrial superoxide production (Figure 7d). These results indicated a potent deleterious effect of ravuconazole on the mitochondrial physiology of promastigotes, consistent with the results obtained by transmission electron microscopy.

Ravuconazole induced the accumulation of lipid bodies

Untreated and treated promastigotes were incubated with Nile red, a phenoxazone dye that binds to neutral lipids and can be used for quantification of lipid bodies.[10,14] Fluorescence

microscopy revealed the presence of lipid bodies randomly distributed throughout the cytoplasm in both untreated and treated promastigotes (Figure 8); however, in treated parasites there was a time-dependent increase in these structures (Figure 8e–h), not seen in control cells (Figure 8c and d). Quantitative fluorimetric analysis revealed that all treatments induced accumulation of lipid bodies after 48 h of treatment (Figure 8a and b), which was not observed in control cells. Lipid bodies were also observed by transmission electron microscopy in promastigotes (Figure 5e) and intracellular amastigotes (Figure 6b and c).

Effects of ravuconazole on L. amazonensis free sterol composition

High-resolution capillary GC coupled with MS was used to analyse the free sterol composition of control and treated promastigotes (Table 2). The free sterols of control (untreated) promastigotes were ergosta-5,7,24(24′)-trien-3β-ol (5-dehydroepisterol) and ergosta-7,24(24′)-dien-3β-ol (episterol), both synthesized *de novo*, which accounted for 76% and 13%, respectively, of the total sterols, while cholesterol, taken by endocytosis from the growth medium, accounted for 11%. When promastigotes were exposed to ravuconazole for 72 h it was found that the concentration-dependent effect on the growth rate of these cells (Figure 1) was associated with marked changes in their sterol content (Table 2): (i) a dramatic reduction of the major sterols, 5-dehydroepisterol and episterol, found in control cells; and (ii) a concomitant accumulation of endogenous 14-methyl-sterols, mainly 14α-methyl-ergosta-8,24(24′)-dien-3β-ol and 4α,14α-dimethyl-ergosta-8,24(24′)-dien-3β-ol (obtusifoliol), which accounted for 82%–87% of total free sterols at the concentrations assessed, whereas cholesterol levels remained constant, at 7%–10%. These results are essentially identical to those observed in our previous study on the effects of itraconazole and posaconazole on this parasite[15] and indicated that the primary target of ravuconazole in *L. amazonensis* promastigotes is the cytochrome P450-dependent sterol C14α demethylase, as expected.

Discussion

Although leishmaniasis has a high socio-economic impact worldwide, there is no commercial interest in developing new pharmaceutical compounds. Nowadays, the treatments available result in several complications for the patients.[9,12,28] We have focused the present and previous studies on *L. amazonensis* as in Brazil it is one of the most significant species that can cause all clinical manifestations of leishmaniasis;[29] also, it is particularly important for the epidemiology of leishmaniasis in the Amazon region.[30] When the immune system fails to mount an appropriate response against the parasite it can lead to diffuse cutaneous leishmaniasis, which is a serious public health problem in Brazil because the lesions cover a large part of the body, with devastating effects on the patients, and it is incurable using currently available treatments.[30,31] Thus, there is an urgent need for development of new chemical entities that could be used for treatment of leishmaniasis. One of the interesting tools in medicinal chemistry is drug transposition, in which molecules with known activity for related diseases are tested for neglected diseases. From this perspective, azole derivatives, which are known inhibitors of sterol C14α-demethylase and used for the treatment of fungal infections, have been tested against protozoan parasites such as *Leishmania* spp. and our previous studies have shown that *L. amazonensis* is particularly susceptible to such inhibitors.[14,15,32,33]

Ravuconazole is a second-generation triazole that has been developed for the treatment of invasive fungal infections.[34] Against intracellular amastigote forms of *Trypanosoma cruzi*, ravuconazole showed potent activity *in vitro* with an MIC of 1 nM after 96 h of treatment.[16] In animal models of Chagas' disease the anti-*T. cruzi* activity of ravuconazole was limited by the short serum half-life of the drug in mice and dogs,[16,17] but the much longer half-life in humans suggests that ravuconazole should be efficacious for treatment of human Chagas' disease, with appropriate doses and treatment duration.[17,19]

In the present study, we found a potent *in vitro* effect of ravuconazole against promastigotes and intracellular amastigotes of *L. amazonensis*. Comparing the effects on intracellular amastigotes, the activity of the drug against *L. amazonensis* was lower than that against *T. cruzi*, as the IC_{50} value found in our study after 72 h of treatment (1.65 μM) was significantly higher than that for *T. cruzi*.[16] For *L. amazonensis*, itraconazole was more potent than ravuconazole against promastigotes after 72 h of treatment,[14] but ravuconazole had better activity than posaconazole after the same incubation time; however, for intracellular amastigotes, the IC_{50} values were similar.[14]

We also evaluated the viability of promastigotes by different methods to investigate the cellular mechanism of action of ravuconazole. Using the MTS/PMS metabolic assay and the fluorescent marker 7-AAD for DNA we found that ravuconazole affected the shape, plasma membrane integrity and cell viability after 72 h of incubation at >0.5 μM. GC-MS analysis showed that the alterations mentioned above were associated with drastic changes in the composition of parasite sterols as the main sterols (episterol and 5-dehydroepiserol) were almost completely replaced by 14α-methyl sterols. Thus, alterations in the lipid composition of the plasma membrane seem to result in significant changes in its permeability properties, leading to cell death of the parasites by a mechanism similar to necrosis in human cells. Moreover, when the parasites were treated with the same levels of ravuconazole, $\Delta\psi_m$ decreased significantly, resulting in a reduction of intracellular ATP levels and an increase in mitochondrial ROS production (Figure 7). Consistently, transmission electron microscopy images indicated that the mitochondrion ultrastructure was dramatically altered in both developmental stages of the parasite (Figures 5 and 6). We also observed some structures similar to autophagic vacuoles close to mitochondria, indicating a possible induction of the mitophagy process. Several studies from our group showed the effects of EBIs on the *L. amazonensis* and *T. cruzi* mitochondrion.[14,15,25,26,35] As published previously, the single mitochondrion of trypanosomatids has a special sterol composition,[36] confirming that this organelle is a primary target for EBIs. The mitochondrion alterations were accompanied by accumulation of lipid bodies in the cytoplasm of the parasites (Figure 8). Several studies had reported these lipid bodies as a place of abnormal accumulation of endogenous intermediates, most probably resulting from the inhibition of ergosterol biosynthesis.[26]

In conclusion, ravuconazole has potent antiproliferative effects on promastigotes and intracellular amastigotes of *L. amazonensis*. Ravuconazole is able to alter the composition of free sterols,

physiology, morphology and ultrastructure of promastigotes, leading to cell lysis. Together, these results indicate that ravuconazole could be a promising treatment for leishmaniasis, alone or in combination with other drugs, and support future *in vivo* studies in murine models infected with *L. amazonensis*.

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Transparency declarations

None to declare.

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