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Anti-leishmanial evaluation of C2-aryl quinolines: Mechanistic insight on bioenergetics and sterol biosynthetic pathway of *Leishmania braziliensis*

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ABSTRACT

A series of diverse simple C2-aryl quinolines was synthesized de novo via a straightforward synthesis based on the acid-catalyzed multicomponent imino Diels–Alder reactions. Seven selected quinolines were evaluated at different stages of *Leishmania braziliensis* parasite. Among them, the 6-ethyl-2-phenyl-quinoline **5f** was able to inhibit the growth of promastigotes of this parasite without affecting the mammalian cells viability and decreasing the number of intracellular *L. braziliensis* amastigotes on BMDM macrophages. The mechanism of action studied for the selected compound consisted in: (1) alteration of parasite bioenergetics, by disrupting mitochondrial electrochemical potential and alkalinization of acidocalcisomes, and (2) inhibition of ergosterol biosynthetic pathway in promastigote forms. These results validate the efficiency of quinoline molecules as leishmanicide compounds.

1. Introduction

Leishmaniasis is one of the six more important parasitic diseases worldwide, affecting 14 million people and 350 million on risk of contracting the disease.[1] Leishmaniasis includes three different clinical forms: cutaneous (CL), muco-cutaneous (ML) and visceral (VL), being the last one, which causes more mortality in humans. The first-line recommended therapy is based on pentavalent antimonials drugs like Glucantime® and Pentostam®. However, these drugs generate severe side-effects on heart, liver and kidneys.[2] Moreover, antimonial-resistant parasites have emerged in endemic areas that have limited treatment and promotes the used of second-line alternatives.[3] At present, the alkyl-lysophospholipid Miltefosine® appears to represent a major advance in the treatment of visceral and cutaneous leishmaniasis, despite its teratogenic characteristics, which limits its use during pregnancy.[4]

Cutaneous leishmaniasis is a disfiguring and stigmatizing disease with non-fatal consequences for the patient.[5] In South America, 300,000 new cases of CL appear annually. *Leishmania braziliensis* is responsible for nearly 90% of all CL cases, and absence or incomplete treatment is associated with the subsequent development of mucocutaneous leishmaniasis.[6] The treatment of CL is based on the same mentioned drugs, but it has been proved the inconsistence in their effectiveness against different *Leishmania* species.[3]

The use of natural and synthetic compounds is a major strategy against parasitic diseases, and an example is antimalarial agent chloroquine.[7] Quinoline derivatives have also revealed a wide spectrum of biological activities such as antibacterial,[8] cytotoxic and antineoplastic,[9] antimycobacterial,[10] and antiviral behavior.[11] Quinolines have been widely used as leishmanicides since ancient times. The Bolivian Indians have used extracts from plants of the *Rutacea* family to treat the wounds of CL and a study of these plants revealed the presence of diverse simple 2-substiuted quinolines that may affect the viability of different Leishmania species.[12] Since

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this discovery, compounds with a quinoline skeleton became to be considered attractive scaffolds to develop new anti-protozoal agents.[13,14]

Sitamaquine, an 8-aminoquinoline, has been proposed as oral treatment alternative against VL, but its efficacy for CL was very poor.[15] The mechanism of action of this experimental drug on *Leishmania donovani* promastigotes consists in the inhibition of complex II of the respiratory chain. This effect produces a depletion in mitochondrial electrochemical potential, a drop in ATP levels, and finally an apoptosis-like lethal response.[16] Although some biological details against the quinoline mechanism on *Leishmania* are now available, further studies of the quinoline structure-activity relationship, and mechanistic insight are urgently needed. Furthermore, there are few studies that evaluate this family compounds as a possible alternative for the treatment of CL caused by *L. braziliensis*.[17–19]

In the course of our ongoing screening program for new biologically actives *N*-heterocycles compounds, we have previously reported the antiparasitic and antifungal effects of different substituted quinolines.[20,21] Herein, we report the anti-leishmanial properties of diverse C2-aryl quinoline derivatives that can modify bioenergetics of *L. braziliensis* through disrupting mitochondrial electrochemical potential and alkalinization of acidocalcisomes.

2. Results and discussion

2.1. Chemistry

Selected diverse quinoline compounds **5** were prepared de novo using a straightforward synthesis based on acid-catalyzed imino Diels–Alder (imino DA) reactions[22] starting with commercial and inexpensive reagents. Performed imino DA cycloaddition of substituted anilines **1**, *N*-vinylpyrrolidin-2-one **2** and diverse benzaldehydes **3** in refluxing MeCN in the presence of 20% mol $BiCl_3$ conducted to the formation of the tetrahydroquinolines **4**, which were used as crude, without further purification, to obtain the final quinoline products **5a-g** (Table 1) via an oxidation-aromatization process. These quinolines were obtained as stable solids in good yields (45–70%) after their column chromatography purification[21] (Fig. 1).

2.2. Biology

2.2.1. Antiparasitic and cytotoxicity activity

Seven C2-aryl substituted quinolines **5a-g** were selected for evaluation on *L. braziliensis* promastigotes viability, and cytotoxicity toward mouse bone marrow-derived macrophages (BMDM). Macrophages BMDM are commonly used as an in vitro infection model for anti-leishmanial drugs assays on the intracellular amastigotes stage.[23] In the first screening we found that among quinoline compounds there were two molecules, 6-ethyl-2-phenylquinoline **5f** and 5,7-dimethyl-2-phenylqunoline **5g**, that were able to inhibit considerably the viability of promastigotes (IC_{50} $\leqslant$50 μM) while five quinoline molecules **5a-e** with a 2-(3,4-methylenedioxyphenyl) moiety (dubamine analogs) were inactive. The results also displayed that the quinoline **5g** was highly cytotoxic toward BMDM macrophages (Table 2).

Employing the Molinspiration software and software Sparc v4.6,[24] the selected quinolines **5a**–**e** were subjected to the Lipinski's rule of five analysis (drug-likeness), which helps to predict and explain biological behavior of small molecules such as quinoline compounds. Calculated log*P* parameters (Table 2) indicated that more lipophilic molecules **5c**,**e** (inactive) and **5f**,**g** (active) have the different log*P* values from 3.87 to 5.19 that reflects nature R and Ar substituents dependence. However, the latter active quinolines **5f**,**g**, being formula isomers with similar high log*P* (5.12 and 5.19), have drastic behavior on BMDM macrophages. It should be also noted that experimental drug sitamaquine, which was selected as structural analog, is even less lipophilic than the studied quinolines. Miltefosine, reference drug in this study, resulted be also less lipophilic molecule. We also addressed to another quantitative parameter for assessing lipophilicity,—log*D* depends on the partitioning of the neutral portion of the molecule population plus the partitioning of the ionized portion of the molecule population, that is it is a pH dependent function. Calculated values demonstrated good permeability, but low absorption for compounds **5f**,**g** and experimental drug sitamaquine ($3 < \log D_{7.4} < 5$), and miltefosine ($\log D_{7.4} < 1$).[25]

Having these results, we selected quinoline **5f** by its adequate in silico cell permeability and antiparasitic effect. We determined the EC_{50} of the chosen compound on promastigotes and intracellular amastigotes of *L. braziliensis* evaluating its effect on **5f** cellular bioenergetics and sterol biosynthetic pathway in promastigotes of this parasite.

The quinoline **5f** affected the viability of *L. braziliensis* promastigotes, with an EC_{50} value of 6.0 μM (Fig. 2). This value is similar to sitamaquine, evaluated on this and other parasite specie.[17] It was noted that the substituents nature on the quinoline ring are essential for leishmanicidal activity of quinoline derivatives.[12,26,27]

Paloque et al.[27] demonstrated that a series of quinoline derivatives with substitutions at C2 position, affected the viability of promastigotes of *L. donovani* with an EC_{50} of 6.6 μM. This effect is also similar to that obtained in this study with compound **5f**.

2.2.2. Intracellular amastigotes evaluation

Leishmanicidal effect of quinoline **5f** on the clinically relevant stage was realized thought the design of an in vitro infection model with macrophages BMDM and *L. braziliensis* amastigotes. Using this model, a 40% maximum infection was obtained, similar to previously results.[28] The effect of **5f** was evaluated at 96 hours post-treatment. Figure 3 shows that as the molecule **5f** concentration is increased, a concomitant decrease in the number of intracellular amastigotes was detected with an EC_{50} value of 20 μM. Similar values were reported for intracellular amastigotes of *Leishmania major* exposed to other quinoline derivatives.[29] Furthermore, we found

Table 1
Physico-chemical data of 2-aryl quinolines **5a**–**g**

Compd **5**	R_1	R_2	R_3	Ar	MF	MW	Mp (°C)	Yield (%)
a	H	H	H	3,4-$(OCH_2O)C_6H_3$	$C_{16}H_{11}NO_2$	249.26	90–92	50
b	H	Me	H	3,4-$(OCH_2O)C_6H_3$	$C_{17}H_{13}NO_2$	263.29	166–168	45
c	H	Et	H	3,4-$(OCH_2O)C_6H_3$	$C_{18}H_{15}NO_2$	277.32	175–177	45
d	H	MeO	H	3,4-$(OCH_2O)C_6H_3$	$C_{17}H_{13}NO_3$	279.29	139–141	60
e	Me	Me	Me	3,4-$(OCH_2O)C_6H_3$	$C_{18}H_{15}NO_2$	277.11	170–173	62
f	H	Et	H	C_6H_5	$C_{17}H_{15}N$	233.31	63–66	55
g	Me	H	Me	C_6H_5	$C_{17}H_{15}N$	233.31	71–74	70

MF: molecular formula; MW: molecular weight; Mp: melting point.

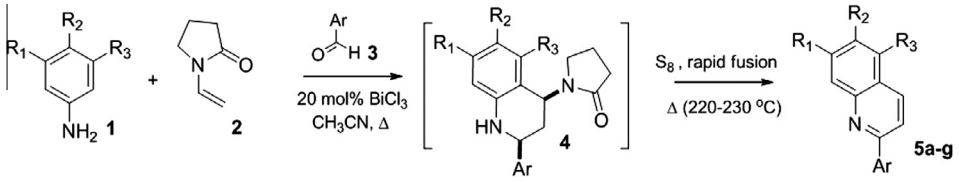
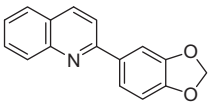
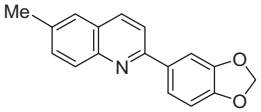
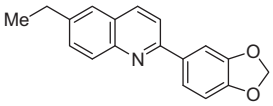
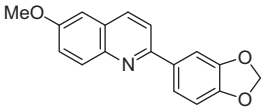
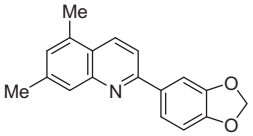
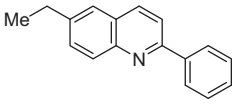
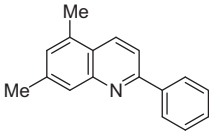
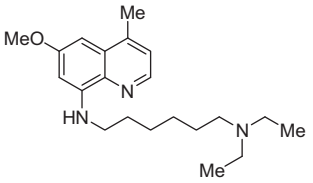
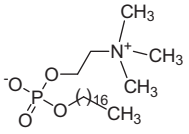


Figure 1. Scheme of synthesis.

Table 2
Evaluation of C2-aryl substituted quinolines **5a–e** on viability of *L. braziliensis* promastigotes and BMDM macrophages

Compd	Structure	Physico-chemical properties in silico			IC_{50} (μM)	
		log*P*	log D5.2	log D7.4	*L. braziliensis*	BMDM macrophages
5a		4.00	3.40	3.46	⩾50	NT
5b		4.48	3.73	3.94	⩾50	NT
5c		4.90	4.28	4.42	⩾50	NT
5d		3.87	3.78	3.95	⩾50	NT*
5e		4.97	4.05	4.38	⩾50	NT
5f		5.12	4.85	5.04	⩽**50**	⩾**200**
5g		5.19	3.65	5.10	⩽50	⩽100
Structural analog	 Sitamaquine	4.04	1.78	3.52	⩽50[24]	⩽100[17a]
Reference drug	 Miltefosine	−0.212	0.12	0.12	⩽50	⩾200

* NT—not tested on macrophages BMDM, very low activity against promastigotes of *L. braziliensis*.

that this effect is specific for intracellular amastigotes without affecting the viability of host cells (BMDM macrophages) in the concentration ranges tested.

2.2.3. Mechanisms of action studies

2.2.3.1. Parasite bioenergetics. To probe the molecular basis of the antiparasitic activity generated by quinoline **5f**, its effect on the

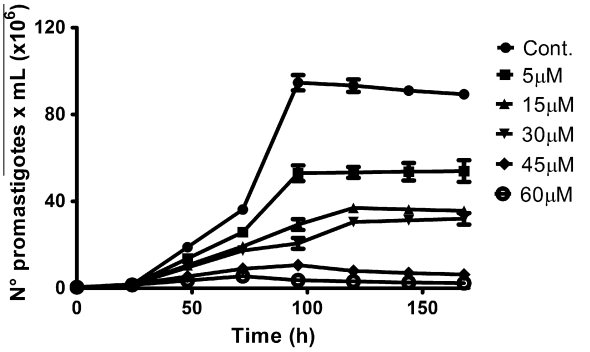


Figure 2. Evaluation of **5f** on *L. braziliensis* promastigotes. Growth curve of *L. braziliensis* promastigotes, exposed to crescent concentrations of **5f**. Each condition was made for triplicate. EC_{50} 6 μM (±2 μM).

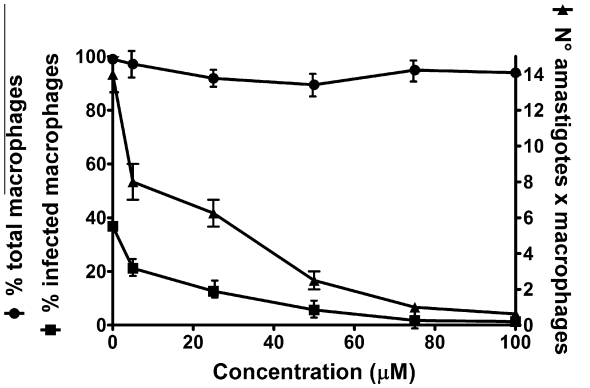


Figure 3. Quinoline **5f** evaluation on intracellular amastigotes of *L. braziliensis*. Dose–response curve, BMDM macrophages were infected with promastigotes of *L. braziliensis* (1:10) and incubated with **5f** for 96 h. ● % macrophages total, ■ % infected macrophages and ▲ N° amastigotes × macrophages. EC_{50}: 20 μM (±3 μM) calculated based on N° de amastigotes × macrophages. Each experiment was made by triplicate.

mitochondrial electrochemical potential and the accumulation of H^+ in acidocalcisomes was analyzed. Parasites loaded with rhodamine 123 were exposed to the quinoline **5f** (Fig. 4), showing a rapid increase in fluorescence similar to that obtained with trifluorocarbonylcyanide phenylhydrazone (FCCP), a classical protonophore uncoupler that dissipates the mitochondrial H^+ gradient. The uncoupling of mitochondrial H^+ gradient was previously reported with the 8-aminoquinoline sitamaquine on promastigotes of *L. donovani* parasites.[30] The authors suggested that the cationic nature of this drug generates an activation of an electrophoretic mechanism causing the failure of mitochondrial potential, compromising viability of parasites.[30] Moreover, the tafenoquine generates a loss of mitochondrial potential on *L. donovani* by inhibition of cytochrome C reductase of the mitochondrial respiratory chain,[31] while the sitamaquine inhibits electron transport chain, at succinate deshydrogenase level.[16]

When the promastigotes were loaded with acridine orange (Fig. 5), it was observed that quinoline **5f** generated a rapid alkalinization of the parasite acidocalcisomes. These results were corroborated with the addition of nigericin, a K^+/H^+ exchanger capable to alkalinize these compartments.[32] The same effect was reported in *L. donovani* exposed to sitamaquine and chloroquine.[33]

2.2.3.2. Sterol biosynthetic pathway. The sterol biosynthetic pathway has been extensively validated as an important chemotherapeutic target.[34] In this study, we demonstrated that quinoline **5f** interfere this pathway in promastigotes of *L. braziliensis*. Indeed, we were able to show an accumulation of squalene and a depletion of 5-dehydroepisterol in treated parasites (Table 3). A similar effect was observed in promastigotes of *Leishmania mexicana* exposed to miltefosine at the same period of time, affecting the viability of this species of parasites.[35] This is a first report that demonstrated the effect of aryl-quinoline compounds on the viability of *L. braziliensis*, through a blockade of sterol biosynthesis pathway.

3. Conclusion

This study demonstrates the effect of the 2-phenylquinoline **5f** on *L. braziliensis* promastigotes and intracellular amastigotes, without affecting the viability of the host cells. This confirms the effectiveness of aryl quinolinic structures against this parasite species, especially if we consider that the mechanisms of action demonstrated in this study have been previously validated with other compounds.[30,33,34,35]

Given the proven effectiveness of quinolines on VL,[9] in addition to the studies shown in this paper, we suggest that 2-phenylquinoline compounds (such as the compd **5f**) could represent an alternative for conducting more advanced studies, in the search of alternative treatments for patients with CL by *Leishmania braziliensis*.

4. Experimental

4.1. Chemistry

The melting points (uncorrected) were determined on a Fisher–Johns melting point equipment. The IR spectra were recorded on a

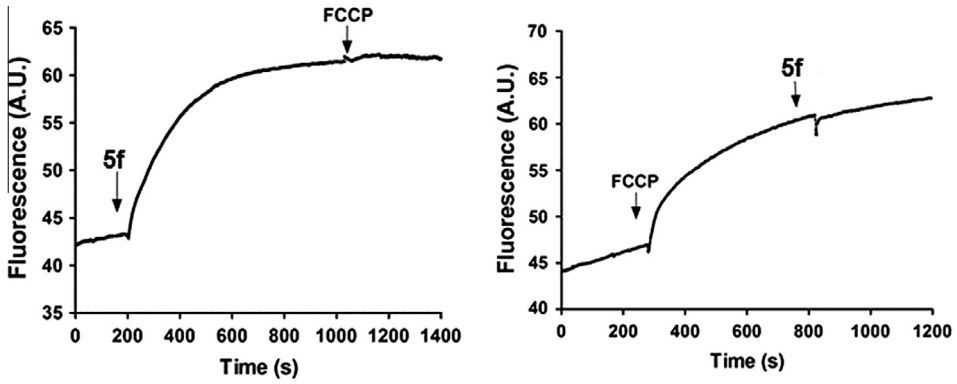


Figure 4. Action of **5f** on mitochondrial electrochemical potential in *L. braziliensis*. Promastigotes were loaded with rhodamine 123 for 45 min. Letf panel: compound **5f** at 6 μM (arrow), followed by the addition of FCCP at 1 μM (arrow). Right panel: FCCP at 1 μM (arrow), followed by the addition of the compound **5f** at 6 μM (arrow). The experiment shown is typical of at least three repeats.

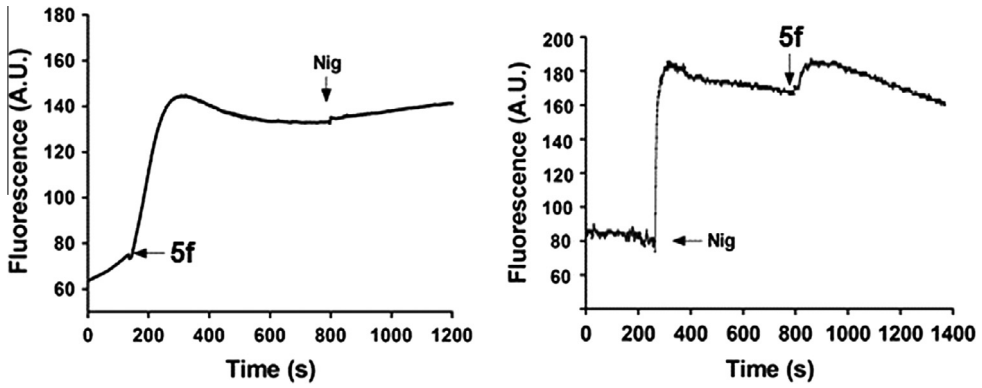


Figure 5. Evaluation of **5f** on acidocalcisomes alkalinization in *L. braziliensis*. Promastigotes were loaded with acridine orange for 5 min. Left panel: compound **5f** at 6 μM (arrow), followed by the addition of nigericine (Nig) at 2 μM (arrow). Right panel: Nig at 2 μM (arrow), followed by the addition of the compound **5f** at 6 μM (arrow). The experiment shown is typical of at least three repeats.

Table 3
Effect of quinoline **5f** on the biosynthesis of free sterol in *L. braziliensis* promastigotes

Sterols	Retention time (min)	Mass percent after treatment with		*p*-Value
		Control	Compd **5f** at 6 μM	
Exogenous cholesterol	24.2	13.6 ± 0.51	12.3 ± 1.87	0.0232
Endogenous 14-methyl squalene	20.5	22.7 ± 0.12	64.3 ± 0.89	<0.0001
Endogenous 14-desmethyl 5-dehydroepisterol	28.9	63.7 ± 1.02	23.4 ± 0.22	<0.0001

Mass percent of squalene and 5-dehydroepisterol, in this biosynthetic pathway. Parameters determined by Gas Chromatography and High Resolution Mass Spectrometry. Was made a '*t*-test' for statistics analysis, using InfoStat 2012 version,[36] (p significance $\leqslant 0.05$).

Lumex infralum FT-02 spectrophotometer in KBr. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AC-200 or Bruker AC-400 spectrometers. Chemical shifts are reported in ppm (d) relative to the solvent peak ($CHCl_3$ in $CDCl_3$ at 7.24 ppm for protons). Signals are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; ddd, doublet of doublets of doublets; t, triplet; td, triplet of doublets; q, quartet; m, multiplet; br., broad. A Hewlett–Packard 5890a series II Gas Chromatograph interfaced to an HP 5972 mass selective detector (MSD) with an HP MS Chemstation Data system was used for MS identification at 70 eV using a 60 m capillary column coated with HP-5 [5%-phenyl-poly(dimethyl-siloxane)]. Elemental analyses were performed on a Perkin–Elmer 2400 Series II analyzer, and were within ±0.4 of theoretical values. The reaction progress was monitored using thin layer chromatography on a silufol UV254 TLC aluminum sheet.

The chemical purity of obtained 2-aryl quinolines **5** was confirmed using elemental analyses, performed on a Perkin Elmer 2400 Series II analyzer, that were within ± 0.4 of theoretical values. Their spectral and physical properties were in agreement with those reported in literature.[21]

4.2. Biology

4.2.1. General

Promastigotes of *Leishmania* (*V.*) *braziliensis* strain *MHOM/CO/87/UA301* (provided by Dr. Carlos Muskus. Programa de Estudio y Control de Enfermedades Tropicales PECET, Universidad de Antioquia, Colombia) were isolated from footpad lesions in BalbC mice previously infected. For differentiation and culture maintenance was used medium LIT (tryptose 15 g/L, yeast extract 5 g/L, liver extract 2 g/L, hemin–NaOH 0.02 g/L, glucose 4 g/L, NaCl 9 g/L, KCl 0.4 g/L Na_2HPO4, 7.5 g/L, pH 7.4) supplemented with 10% fetal calf serum and maintained at 29 °C. Macrophages BMDM were obtained from mouse bone marrow and differentiated in a conditioned medium of mouse lung fibroblasts (medium L-929), as previously reported methodologies.[23,37]

4.2.2. Anti-leishamial activity and cytotoxicity

In order to evaluate the effect of seven 2-aryl quinoline derivatives on the promastigotes viability, a colorimetric test, as reported by Saint–Pierrre–Chazalet,[38] was performed with modifications. Briefly, 200,000 parasites/well were seeded in a 96-well plate, adding a unique concentration of 50 μM of each compound, and it was incubated for 96 h at 29 °C. After the incubation, 1 μg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added and than it was incubated in darkness for 4 h. After this time, the parasites were lysed with acidic isopropanol and the plate was read at 570 nm in a spectrophotometer Synergy HT (Biotek) with the software KC4 3.4 Rev. 21. Miltefosine® was used as reference drug.

Compounds that generated a 50% or more inhibition of viability (compared to untreated parasites) were selected for further evaluation. The citotoxicity on mammalian host cells (macrophages BMDM) was evaluated by the same assay, with few modifications. For this case, 5000 cells/well were seeded and the concentrations of the compounds 100, 200 and 300 μM were tested.

The EC_{50} calculation of selected compounds was performed using growth curves in LIT medium, as previously reported.[39] Briefly, the cultures were established at 1×10^6 promastigotes/mL, and were added increasing concentration of quinoline **5f** after 24 h at 29 °C. Parasite proliferation was monitored daily by direct counting in a Neubauer chamber, three independent experiments were performed.

4.2.3. Intracellular amastigotes infections

The effect of selected compound on intracellular amastigotes was performed by the protocol previously described.[40] Briefly, a mixture of macrophages BMDM and promastigotes of *L. braziliensis*, in a proportion 1:10 diluted in DMEM +10% FBS, was prepared. The mixture were placed on glass coverslips and keeping in a moist chamber for 18 h at 37 °C and 5% CO_2. After, the medium was removed, adding new medium with the tested condition and incubated for 96 h in same condition. Then, macrophages were stained with Giemsa and counted.

4.2.4. Bioenergetic determinations

The mitochondrial membrane potential estimations were carried out with the fluorescent dye rhodamine 123, that presents maximum peaks in its excitation and emission spectra at 488 and 530 nm, respectively. Rhodamine 123, is a mitochondrion-specific cationic dye that allows visualization of electrochemical potential on this organelle. As described by Serrano-Martín and co-workers,[40] 1×10^8 parasites were loaded with 10 μg/mL of rhodamine 123 in buffer 130 mM KCl, 1 mM $MgCl_2$, 2 mM KH_2PO_4, 20 mM Tris–HCl, pH 7.4 for 30 min at 29 °C. All measurements were performed in a Hitachi 7000 spectrofluorimeter at 29 °C, under continuous agitation.

The accumulation of acridine orange in the acidocalcisomes was used as a probe for alkalinization, reported by Docampo and coworkers.[33] Initially, promastigotes (10^9cells/mL) were washed twice with the same buffer and loaded with 2 μM of acridine orange for 5 min at 29 °C. Measurements were performed at an excitation λ of 488 nm and an emission λ of 530 nm in a Hitachi 7000 spectrofluorimeter under continuous agitation in a stirred cuvette at 29 °C.

4.2.5. Free sterol contents

Free sterol contents were determined by gas-liquid chromatography coupled with high-resolution mass spectrometry, as described previously.[40,41] Briefly, for the extraction and the separation of neutral lipids, *L. braziliensis* was cultured in the presence of quinoline **5f**, and total lipids were extracted with chloroform-methanol (2:1, vol/vol). The extract was dried and resuspended in a minimum volume of chloroform. The suspension was applied to a silicic acid column (1.5 by 4 cm) and washed with chloroform to separate neutral lipids from other lipid fractions.

For the quantitative analysis of free sterols and structural assignment, neutral lipids were then separated in a capillary high-resolution column (25 m by 0.20 mm [inner diameter]; Ultra-2; 5% phenyl-methyl-siloxane; film thickness, 0.33 μm) in an Agilent Technologies 7890A gas chromatograph equipped with mass-sensitive detector Agilent Technologies 5975C. The lipids were dissolved in chloroform and injected into the column at an initial temperature of 50 °C (1 min), followed by a temperature increase to 280 °C at a rate of 25 °C/min and a further rise to 300 °C at a rate of 1 °C/min. The flow rate of the carrier gas (He) was kept constant at 0.6 mL/min. The injector temperature was 250 °C; the detector was kept at 280 °C.

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