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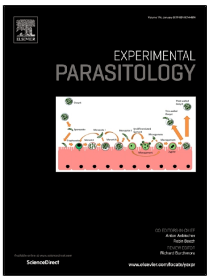

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PII:	S0014-4894(18)30341-2
DOI:	10.1016/j.exppara.2019.01.011
Reference:	YEXPR 7653
To appear in:	*Experimental Parasitology*
Received Date:	18 July 2018
Accepted Date:	20 January 2019

Please cite this article as: Jacques Leañez, Jorge Nuñez, Yael García-Marchan, Felipe Sojo, Francisco Arvelo, Daniel Rodriguez, Ignacio Buscema, Alvaro Alvarez-Aular, Josué S. Bello Forero, Vladímir V. Kouznetsov, Xenón Serrano-Martín, Anti-leishmanial effect of spiro dihydroquinoline-oxindoles on volume regulation decrease and sterol biosynthesis of Leishmania braziliensis, *Experimental Parasitology* (2019), doi: 10.1016/j.exppara.2019.01.011

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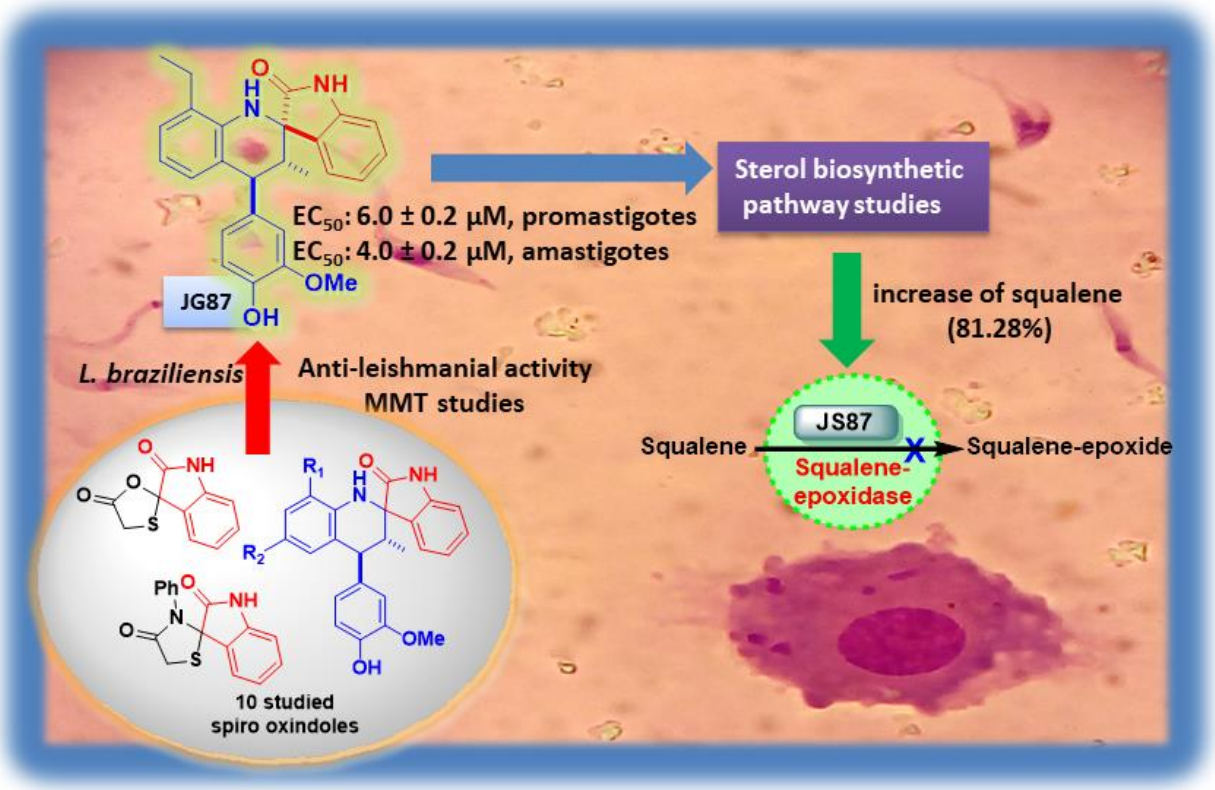


Anti-leishmanial effect of spiro dihydroquinoline-oxindoles on volume regulation decrease and sterol biosynthesis of *Leishmania braziliensis*

Jacques Leañez[a], Jorge Nuñez[a], Yael García-Marchan[a], Felipe Sojo[b,c], Francisco Arvelo[b,c], Daniel Rodriguez[d], Ignacio Buscema[e], Alvaro Alvarez-Aular[f], Josué S. Bello Forero[g], Vladímir V. Kouznetsov[g], Xenón Serrano-Martín[a,*]

[a] *Laboratorio de Biología y Quimioterapia de Parasitosis Tropicales, Área de Salud, Instituto de Estudios Avanzados (IDEA), Caracas, República Bolivariana de Venezuela.*

[b] *Laboratorio de Cultivo de Tejidos y Biología de Tumores, Instituto de Biología Experimental (IBE), Universidad Central de Venezuela, Caracas, República Bolivariana de Venezuela.*

[c] *Laboratorio de Ingeniería de Tejidos Humanos, Área de Salud, Instituto de Estudios Avanzados (IDEA), Caracas, República Bolivariana de Venezuela.*

[d] *Laboratorio de Pesquisa Selectiva, Área de Salud, Instituto de Estudios Avanzados (IDEA), Caracas, República Bolivariana de Venezuela.*

[e] *Laboratorio de Fitoquímica, Área de Agricultura y Soberanía Alimentaria, Instituto de Estudios Avanzados (IDEA), Caracas, República Bolivariana de Venezuela.*

[f] *Laboratorio de Síntesis Orgánica y Compuestos Naturales, Centro de Química, Instituto Venezolano de Investigaciones Científicas (IVIC), Caracas, República Bolivariana de Venezuela.*

[g] *Laboratorio de Química Orgánica y Biomolecular, CMN, Universidad Industrial de Santander, Parque Tecnológico Guatiguará, Km 2 Vía Refugio, Piedecuesta 681011, Colombia.*

*Correspondig author: Dr. Xenon Serrano-Martín. Laboratorio de Biología y Quimioterapia de Parasitosis Tropicales. Área de Salud. Instituto de Estudios Avanzados IDEA. Carretera Nacional Hoyo de la Puerta. Valle de Sartenejas. Baruta. Caracas, República Bolivariana de Venezuela. Teléfono: +58 212 9035095. e-mail: xenonserrano@gmail.com

Abstract

Diverse spiro dihydroquinoline-oxindoles (**JS** series) were prepared using the BF_3•OEt_2-catalyzed imino Diels-Alder reaction between ketimine-isatin derivatives and *trans*-isoeugenol. Ten spiro-oxiindole derivatives were selected and evaluated at different stages of the life cycle of *Leishmania braziliensis* parasites, responsible for cutaneous leishmaniasis in South America. Among them, the 8'-ethyl-4'-(4-hydroxy-3-methoxyphenyl)-3'-methyl-3',4'-dihydro-1'H-spiro[indoline-3,2'-quinolin]-2-one called **JS87** was able to inhibit the growth of promastigotes without affecting the mammalian cells viability, and to decrease the number of intracellular amastigotes of *L. braziliensis*. This spiro compound was found to act through the alteration of parasite internal regulation by disrupting the regulatory volume decrease (RVD), and to affect the sterol biosynthetic pathway at level of squalene epoxidase (SE) enzyme. These results revealed that the spiro annulation between quinoline and oxindole scaffolds enhances the anti-leishmanial activity, and could assist in the development of potent quinoline-oxindole hybrids against *Leishmania braziliensis*, the main etiological agent of cutaneous leishmaniasis in South America.

Keywords: *chemotherapy, Leishmania braziliensis, spiro dihydroquinoline-oxindoles, parasite internal regulation, sterol pathway, squalene epoxidase inhibitors*

1. Introduction

Leishmaniasis is one of the most neglected diseases in the world, with a significant impact on people in developing countries. It has been ranked between the six more important parasitic diseases worldwide, and it is estimated that over one billion people are at risk in endemic countries[1]. There are three different clinical forms of Leishmaniasis: cutaneous (CL), muco-cutaneous (ML) and visceral (VL), being the first one, the most common. The first-line recommended therapy since 1929 is based on pentavalent antimonials drugs like Glucantime® and Pentostam®. However, these drugs generate severe side-effects on heart, liver and kidneys[2], they are expensive and require repeated parental administration[3]. Moreover, antimonial-resistant parasites have emerged in endemic areas that have limited access to treatment, which promotes the use of second-line alternatives, including amphotericine B and miltefosine[4-6]. In South America the most prevalent clinical form is cutaneous leishmaniasis, a disfiguring and stigmatizing disease with non-fatal consequences for the patient. The parasite responsible for nearly 90% of all CL cases is *Leishmania braziliensis*[7]. This parasite has adapted to deforestation, finding new vectors and reservoirs hosts, leading to increased numbers of urban cases of cutaneous and muco-cutaneous leishmaniasis[8]. The treatment of CL is based on the same mentioned drugs, but it has been proved inconsistence in their effectiveness against different Leishmania species, including *L. braziliensis*[4].

Native Bolivians have used extracts from Galipea longiflora, a plant of the Rutacea family, to treat the wounds of CL[9]. A study of these plant extracts revealed the presence of diverse simple 2-substituted quinolines, especially 2-n-propylquinoline A (Figure 1), that affect the viability of different Leishmania species[9]. Due to this finding, synthetic molecules containing a quinoline skeleton became to be considered attractive platforms to develop new anti-protozoal agents[10,11]. Generally, quinolines and their reduced (dihydro-, tetrahydro-) derivatives have specific and general biological properties such as antitumoral [12], anti-inflammatory[13], antimalarial[14] activities. Some of the specific activities of quinoline derivatives are melanocotrin receptor agonists, acetylcholisterase inhibitors, ligands for estrogen receptors, and protein farnesyltranferase inhibitors[15]. Particularly, the 6-methoxy-8-(6-diethylaminohexylamine)-4-methylquinoline (Sitamaquine, B), developed for oral treatment against VL caused by *L. chagasi*, inhibits complex II (succinate dehydrogenase) of the respiratory chain in digitonin-permeabilized promastigotes[16]. However, the clinical development of this agent has been abandoned because of its low efficacy[17]. Recently, active C2-aryl quinoline molecules on *L. braziliensis* parasites, especially the 6-ethyl-2-phenylquinoline C were found to disrupt considerably parasite bioenergetics and sterol biosynthesis pathway[18]. Interestingly, tetrahydrogenated quinolines, the 2,4-diaryl-1,2,3,4-tetrahydroquinoline D (Figure 1) resulted also active against *L. chagasi* promastigotes, but could not access intracellular parasites forms[19].

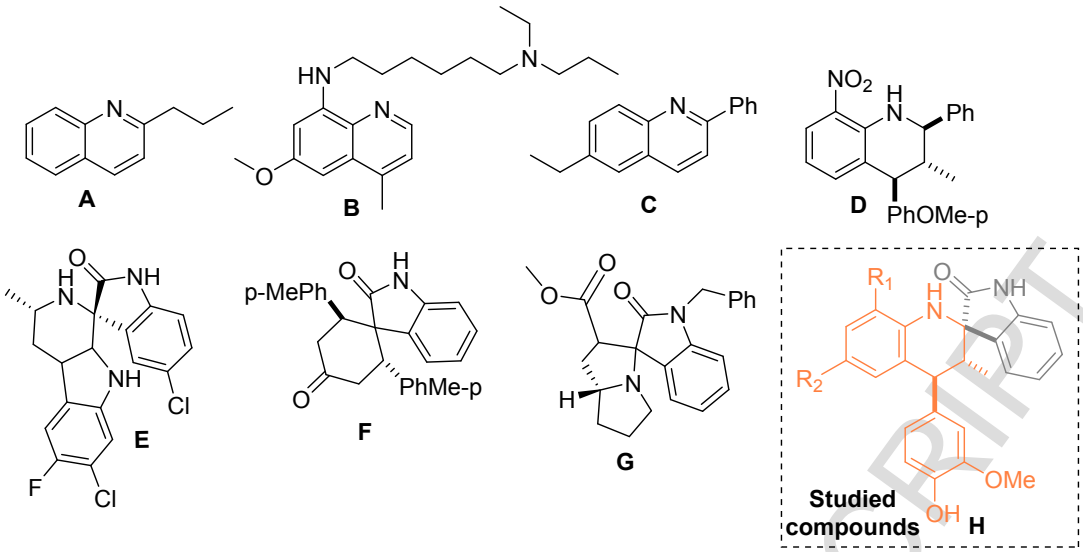


Figure 1. Chemical structures of selected quinoline and oxindole molecules possessing anti-leishmanial activity and the studied compounds. A: 2-n-propylquinoline, B: 6-methoxy-8-(6-diethylaminohexylamine)-4-methylquinoline, C: 6-ethyl-2-phenylquinoline, D: 2,4-diaryl-1,2,3,4-tetrahydroquinoline, E: spiro-indolone NITD609, F: spiro[cyclohexanone-oxindoles], G: spiro[indole-3,3'-pyrrolizidine]-2-one, H: spiro dihydroquinoline-oxindoles, examined compounds.

On the other hand, the indole, especially oxindole rings including spirocyclic oxindoles (spirooxindoles), are some of the most common heterocycles present in nature, and several biologically active natural products indole-based as well as indole-derived drugs are known to possess a variety of bioactivity profiles including anti-cancer, anti-HIV, anti-diabetic, antibacterial, antioxidant, kinase inhibitory, AChE inhibitory, anti-leishmanial, β3 adrenergic receptor agonistic, phosphatase inhibitory, analgesic, spermicidal, and vasopressin antagonists[20]. Most importantly, spiro-oxindoles are also effective anti-protozoal agents. For example, spiro-indolone NITD609 E is a potent antimalarial drug candidate[21] and spiro[cyclohexanone-oxindoles] like molecule F can stop proliferation of both promastigote and axenic amastigote forms of *L. infantum* in a dose-dependent manner[22]. Moreover, it was recently found that spiro[indole-3,3'-pyrrolizidine]-2-one G (Figure 1) acts as a catalytic inhibitor of the unusual bisubunit DNA topoisomerase IB of *L. donovani* and it holds a strong anti-leishmanial efficacy in the BALB/c mouse model of leishmaniasis[23]. It is noteworthy that anti-protozoal properties of spiro dihydroquinoline-oxindoles have not yet been studied.

Based on these findings we report the anti-leishmanial properties of diverse spiro dihydroquinoline-oxindoles H, which were easily prepared using the BF3•OEt2-catalyzed imino Diels-Alder reaction between ketimine-isatin derivatives and trans-isoeugenol.

In our study, it was demonstrated that one of these compounds (JS87) causes a profound effect on promastigotes and intracellular amastigotes viability. Also, the internal regulation of *L. braziliensis* was

affected in two ways: (i) by disruption of the RVD, and (ii) by alteration of the ergosterol biosynthetic pathway. Findings that can be helpful in further researches focused on mechanistic details of selective *L. braziliensis* growth inhibition.

2. Experimental procedures

2.1. Chemical materials. We previously published the synthesis and characterization of spiro oxindoles **6-13** tested herein[24]. Two simpler spiro oxindoles **14**,**15** were prepared following by literature reports[25,26] (Scheme 1, Table 1). The identity of these compounds was determined by ^{1}H and ^{13}C NMR as well as by IR and mass spectrometry. All tested molecules were obtained as stable crystalline substances with their respective well-defined melting points (uncorrected) (Table 1) that were determined on a Fisher–Johns melting point equipment. Their combustion analysis performed on a Perkin Elmer 2400 Series II analyzer were within ±0.4 of theoretical values that confirm their chemical purity (≥95%). The IR spectra were recorded on a Lumex infralum FT-02 spectrophotometer in KBr. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC-400 spectrometer. Chemical shifts are reported in ppm (d) relative to the solvent peak ($CHCl_3$ in $CDCl_3$ at 7.24 ppm for protons). 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)]. All the chemicals used in this study for synthesis that we used as a control) were purchased from SigmaAldrich (St. Louis, MO) or AK Scientific (Union City, CA) and used without any further purification. All purchased compounds were all ≥95% as per the suppliers. All compounds were dissolved in 0.01% DMSO.

2.2. Biological material. 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 Balb/C mice previously infected LIT medium (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_2HPO_4, 7.5 g/L at pH 7.4) supplemented with 10% fetal calf serum and maintained at 29 °C was used for parasite differentiation and maintenance. BMDM macrophages were obtained from mouse bone marrow and differentiated in a conditioned medium of mouse lung fibroblasts (medium L-929), as previously reported [27,28].

2.3. Anti-leishmanial activity and cytotoxicity. The effect of ten spiro oxindoles **6-15** was evaluated on the viability of *L. braziliensis* promastigotes through colorimetric test, as reported by Saint-Pierrre-Chazalet and co-workers [29] with minor modifications. Briefly, $2x10^6$ parasites/mL were seeded in a 96-well plate, adding 50 µM of each derivative, and incubated for 96h at 29°C. Then, 1µg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added and incubated in darkness for 4 h. After this time, acidic

isopropanol (4N) was added and the plate was read at 570 nm in a spectrophotometer Synergy HT (Biotek). Miltefosine was used as reference drug.

Compounds that generated a viability inhibition ≈ 50% or more were selected, for further evaluation on the viability of mammalian host cells (macrophages BMDM). On these host cells, we used MTT assay with few modifications, seeded 5000 cells/well and the compounds concentration tested was 300 μM. From these values were determined ranges of EC_{50} (half maximal effective concentration) and CC_{50} (half maximal cytotoxic concentration) for promastigotes and BMDM, respectively.

The EC_{50} calculation of a selected compound was performed using growth curves in LIT medium, as previously reported[30,31]. Briefly, the cultures once established ($1x10^6$ promastigotes/mL) increasing concentration of **JS87** (and miltefosine as reference drug) were added after 24 h at 29°C. Parasite proliferation was monitored daily by direct counting in a Neubauer chamber; three independent experiments were performed for each condition. EC_{50} index was calculated as previously reported [30,31]. The selectivity index was calculated with $SI = CC_{50} / EC_{50}$, where CC_{50} was the maximum concentration range of JS87 used to evaluate cytotoxicity in BMDM.

2.4. Intracellular amastigotes infections. The effect of the selected compound on intracellular amastigotes was evaluated according to previously described methods [16]. Briefly, a mixture of macrophages BMDM and promastigotes of *L. braziliensis* was prepared, in a proportion 1:10 diluted in DMEM +10% FBS. This mixture was incubated for 18 h at 37 °C and 5% CO_2. After this time, new medium with different derivate concentrations was added and incubated for 96 h in same conditions. Then, macrophages were stained with Giemsa and counted: % total of macrophage, % of infected macrophages and number of amastigotes by macrophages.

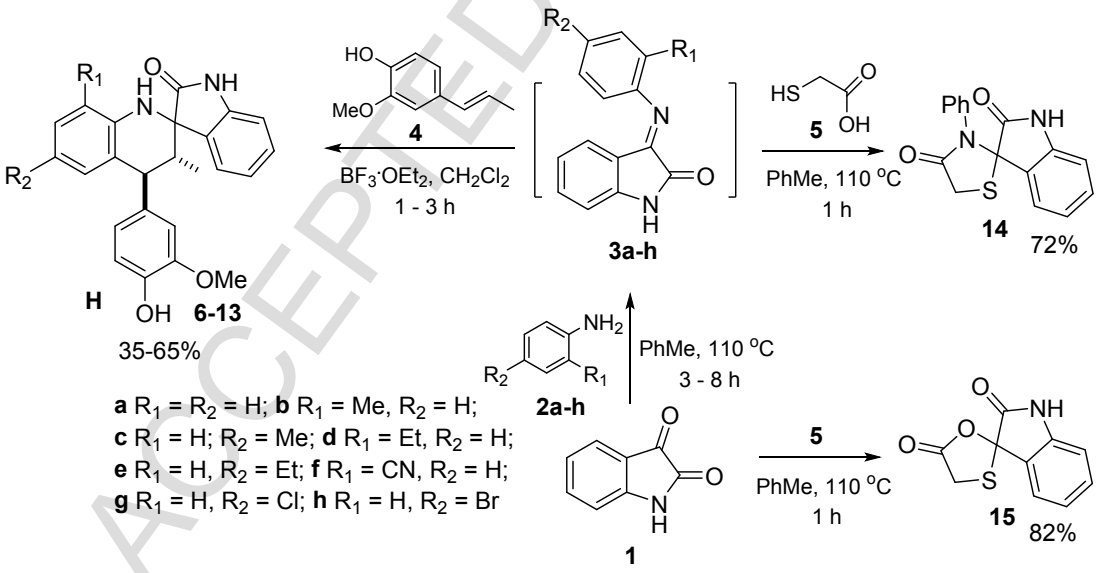
2.5. Sterol biosynthesis contents. Free sterol contents were determined by High-Resolution Gas-Liquid Chromatography coupled with High-Resolution Mass Spectrometry (HRGLC/HRMS), as described previously[32]. Briefly, for the extraction and the separation of neutral lipids, *L. braziliensis* was cultured in the presence of **JS87** for 96 h, and lipids were extracted with chloroform-methanol (2:1, vol/vol). The extract was dried and suspended in a minimum volume of chloroform. The chloroform suspension was applied to a silicic acid column (1.5 by 4 cm) and washed with chloroform to separate the neutral lipids. For the quantitative analysis of free sterols and structural assignment, the neutral lipids were separated in a high-resolution capillary column (25 m by 0.20 mm [inner diameter]; Ultra-2; 5% phenyl-methyl-siloxane; film thickness) in an Agilent Technologies 7890A (Agilent)) 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

177 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.
178 The injector temperature was 250°C; the detector was kept at 280°C.

179 **2.6. RVD studies.** For the experiments involving induction of hypo-osmotic cell stress, the method described
180 by Rohloff and co-workers,[33] was used. Briefly, promastigotes were washed with buffer Iso-Cl (137 mM
181 NaCl, 4 mM KCl, 1: 5 mM KH_2PO_4, 8.5 mM Na_2PO_4, 20 mM Hepes, 11 mM glucose, 1 mM $CaCl_2$, 0.8 mM
182 $MgSO_4$, pH 7.4, 300 mOsm) and resuspended in the same buffer at $1x10^8$ - $1x10^9$/mL. Suspensions were
183 distributed in a 96-well plate, 100 µL per well, in triplicate, and hypo-osmotic stress was induced with a 1:1
184 dilution of sterile deionized water. The internal volume changes were measured by light scattering method, at
185 550 nm absorbance and recorded every 10s for 10 min, using a spectrophotometer (Synergy HT, Biotek).

186 **3. Results and Discussion**

187 **3.1. Synthesis of spiro-oxindoles.** The preparation of selected dihydrospiro[indoline-3,2'-quinolin]-2-ones **6-**
188 **13** (molecules **H**) was performed using a straightforward synthesis based on BF_3•OEt_2-catalyzed imino Diels-
189 Alder reaction of preformed ketamines **3** derived from isatin **1** and anilines **2a-g**, and *trans*-isoeugenol **4** in
190 anhydrous CH_2Cl_2 as solvent, at room temperature from 1 to 3 h,[24] while related 3'-phenylspiro[indoline-
191 3,2'-thiazolidine]-2,4'-dione **14** was easily synthetized via a three-component condensation reaction of isatin **1**,
192 aniline **2a** and thioglycolic acid **5** in anhydrous toluene at reflux for 1 h[25] and spiro compound **15** was simply
193 obtained through heating only two components **1** and **5** under the same reaction condition[26] (Scheme 1).



194

195 **Scheme 1.** Synthesis of selected dihydrospiro[indoline-3,2'-quinolin]-2-ones **6-13** and related spiro oxindole
196 compounds **14**,**15**.

197

These spiro oxindoles were obtained as stable solids in good yields (35–82%) after chromatography column purification. Their structures were confirmed by various spectroscopic techniques such as EI-MS, ^{1}H NMR, ^{13}C NMR and IR, testing each sample as a pure chemical substance in the following biological experiments.

3.2. Preliminary antiparasitic and cytotoxicity activity: MTT studies. The obtained de *novo* spiro oxindoles **6-15** identified as series **JS** were evaluated on *L. braziliensis* promastigotes proliferation and cytotoxicity assays on mouse bone marrow-derived macrophages (BMDM). In the first screening through MTT assays, we observed that almost all compounds showed an appreciable biological activity (EC_{50} < 50 μM) on parasite proliferation (Figure 2) and low BMDM cytotoxicity (CC_{50} > 300 μM) (Table 1). It was found that only one compound, spiro[indoline-3,2'-[1,3]oxathiolane]-2,5'-dione **15** (**JS61**) resulted to be inactive (EC_{50} > 50 μM) against *L. braziliensis* promastigotes. Low toxicity of dihydrospiro[indoline-3,2'-quinolin]-2-ones **6-13** on mammalian cells has also been reported for other cells lines[34,35].

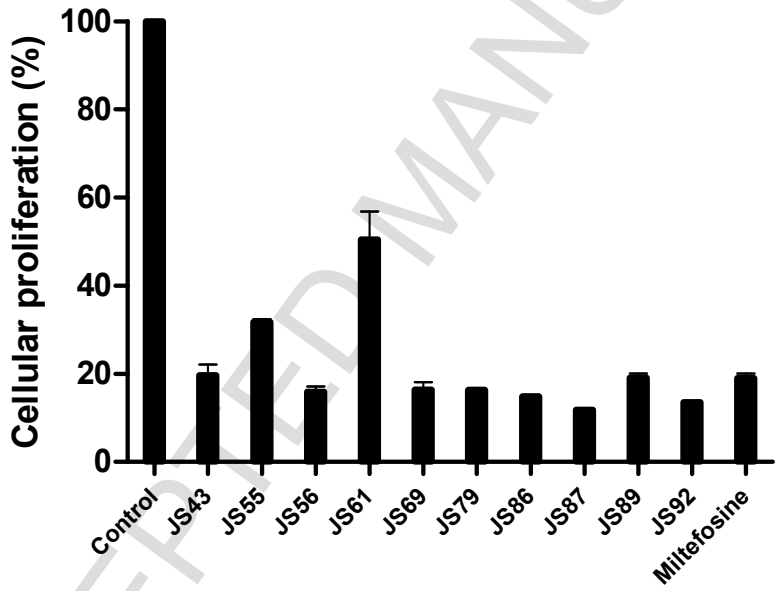
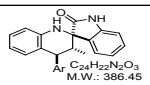
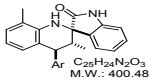
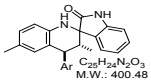
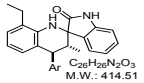
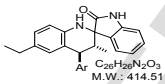
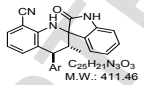
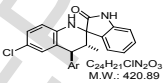
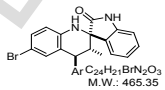
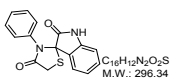
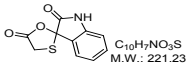
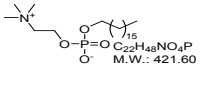


Figure 2. Evaluation of **JS** compounds on *L. braziliensis* promastigotes proliferation. Screening of compounds using MTT assay with *L. braziliensis* promastigotes exposed to 50 μM of each spiro derivative and Miltefosine. Each condition was done in triplicate.

Among the active dihydrospiro[indoline-3,2'-quinolin]-2-ones **6-13**, the 8'-ethyl-4'-(4-hydroxy-3-methoxyphenyl)-3'-methyl-3',4'-dihydro-1'H-spiro[indoline-3,2'-quinolin]-2-one **9**, code **JS87**, was the most active antiparasitic compound. Interestingly, its regio-isomer in position C-6' (**JS86**) resulted less active, but both molecules showed higher activity than miltefosine, the reference drug.

The same level of anti-leishmanial activity was obtained with the unsubstituted spiro indoline-3,2'-quinolin-2-one **6** (J**S43**) compared to 6-chlorosubstituted analog **12** (**JS89**). The substitution of Cl atom with bromine atom (comp.**13**, code **JS92**) enhanced slightly the biological effect. However, the most notable differences in activity were observed when the activity of spiro[indoline-3,2'-[1,3]oxathiolan]-2-one **15** and of spiro[indoline-3,2'-quinolin]-2-ones **6-13** or spiro[indoline-3,2*'*-thiazolidine]-2,4*'*-dione **14** were compared.

Table 1. Evaluation of Spiro Oxindoles 6-15 on the Viability of *L. braziliensis* Promastigotes and BMDM Macrophages.

Compound	Code	Structure, Mol. Formula, M.W.[a]	Physicochemical properties *in silico*[b]			EC_{50} (μM)	CC_{50}(μM)
			cLogP	cLogS	pKa	*Leishmania braziliensis*[c]	BMDM macrophages[d]
6	**JS43**	 $C_{24}H_{22}N_2O_3$ M.W.: 386.45	2.7676	-4.722	8.958	< 50	> 300
7	**JS69**	 $C_{25}H_{24}N_2O_3$ M.W.: 400.48	3.1115	-5.066	8.949	< 50	> 300
8	**JS79**	 $C_{25}H_{24}N_2O_3$ M.W.: 400.48	3.1115	-5.066	8.949	< 50	> 300
9	**JS87**	 $C_{26}H_{26}N_2O_3$ M.W.: 414.51	3.5271	-5.225	8.943	< 50	> 300
10	**JS86**	 $C_{26}H_{26}N_2O_3$ M.W.: 414.51	3.5271	-5.225	8.949	< 50	> 300
11	**JS56**	 $C_{25}H_{21}N_3O_3$ M.W.: 411.46	2.6032	-5.495	8.922	< 50	> 300
12	**JS89**	 $C_{24}H_{21}ClN_2O_3$ M.W.: 420.89	3.3736	-5.458	8.935	< 50	> 300
13	**JS92**	 $C_{24}H_{21}BrN_2O_3$ M.W.: 465.35	3.4928	-5.556	8.931	< 50	> 300
14	**JS55**	 $C_{16}H_{12}N_2O_2S$ M.W.: 296.34	1.9324	-4.461	nd	< 50	> 300
15	**JS61**	 $C_{10}H_7NO_3S$ M.W.: 221.23	0.3287	-3.114	nd	> 50	> 300
Miltefosine	**Drug reference**	 $C_{22}H_{48}NO_4P$ M.W.: 421.60	0.5768	-2.66	nd	< 50	> 200

[a] Ar is 2-methoxy-4-methylphenyl, M.W. - molecular weight. [b] Theoretical values cLogP, cLogS were calculated using commercially available the OSIRIS Property Explorer software, and pKa were calculated with ChemDraw 15.0 program: cLogP = n-octanol/water partition coefficient. The lipophilicity optimum interval is 0 < Log P < 3; cLogS = aqueous solubility. [c] Compounds

with EC_{50} > 50 µM were classified as inactive for *L. braziliensis* protozoan. [d] Compounds with CC_{50} < 100 µM were classified as toxic for the mammalian cell lines.

From these results it can be concluded that spiro annulation of indoline and tetrahydroquinoline nucleus is responsible for anti-leishmanial activity in this series of molecules. In order to understand the biological behavior of this series, the physicochemical properties of the tested compounds **6-15** were calculated using the Osiris Property Explorer software. In general, the values of calculated partition coefficient (cLog P) of the active compounds **6-14** are near the optimal range of the lipophilicity (1.9324 < cLog P < 3.5271), thus these compounds are all lipophilic molecules with good enough water solubility at pH 7.4 (-4.461< cLogS < -5.556). It should be noted that more than 80% of the drugs have an estimated logS value greater than -4.

Miltefosine, the reference drug in this study, resulted to be a lower lipophilic molecule (cLog P = 0.5768) with higher water solubility (cLogS = -2.66), but the spiro[indoline-3,2'-[1,3]oxathiolane]-2,5'-dione **15**, which was inactive, has similar parameters (cLog P = 0.3287, cLogS = -3.114). Hence, these results obtained cannot explain clearly the structure-antileishmanial activity relationship of the tested spiro oxindoles in this work. Nevertheless, the most active antiparasitic compound **JS87** was selected for subsequent studies.

3.3. Anti-leishmanial activity of JS87: Growth curve and EC_{50} determination. The compound **JS87** affected the viability of *L. braziliensis* promastigotes with an EC_{50}: 6.0 ± 0.2 µM (Figure 3). A similar result was obtained with the 6-ethyl-2-phenylquinoline on *L. braziliensis* promastigotes (EC_{50}: 6.0 ± 0.2 µM)[16]. Both nitrogen-containing heterocycles were more effective than miltefosine (EC_{50}: 21 ±0.2 µM) on the viability of these parasites species. From the EC_{50} it was possible to calculate the selectivity index of the JS87 obtaining an SI> 50, resulting that the JS87 is at least 50 times more active on the parasites than on the host cell.

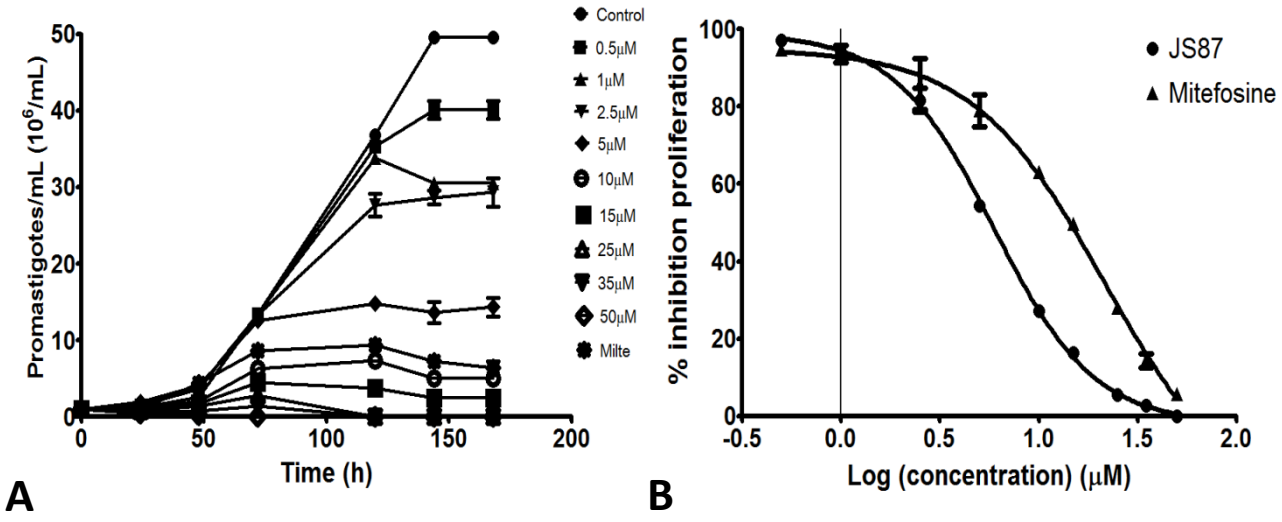


Figure 3. A) Evaluation of **JS87** on *L. braziliensis* promastigotes. Growth curve of *L. braziliensis* promastigotes, exposed to crescent concentrations of **JS87**. **B**) Determination EC_{50} of JS87 (6.0 ± 0.2 µM) and Miltefosine (21.0 ± 0.2 µM) at 96 h exposure. Each condition was done in triplicate.

3.4. Effect of JS87 on intracellular amastigotes: EC_{50} determination. Leishmanicidal effect of **JS87** on the clinically relevant stage was studied through the design of an *in vitro* infection model with macrophages BMDM and *L. braziliensis* amastigotes. With this model, a maximum infection of 65% was obtained; these results are similar to that reported by Zauli-Nascimento and co-workers [36]. The effect of **JS87** was evaluated at 96 h post-treatment. Figure 4 shows that as the **JS87** concentration increased, a concomitant decrease in the number of intracellular amastigotes was observed with an EC_{50} value of 3.3 ± 0.2µM. Analogous values were reported for intracellular amastigotes of *L. major* and *L. braziliensis*, exposed to similar quinoline molecules[16,19].

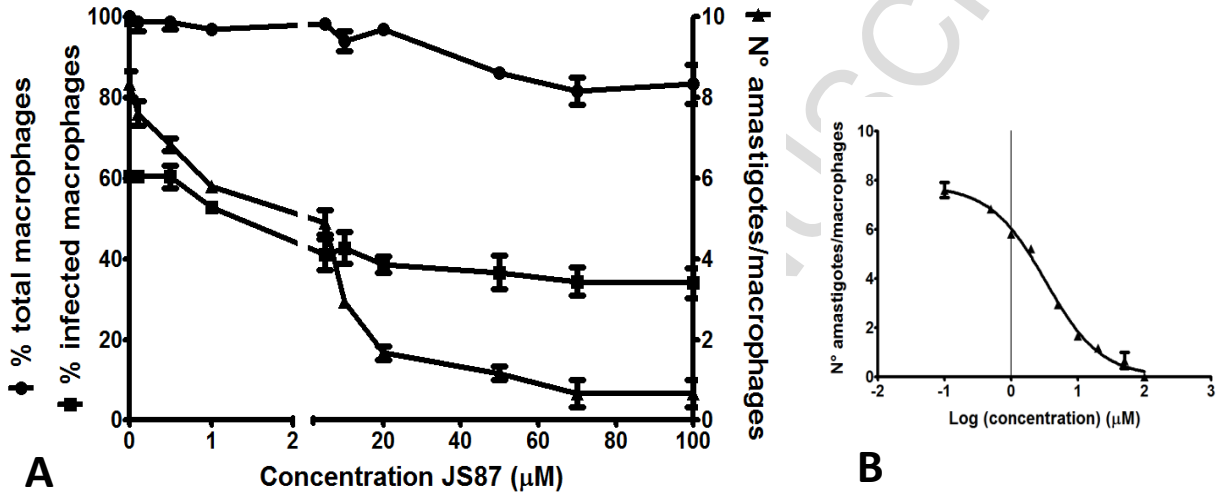


Figure 4. Evaluation of JS87 on intracellular amastigotes of *L. braziliensis*. **A**) BMDM macrophages were infected with promastigotes of *L. braziliensis* (1:10) and incubated with JS87 for 96 h. ● % macrophages total, ■ % infected macrophages and ▲ N° amastigotes x macrophages. **B**) Determination EC_{50} of JS87 (3.3 ± 0.2µM) calculated with N° de amastigotes per macrophages. Each experiment was done in triplicate.

Furthermore, we showed that this effect was specific for intracellular amastigotes without affecting the viability of host cells (macrophages BMDM) in the concentration ranges tested. When calculating SI> 90, we observed that JS87 is at least 90 times more effective on intracellular amastigotes than on BMDM, and 1.8 times more effective than on promastigotes of *L. braziliensis*. It is also evident that at the highest concentration tested (100µM), there is still 40% of infected macrophages remaining. Perhaps (and in spite of its anti-parasitic activity), this effect could attribute to limited access of **JS87** to intra-vacuolar compartments, where the amastigote proliferates within the host cell. This could affect the "in vivo" activity of the compound. It would be pertinent to take this factor into consideration, in order to make some structural modifications and improve the penetration capacity, while maintaining the anti-parasitic potency.

3.5. Sterol biosynthetic pathway. The sterol biosynthetic pathway has been extensively validated as an important chemotherapeutic target[37]. After quantification of the neutral lipid fraction in a gas chromatograph (GC) equipped with a mass selective detector (MSD), it was possible to determine that **JS87** affected this biological pathway in *L. braziliensis* (Table 2).

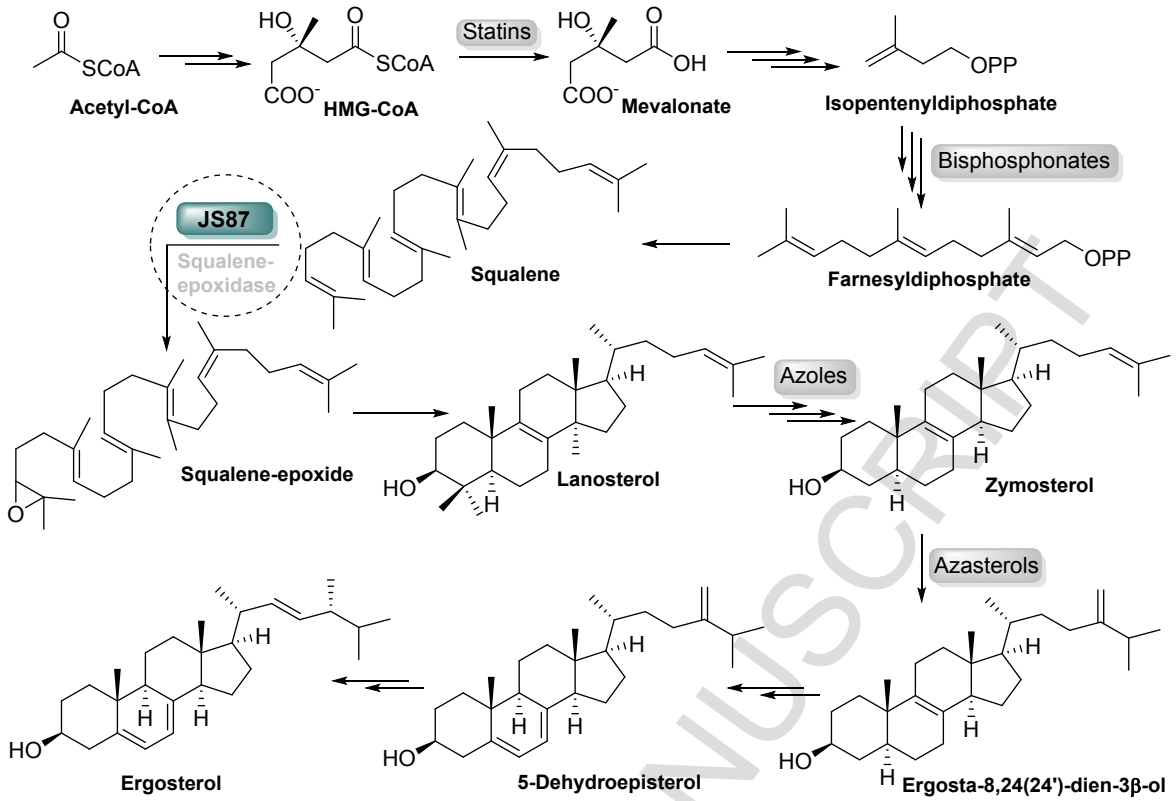
Indeed, we were able to show an increase of squalene (81.28%), concordant with: 1) a significant depletion of remainder sterols, and 2) an accumulation of exogenous cholesterol being a possible compensation mechanism like the ones proposed for *L. amazonensis*[38] and *L. donovani* [39].

These interesting outcomes suggest an affectation, on the natural behavior of squalene-2,3-epoxidase enzyme (Scheme 2), which is essential for sterols biosynthesis[40], and they demonstrate the biological effect of dihydrospiro[indoline-3,2'-quinolin]-2-ones on *L. braziliensis*, through an alternation of sterol biosynthesis pathway. The same effect was reported previously by our work group, but with aryl-quinolines[16]. Currently, we do not have enough evidence to support a direct action of **JS87** on Squalene Epoxidase. However, considering the accumulation of Squalene and the non-detection of Squalene Epoxide, we can presume that the compound affected the natural functioning of the enzyme.

Table 2. Effect of JS87 on the biosynthesis of free sterols in *L. braziliensis* promastigotes.[a]

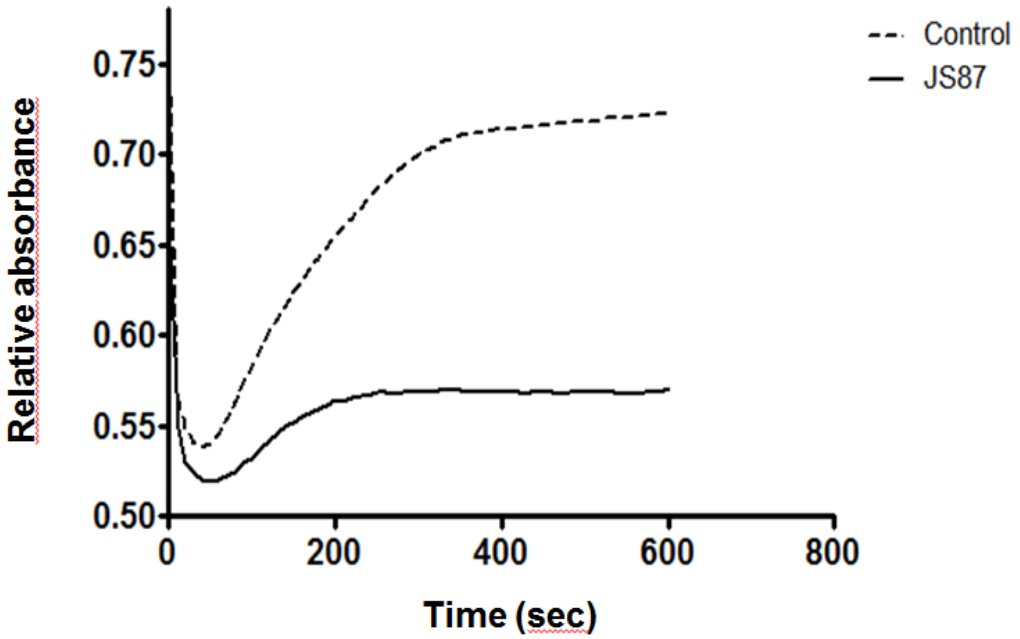
Sterol	Retention time (min)	Mass percent after treatment with:		p-value
		Control	**JS87** (6 μM)	
Exogenous cholesterol	32.72	4.83	18.72	0.0232
Squalene	26.95	18.76	**81.28**	<0.0001
Squalene epoxide	33.52	4.45	ND[b]	<0.0001
Lanosterol	35.74	8.98	ND	<0.0001
Ergosterol	35.98	76.41	ND	<0.0001

[a] Parameters determined by Gas Chromatography and High Resolution Mass Spectrometry; p significance ≤ 0.05, through "t test" (InfoStat 2012). **JS87** added at EC_{50} value. [b] ND, not detected.



Scheme 2. Fragment of sterol biosynthetic pathway, showing the possible site of action of **JS87**. (Modified using the work by Serrano-Martín and co-workers[30]).

3.6. Regulatory volume decrease (RVD). Few researches have been reported regarding RVD in trypanosomatids[41-43]. Due to its digenetic cycle, *Leishmania* spp are exposed to multiple extracellular osmotic stress conditions that involve cell swelling. In these conditions, a series of adaptive mechanisms oriented toward restoring cell volume, are activated[43-45] and if these replies fail, cell death could occur either by necrosis or apoptosis. Using a light scattering technique we registered the relative volume alteration over time, where changes in parasites absorbance are related to cell volume. In this sense, we demonstrated that **JS87** (6.0 μM) affected significantly the RVD of *L. braziliensis* under hypo-osmotic stress, compared to untreated controls (Figure 5).



307

308 **Figure 5.** Effect of **JS87** on *L. braziliensis* RVD. Promastigotes of *L. braziliensis* were incubated 96 h with 6
309 µM of **JS87** (continuous line). Parasites not exposed to **JS87** (control) are shown with a discontinued line.
310 After incubation both parasites populations, were subjected to hypo-osmotic stress. Volume recovery was
311 followed by light scattering. Each experience was done in triplicate.
312

313 Subsequent to a hypo-osmotic shock, untreated controls showed a decrease in absorbance indicating cell
314 swelling, followed by a steady rise near to original level indicating normal volume recovery. Nevertheless,
315 parasites previously treated with **JS87** showed a decrease in absorbance without the prominent rise, indicating
316 no cell volume recovery. Similar results have been obtained with *T. cruzi*[41] and *L. mexicana*[43].

317 This result represents the first report that shows the effect of spiro dihydroquinoline-oxindoles on the RVD,
318 in *L. braziliensis*. It is possible that sterol depletion was involved in a destabilization of the plasma membrane,
319 implicating affection in the natural RVD process. Currently our work group is performing experiments to
320 correlate these results to find a possible connection between both biological events.

321 **4. Conclusion**

322 Currently, treatment of leishmaniasis is far from satisfactory; all antileishmanial drugs available for
323 leishmaniasis treatment are toxic, expensive, and frequently ineffective. Due to these limitations of the existing
324 treatments, better drugs are urgently needed[45,46]. A series of spiro dihydroquinoline-oxindoles was prepared
325 via straightforward synthesis based on the $BF_3 \cdot OEt_2$-catalyzed imino Diels-Alder reaction of ketamine-isatin
326 and *trans*-isoeugenol that allowed evaluating leishmanicidal activity of 10 spiro derivatives. Among the
327 synthetized compounds, the spiro oxindole **JS87** was selected, according to its potent leishmanicidal capacity
328 and low toxicity on the host cell. It was demonstrated that **JS87** generated a profound *in vitro* activity on the

proliferation of *L. braziliensis* promastigotes at micro-molar levels, more active than the standard and alternative drug, miltefosine. Additionally, it was determined that this compound generated a selective effect on intracellular amastigotes. This effect was slightly more potent than promastigotes and did not affect the host cells (BMDM macrophages). It is also evident that at the highest concentration tested (20μM), there is still 40% of infected macrophages remaining. Perhaps (and in spite of its anti-parasitic activity), this effect could attribute to limited access of **JS87** to intra-vacuolar compartments where the amastigote proliferates within the host cell. Studying this effect, some specific biological factors were revealed that could explain the high anti-parasitic activity through a destabilization in the intracellular RVD mechanism and/or an affectation in normal activity of squalene epoxidase enzyme, which is essential for the sterol biosynthesis route. It is possible that both factors could affect synergistically against the normal functioning of the plasmatic membranes (externals and/or internal). Furthermore, it is possible that the destabilization of sterol biosynthesis could lead to the alteration of the RVD mechanism. In this sense, actually we development some assays, evaluating the effect of classical sterol biosynthesis inhibitors on RVD capacity. However, the dihydroquinoline-oxindole **JS87** could represents a promising candidate for more advanced studies in the search of an alternative drug against *L. braziliensis*, the main etiological agent of cutaneous leishmaniasis in South America.

Acknowledgments

The authors gratefully acknowledge critical comments of Lilia Ramírez, Jaime Charris and Cecilia Castillo. Financial support: this work was supported the Fondo Nacional de Ciencia Tecnología e Innovación (FONACIT), Ministerio del Poder Popular para Educación Universitaria, Ciencia y Tecnología, República Bolivariana de Venezuela (Project to XS-M: 2021002132, 2012000784 and 2012001961). V.V.K. thanks the financial support of Colombian Institute for Science and Research (COLCIENCIAS) under the project No. RC-007-2017.

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- Quinoline-oxindoles affects *Leishmania braziliensis* proliferation
- Spiro-quinolin alters parasite regulation, disrupting the regulatory volume decrease
- Quinoline-oxindoles affect the sterol biosynthesis in *Leishmania braziliensis*
- Spiro-quinolin is 90 times more effective on intracellular amastigotes than on BMDM