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New Licarin A Derivative is Effective against *Leishmania* (*Leishmania*) *amazonensis* Promastigotes and Intracellular Amastigotes

Marcilene A. Alves,[a] Patrícia F. Espuri,[b] Dalila J. Alvarenga,[c] Thalles H. F. Souza,[a] Matheus F. Alves,[a] Diogo T. Carvalho,[c] Marcos J. Marques[b] and Eduardo F. Peloso*[*,a]

[a]*Departamento de Bioquímica, Instituto de Ciências Biomédicas, Universidade Federal de Alfenas, 37130-001 Alfenas-MG, Brazil*

[b]*Laboratório de Parasitologia, Departamento de Patologia e Parasitologia, Instituto de Ciências Biomédicas, Universidade Federal de Alfenas, 37130-001 Alfenas-MG, Brazil*

[c]*Laboratório de Pesquisa em Química Farmacêutica, Faculdade de Ciências Farmacêuticas, Universidade Federal de Alfenas, 37130-001 Alfenas-MG, Brazil*

New therapeutic options against leishmaniasis are necessary, especially those of natural origin, like licarin A, a neolignan with activity against *Leishmania major*. The effect of licarin A (DL01) and its derivatives (DL03, DL10, DL17 and DL21) was evaluated against *Leishmania amazonensis* promastigotes and intracellular amastigotes. Promastigote forms were assayed in different incubation periods and the 50% effective concentration (EC_{50}) was determined. Cytotoxicity was assessed in murine peritoneal macrophages by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay to determine the 50.0% cytotoxicity concentrations (CC_{50}). Anti-amastigote activity was evaluated through the effective concentration to amastigotes (EC_{50ama} and EC_{90ama}), and selectivity indexes (SI) were calculated. Lipophilicity (LogP) and mitochondrial membrane potential ($\Delta\Psi$) were analyzed. DL21 showed a significant anti-promastigote (EC_{50pro}: 4.68 μM) and anti-amastigote (EC_{50ama} and EC_{90ama}: 0.42 and 15.91 μM, respectively) activity, and substantial SI (94.73) to amastigotes and an adequate Log P (5.54), while not changing $\Delta\Psi$. DL21 is a promising drug candidate and further studies are necessary for better understanding licarin A mechanisms of action.

Keywords: leishmaniasis, new drugs, derivatives, licarin A

Introduction

Neglected tropical diseases are a group of infectious or parasitic diseases considered endemic in tropical and subtropical regions, affecting currently more than 1 billion people worldwide, especially the poorest.[1] Among such, leishmaniasis is recognized as one of the main public health problems in Brazil and around the world.[1] Leishmaniasis groups a diverse range of parasitic diseases caused by different species of flagellated protozoa of the genus *Leishmania*, which are transmitted to humans through the bites of infected female sandflies of the genus *Lutzomyia* or *Phlebotomus*.[1] The prevalence of these diseases is estimated at 12 million people around the world currently infected, with about 1 million new records and more than 26 thousand deaths registered annually. They are considered endemic in 98 countries, in which Brazil is one of the most affected.[1] *Leishmania* protozoa infections have a wide spectrum of clinical manifestations, which vary according to the species involved and the patient's immunological response.[2,3] Different clinical forms of leishmaniasis are classically divided into visceral or Calazar (VL) and cutaneous (CL), which are subdivided into localized, disseminated, diffuse or mucosal.[2] CL forms are the most common across the world and range from skin lesions that heal spontaneously to chronic or erosive ulcers on the mucosa that lead to severe facial destruction, which in turn increases the risk of systemic infection. On the other hand, VL is mostly defined by the increase in liver and spleen volume, being lethal in up to 95% of untreated symptomatic cases.[1]

Regarding treatment, the first-line drugs of choice are pentavalent antimonials, which were introduced to the

*e-mail: eduardo.peloso@unifal-mg.edu.br
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market more than 70 years ago and display a crescent number of therapeutic failures, relapses and resistance cases, in addition to a high associated toxicity.[2-4] Other drugs are indicated as second-line therapies, such as amphotericin B and its liposomal formulations, pentamidine and miltefosine, all which also have a high cost and toxicity, along with many cases of resistance.[5] The high incidence, morbidity and mortality of leishmaniasis, as well as the limitations of available drugs have motivated countless studies searching for new therapeutic alternatives. In the search for new treatments, natural compounds and their derivatives have been considered very promising and represent about 50% of the antiparasitic drugs currently produced.[6] Among several natural compounds with antiparasitic effect, licarin A stands out, showing good activity against *Trypanossoma cruzi* and *Shistossoma mansoni*, in addition to a potent leishmanicidal action against *L. major*, which has been little studied.[7-11]

Licarin A is a compound of natural origin from the group of neolignans, which are substances present in several species of vascular plants of the lignoid class.[12] Neolignans have two phenylpropane units linked by 5-5' carbons, and in some compounds, this bond between the two phenylpropane units can be promoted by an oxygen atom.[13] The effect of neolignans against different species of leishmania has been reported, such as surinamensin, isolated from *Virola surinamensis*, which showed activity against *L. donovani* amastigotes and promastigotes.[14] The same goes for eupomatenoid-5, isolated from leaves of *Piper regnellii var pallescens*, which presents dose-dependent activity against *L. amazonensis* promastigotes and axenic amastigotes.[15] In parallel, structural modification, also called molecular variation or manipulation, is a technique often used in natural compounds that can result in greater biological activity and less toxicity.[16] Meleti *et al.*[8] verified a fourfold increase in schistosomicidal activity of acetylated licarin A compared to its unmodified version, promoting mortality of *Schistossoma mansoni* in concentrations of 50 and 200 μM, respectively. According to the same study, during the evaluation of trypanocidal activity against *Trypanosoma cruzi* trypomastigostes, licarin A was more effective than its modified derivatives, displaying a 50% effective concentration (EC_{50}) value of 100.8 μM, while its most active derivative showed an EC_{50} of 378.4 μM.[8]

Even though the leishmanicidal activity of licarin A has been known for some time, it had only been assessed against *L. major* promastigotes.[11] Therefore, our research group, in partnership with the Laboratório de Pesquisa em Química Farmacêutica (LQFar) at UNIFAL-MG, analyzed the activity of licarin A and its derivatives against different life cycle stages of *L. amazonensis*, plus the assessment of its cytotoxicity, LogP and mitochondrial membrane potential (ΔΨ).

Experimental

Compounds

The antileishmanial activity was evaluated from racemic licarin A (DL01) and four derivative compounds (DL03, DL10, DL17 and DL21) (Figure 1). These were acquired in collaboration with researchers from the LQFar at UNIFAL-MG, according to the methodologies the group had described in the study by Alvarenga *et al.*[17] In summary, licarin A was obtained from isoeugenol by an oxidative coupling methodology with hydrogen peroxide and coconut water (from fresh green coconuts, *Cocos nucifera* L.) as reported by Rodrigues *et al.*[18] Licarin A derivatives were

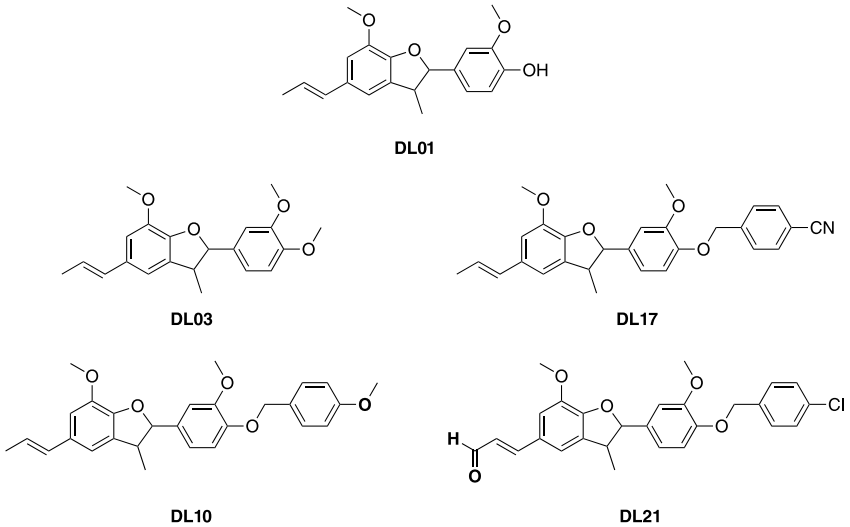

Figure 1. Chemical structure of licarin A (DL01) and its four derivative compounds (DL03, DL10, DL17 and DL21).

synthesized by etherification, epoxidation, hydrolysis, and allylic oxidation methods.

General techniques

Reagents and solvents used in this work were purchased from Sigma-Aldrich (São Paulo, SP, Brazil) and were used as purchased. Reactions were monitored by thin layer chromatography (TLC) on silica gel sheets (Duren, Germany) (Macherey-Nagel, DC-Fertigfolien Alugram® XtraSil G/UV254). Column chromatography was used as the purification method using silica gel 60, 70-230 mesh (Sorbiline®, Mumbai, India). The melting point of the compounds was determined on a Microquímica MOAs 301 apparatus (MS Tecnopon, Piracicaba, Brazil). The structures of all compounds were confirmed by spectroscopic and spectrometric techniques: (*i*) Fourier transform infrared (FTIR) spectra were recorded on a Shimadzu Affinity 1 FTIR spectrometer (Shimadzu Co, Kyoto, Japan) using an attenuated total reflectance (ATR) attachment; (*ii*) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC-300 spectrometer (Rheinstetten, Germany) (300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR spectra). Chemical shifts (δ) were reported in parts *per* million (ppm) with reference to the deuterated solvent employed ($CDCl_3$). Coupling constants (*J*) were reported in hertz (Hz); (*iii*) high resolution mass spectra (HRMS) were obtained in a Bruker (Billerica, MA, USA) Daltonics microTOF QII/ESI-TOF equipment. The values of logP were calculated using the Swiss ADME methodology provided by the Swiss Institute of Bioinformatics.[19]

General procedure for the synthesis of licarin A derivatives

The general synthesis method for the ether derivatives (DL03, DL10 and DL17) used in this study was adapted from a method published by Coolen *et al.*[20] To a round bottom flask, it was added licarin A (100.0 mg, 0.31 mmol), dimethylformamide (1.0 mL) and potassium carbonate (339.0 mg, 2.45 mmol). This mixture was kept under magnetic stirring under reflux at 70 °C. After 30 min, the respective benzyl halide (1.23 mmol) was added and the reaction was followed by thin layer chromatography (TLC) using a solvent mixture of hexane/ethyl acetate (8.0:2.0 v/v). After 3 h, the solvent was eliminated by an air stream, the crude residue dissolved with ethyl acetate and the organic solution was washed with a 0.5 mol L^{-1} NaOH solution (3 × 40.0 mL). The organic phase was dried with anhydrous sodium sulfate and the filtrate was concentrated by rotary evaporator. The crude product was purified by column chromatography using a hexanes/ethyl acetate solvent mixture (8.0:2.0 v/v).

The synthesis method used for derivative DL21 was adapted from a method published by Iliefski *et al.*[21] and was as follows: distilled water (0.5 mL) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (50.0 mg, 0.22 mmol) were added in a round bottom flask. The suspension was left under magnetic stirring at room temperature for 5 min and then, it was added to the 4-chlorobenzyl licarin A (50.0 mg, 0.11 mmol) as a solution in dichloromethane (2.0 mL). The reaction was followed by TLC using a solvent mixture of hexanes/ethyl acetate (7.0:3.0; v/v) and was found to be complete after 30 min. Ascorbic acid (40.0 mg, 0.22 mmol) was then added and the mixture was kept under stirring for 10 min. The solids were removed by filtration, the filtrate was washed with saturated sodium bicarbonate solution and the organic phase was dried with anhydrous sodium sulfate. After evaporating the solvent in a rotary evaporator, the crude product was purified by silica gel column chromatographic column using a solvent mixture of hexanes/ethyl acetate (7.0:3.0 v/v).

Proliferation curve determination

Promastigotes (10^6 cells mL^{-1}) were added to 25 cm^2 flasks containing liver infusion-tryptose (LIT) medium and maintained at 26 °C. On a daily basis, an aliquot was removed and the number of promastigotes was determined using a Neubauer's chamber.[22]

Antileishmanial activity against promastigotes

The experiments were carried out as described by Espuri *et al.*[23] Briefly, *L. amazonensis* promastigotes (strain MHOM/BR/71973/M2269) were grown in 24-well plates in LIT medium, supplemented with 10.0% (v/v) heat-inactivated fetal bovine serum and 1.0% penicillin (10.000 UI mL^{-1})/streptomycin (10.0 mg mL^{-1}) (Sigma, USA). Cells were harvested in their log stage, suspended in fresh medium, counted in Neubauer's chamber and the concentration adjusted to 1 × 10^6 cells mL^{-1}, in 24-wells plates. The compounds tested were added to promastigote cultures (1 × 10^6 cells mL^{-1}) in the range of 0.10 to 40.00 μg mL^{-1} and solubilized in dimethyl sulfoxide (DMSO) (0.6%, v/v in every well), following incubation at 25 °C. After periods of 24, 48 and 72 h of incubation, resazurin was used to detect cellular metabolic activity in order to determine the EC_{50} value for promastigotes (EC_{50pro}). Amphotericin B (Sigma, USA) was used as reference drug.

Cytotoxicity evaluation

Murine peritoneal macrophages were obtained from Swiss mice by peritoneal lavage properly approved by the Comitê de Ética da Universidade Federal de Alfenas (CEP/UNIFAL), project No. 30/2019, and in accordance to the guide for care and use of laboratory animals.[24] The experiments were carried out as described by Espuri *et al*.[23] Briefly, a suspension of 8×10^5 murine peritoneal macrophages, in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10.0% heat-inactivated fetal bovine serum and 1.0% penicillin (10.000 UI mL^{-1})/streptomycin (10 mg mL^{-1}) was added to each well in 96-well plates. These were incubated in a 5.0% CO_2 air mixture at 37 °C to allow cell adhesion. 24 h later, non-adherent cells were removed by washing with RPMI 1640 medium. Then, different concentrations of compounds and reference drugs, ranging from 3.91 to 500.00 μg mL^{-1} in DMSO, at the final concentration of 0.6% (v/v), were added to the wells containing cells, upon which these plates were incubated for another 48 h. Afterwards, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) was solubilized in phosphate-buffered saline (PBS) (5.0 μg mL^{-1}), and 10 μL were transferred to RPMI 1640 medium with a final volume of 200.0 μL *per* well, which was submitted to incubation for 4 h.[25] Later, the medium was removed and 100.0 μL of DMSO were added to each well and homogenized for 15 min. Next, the absorbance of each individual well was measured at 570 nm according to the following formula (OD represents optical density) using equation 1:

$$\text{Inhibition } (\%) = OD_{control} - OD_{compounds}/OD_{control} \times 100 \quad (1)$$

The percentage of viable cells was calculated taking into account the cell culture control (medium + cells + DMSO 0.6% v/v). The 50.0% cytotoxicity concentrations (CC_{50}) were determined and the selectivity index (SI) was established by the ratio between the values of CC_{50} and EC_{50} for amastigote forms.

Antileishmanial activity against amastigotes

The experiments were carried out as described in Espuri *et al*.[23] Briefly, murine peritoneal macrophages were maintained in RPMI 1640 medium (Sigma, USA) supplemented with 10.0% heat-inactivated fetal bovine serum in a 5% CO_2 incubator at 37 °C. These macrophage cells were obtained from Swiss mice by peritoneal lavage as described in the cytotoxicity evaluation. The cells were cultured in 24-well plates on glass slides ((13 mm, Nunc, USA, (8×10^5 cells $well^{-1}$)) and infected with late phase promastigotes at a ratio of 10:1 (parasite:macrophage) while incubated in a 5% CO_2-air mixture at 37 °C for 24 h. Non-phagocytosed promastigotes were removed by washing and compounds were solubilized in DMSO (from 0.10 to 40.00 μg mL^{-1}), used at a concentration of 0.6% v/v. After 48 h, chamber slides were fixed in absolute methanol, stained with Giemsa 10% and examined under an optical light microscope with oil immersion. The percentage of infected cells *per* well was calculated taking into account at least 200 macrophages. The effective ratio (EC_{50} for amastigotes (EC_{50ama})) was stablished in comparison to control, only with DMSO. Amphotericin B was used as reference drug.

Evaluation of lipophilicity by logP (oct/water)

Lipophilicity values were estimated through theoretical determination of logP (oct/wat) using the ChemDraw software ultra-version 11.0.[26] Calculated lipophilicity was expressed by the logP (oct/wat) of compounds as described by Gontijo *et al*.[24]

Mitochondrial membrane potential (ΔΨ)

The ΔΨ was assessed by fluorimetry using the JC-10 probe, as described by Inacio *et al*.[27] In short, promastigotes (10^6 cells mL^{-1}) were treated or not (control) with selected compounds at equal concentrations to the EC_{50pro} for 48 h. Afterwards, the concentration of parasites was adjusted to 10^7 cells mL^{-1} and incubated with the JC-10 probe (10 mg mL^{-1}) in phosphate buffer (pH 7.4) for 30 min at 25 °C. Then, promastigotes were washed three times by centrifugation at 1965 × g for 10 min at 4 °C. The supernatant was disposed and the cells were resuspended in phosphate buffer (pH 7.4) and analyzed in a spectrofluorometer (Agilent, CA, USA) (Varian Cary Eclipse Fluorescence Spectrophotometer) at 530 and 590 nm excitation, and 480 nm emission wavelengths. The ratio between the values obtained at 530 and 590 nm was used to determine the ΔΨ.

Statistical analysis

Statistical analysis was performed using non-linear regression to obtain the EC_{50pro}, EC_{50ama} and CC_{50} values, followed by Tukey's tests and variance analyses. Differences in the $EC_{50(pro\ and\ ama)}$ and CC_{50} values between the standard drug and compounds were significant when the *p*-value was less than 0.05. At least three independent experiments were carried out in triplicate.

Results and Discussion

Screenings of compounds with leishmanicidal potential were initiated by *in vitro* studies in both evolutionary forms of the parasite, as well as cytotoxicity studies.[12,23] Thus, it was evaluated initially the anti-promastigote activity of DL01 and derived compounds at different incubation times, cytotoxicity in murine peritoneal macrophages within 48 h of incubation and SI (Table 1).

The results here attained show that among the derivative compounds studied, DL21 presented the best leishmanicidal activity for all incubation times ($EC_{50pro/24h}$: 5.11 ± 0.94 μM; $EC_{50pro/48h}$: 4.68 ± 0.28 μM and $EC_{50pro/72h}$: 5.26 ± 0.45 μM), including in relation to the original prototype (DL01) ($EC_{50pro/24h}$: 91.24 ± 1.01 μM; $EC_{50pro/48h}$: 86.85 ± 1.99 μM and $EC_{50pro/72h}$: 95.49 ± 2.23 μM) (Table 1). Furthermore, in relation to compound DL21, it is noticeable that different incubation times did not influence its activity, and regarding the reference drug, amphotericin B, the values obtained with the compound DL21 were the closest. Néris *et al.*[11] also observed that amphotericin B was more effective than licarin A against *L. major* promastigotes. The assessment of the activity of compounds with antiparasitic effect in different incubation periods can help determine their effectiveness and time required for maximum activity. This is explained by the fact that depending on the metabolic pathway involved in the mechanism of action of a compound, the effects may present themselves through fast or slow responses. However, the main discovery regarding the treatment of leishmania at different incubation times is that there are no significant variations between the results obtained for every compound, despite being statistically different (DL01, DL03 and DL17) and this may be because after 24 h the maximum effect of each compound is already achieved. Since it was decided to incubate the promastigotes with the tested compounds in the log phase of the proliferation curve, the incubation period of 48 h was selected as it guarantees the interval between the second and fifth day of the curve, right before the beginning of the stationary phase (fifth day). Therefore, for comparison purposes, the cytotoxicity and anti-amastigote activity tests were also performed with 48 h of incubation.

The determination of the activity and safety of new drug candidate compounds is of great relevance and must be carried out in the early stages of research.[28] The importance of toxicity testing is justified because compounds that are to be tested should not be toxic to mammalian cells, given amastigotes live within them. In this context, the compounds need to cross cell membranes and reach their target (amastigote forms) without damaging the cell in which it is present.[10] According to the results found, it was possible to observe that from all tested compounds, only DL03 (CC_{50}: 149.38 ± 17.31 μM), DL10 (CC_{50}: 244.10 ± 10.78 μM) and DL17 (CC_{50}: 253.90 ± 26.04 μM) were statistically less toxic than DL01 (CC_{50}: 117.96 ± 9.19 μM) (Table 1). Among these, compounds DL10 and DL17 stood out with toxicity about nine times less than that of the reference drug, amphotericin B (CC_{50}: 27.10 ± 3.19 μM). Regarding DL21, its cytotoxicity (CC_{50}: 39.79 ± 5.21 μM) was the highest among the tested compounds, yet still lower than that of amphotericin B (Table 1).

Data found in the literature regarding the antiparasitic activities and cytotoxicity of licarin A against trypanosomatids differ from one another and between the results described in the current study.[9,10,11,29] Licarin A is an organic compound that has two chiral centers in its structure and different enantiomeric forms. However, stereoselectivity may affect the biological activity of compounds, which causes chiral compounds to have distinct biochemical, pharmacological and physiological properties, including cytotoxicity.[30,31] Additionally, the trypanosomatid species assessed in the different studies are

Table 1. Evaluation of the anti-promastigote activity of DL01 and derived compounds at different incubation times, cytotoxicity in murine peritoneal macrophages with 48 h of incubation and selectivity index (SI) in relation to *L. amazonensis* promastigotes

Compound	EC_{50pro} ± SD / μM			CC_{50} ± SD (48 h) / μM	SI (CC_{50}/EC_{50pro}) (48 h)
	24 h	48 h	72 h		
DL01	91.24 ± 1.01^{A}	86.85 ± 1.99^{A,a,b}	95.49 ± 2.23^{A}	117.96 ± 9.19	1.35
DL03	107.48 ± 5.50^{A}	87.62 ± 2.46^{A,a,c}	82.25 ± 4.40^{A,d,e}	149.38 ± 17.31	1.70
DL10	74.86 ± 5.70^{A}	84.22 ± 2.55^{b,c}	86.66 ± 2.02^{d,e}	244.10 ± 10.78^{B}	2.89
DL17	66.29 ± 1.99^{A}	76.89 ± 6.1^{A}	84.77 ± 2.37^{A}	253.90 ± 26.04^{B}	3.30
DL21	5.11 ± 0.94	4.68 ± 0.28	5.26 ± 0.45	39.79 ± 5.21	8.50
Amphotericin B	1.85 ± 0.05	1.58 ± 0.01	1.40 ± 0.04	27.10 ± 3.19	17.15

$^{A}EC_{50pro}$: analysis between different incubation times with the same compound (statistically different ($p < 0.01$)); $^{B}CC_{50}$: there is no significant difference ($p < 0.05$); $^{a,b,c,d,e,f,g}EC_{50pro}$: analysis between the tested compounds in the same incubation time (there is no significant difference ($p < 0.01$)). EC_{50pro}: 50% effective concentration to promastigotes; CC_{50}: 50.0% cytotoxicity concentrations; SI: selectivity index; DL01: licarin A; SD: standard deviation.

different, as well as the culture medium, treatment periods and analyses methods. Previous studies[7,11,24] have reported leishmanicidal activity of (–) licarin A against *L. major* promastigote forms (EC_{50}: 10.59 μg mL^{-1}, trypanocidal action against *T. cruzi* trypomastigotes (EC_{50}: 7.49 μg mL^{-1}) and trypanocidal activity of unidentified enantiomeric form against *T. cruzi* epimastigote and trypomastigotes (EC_{50}: 150.8 and 312.96 μg mL^{-1}, respectively). Néris *et al.*[11] analyzed the cytotoxicity of (–) licarin A after 24 h treatment by two different methods, MTT and trypan blue exclusion assays, obtaining different CC_{50} values of 729.80 and 308.96 μg mL^{-1}, respectively. Léon-Diaz *et al.*[29] evaluated licarin A cytotoxicity isolated from *Aristolochia taliscana* roots for a period of 48 h using trypan blue exclusion method, with CC_{50} = 312 μg mL^{-1}.

A study[9] reveals that licarin A derivatives or analogues exhibit less toxicity in mammalian cells, presenting CC_{50} higher than 65.2 μg mL^{-1}, which was not observed in our study with the compound DL21. In our experiments, it was possible to determine, from the EC_{50pro} and CC_{50} values, the SI of compounds DL01 (1.35), DL03 (1.70), DL10 (2.89), DL17 (3.30) and DL21 (8.50) (Table 1), all of which are lower than that obtained for amphotericin B (17.15).

Among the screening stages of candidate compounds for new drugs with leishmanicidal action, the activity assays against amastigote forms are considered one of the most important, given they are mainly present in macrophages of the hosts. Different life stages of leishmania show very different metabolic pathways and enzyme expression, which happen due to environmental adaptations, such as osmolarity, pH, nutritional availability, temperature, presence of oxidative species and the immune response of the vertebrate host.[32] Moreover, the activity of DL01 and its derivatives were evaluated against *L. amazonensis* intracellular amastigote forms. The results were expressed through the percentage of infected macrophages (IM, in percentage) (Figure 2) and number of amastigotes *per* infected macrophage (A/IM) (Figure 3).

In Figures 2 and 3 it is possible to see that incubation with DL01 significantly reduced the I/M percentage at the concentration of 30.6 μM, 76% IM; while incubation with DL03 only generated significant decrease at the concentration of 118.4 μM, 24.7% IM. DL10 and DL17 did not show significant reduction in I/M even at the maximum concentration employed, 89.6 and 90.6 μM, 100 and 98% IM, respectively. Conversely, with compound DL21, it is possible to observe a clear decline in IM in all concentrations used, while for those greater than 2.15 μM, it exceeded 50%. As for the number of A/IM, DL21 caused a significant decrease in parasitic concentration in all concentrations, whereas DL01 only did so from a higher

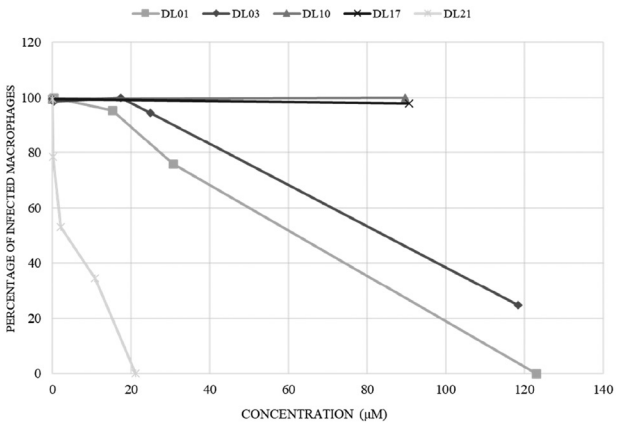

Figure 2. Percentage of infected macrophages (IM) after infection with *L. amazonensis* promastigotes and treatment with DL01 and derivative compounds. The first point of each line corresponds to the untreated control group (IM = 99.6%).

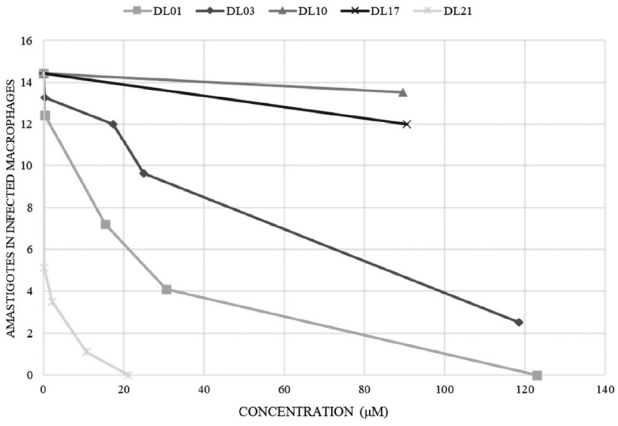

Figure 3. Number of amastigotes in infected macrophages (A/IM) after infection with *L. amazonensis promastigotes* and treatment with DL01 and derivative compounds. Concentration expressed in μM. The first point of each line corresponds to the untreated control group (A/IM = 14.42).

concentration, 15.3 μM, and DL03 only from 24.9 μM (9.65 A/IM). DL10 was considered statistically equal to control and DL17 led to a reduction of only 16.7% in the number of A/IM (12.01) when compared to control. Finally, the 50 and 90% effective concentrations (EC_{50} and EC_{90ama}) of *Leishmania amazonensis* amastigotes treated with DL01 and its derivative compounds were assessed (Table 2).

Regarding the EC_{50} and EC_{90ama} (Table 2), it was possible to verify that the compound DL21 showed the best results, 0.42 ± 0.07 and 15.91 ± 0.14 μM, respectively, with EC_{50ama} lower than that obtained with amphotericin B (1.17 ± 0.08). The other compounds showed EC_{50ama} values higher than that of amphotericin B, and specifically, DL10 and DL17, above 40 μM. The same pattern of values was observed with EC_{90ama}, with the exception of DL21, which presented a result (15.91 ± 0.14 μM) superior to that of amphotericin B (1.90 ± 0.07 μM). In *L. donovani* amastigotes and promastigotes treated with an isolated neolignan from *Virola pavonis*, and

Table 2. 50 and 90% effective concentration of *Leishmania amazonensis* amastigotes treated with DL01 and derivative compounds, selectivity index and lipophilicity by LogP

Compound	EC_{50ama} 48 h + SD / μM	EC_{90ama} 48 h + SD / μM	SI (CC_{50} / EC_{50ama}) 48 h + SD / μM	LogP[a]
DL01	5.25 ± 0.35	36.30 ± 1.2	22.46	4.36
DL03	18.05 ± 1.05	107.51 ± 6.5	8.27	4.94
DL10	> 40	–	< 6.10	6.23
DL17	> 40	–	< 6.34	8.48
DL21	0.42 ± 0.07	15.91 ± 0.14	94.73	5.54
Amphotericin B	1.17 ± 0.08	1.90 ± 0.07	23.16	0.80

[a]Express values of LogP (octanol/water) using ChemDraw Ultra version 11.0 program.[26] DL01: licarin A; SD: standard deviation; EC_{50ama}: 50% effective concentration to amastigote; EC_{90ama}: 90% effective concentration to amastigote; CC_{50}: 50.0% cytotoxicity concentrations; SI: selectivity index. All values are statistically different ($p < 0.05$).

its synthetic analogues, the natural neolignan proved to be active against promastigotes at 100 μM, but inactive against amastigote forms.[14] However, the synthetic analogue, β-ketosulfide (3,4-dimethoxy)-8-(4'-methylthiophenoxy)-propiophenone instead led to a 42% inhibition of *L. donovani* amastigotes in BALB/c mice livers.[14] Similarly, Vendrametto *et al*.[15] treated *L. amazonesis* intracellular and axenic amastigotes with eupomatenoid-5, a neolignan, in which it was possible to observe antileishmanial effect with EC_{50} values of 5.0 and 13.0 μg mL^{-1}, respectively. Besides, the activity against *T. cruzi* intracellular amastigotes treated with eupomatenoid-5 has been reported (EC_{50}: 5.0 μg mL^{-1}).[33] Amaral *et al*.[34] related that a dehydrodieugenol B derivative (1-propyl-3-(1'-propyl-3'-methoxyphenoxy)-5-methoxy-4-benzoyloxybenzene), a neolignan, demonstrated activity against *L. infantum* intracellular amastigotes, eliminating 100% of amastigotes without affecting macrophage viability and with a SI approximately 3 times higher than the original neolignans. The SI presented by compound DL21 (94.73) (Table 2) indicates a selectivity 4.21 times greater than DL01 and 4.01 times higher than amphotericin B (23.16); suggesting DL21 as a promising candidate for a leishmanicidal drug. According to Don and Ioset,[35] in Kinetoplastids, compounds with SI values greater than 20.0 are considered excellent candidates for new drugs, that is, a high SI value shows that such molecules present themselves with less toxicity and greater activity. DL01 exhibited a SI (22.46) close to that of amphotericin B, while the other compounds had much lower values, therefore, showing low leishmanicidal potential, getting discarded for experiments of mechanism of action.

The study of lipophilicity has been widely used to correlate physical, chemical and biological properties of compounds with respect to their toxicity and solubility. In the case of drugs, it is essential to have a balance between logP values so that there is an equilibrium between permeability and solubility. Thus, very hydrophilic drugs have little permeability through cell membranes, while the ones of lipophilic nature have low dissolution in the biological environment.[36] Bioactive compounds are capable of producing a biological response that is directly associated to mechanisms of absorption, distribution and intrinsic activity. Lipophilicity predicts these properties in order to investigate the behavior of new drug candidates, increasing the chances of success in introducing new drugs in the therapeutic arsenal.[37] According to the logP data obtained (Table 2), compounds DL10 and DL17 had very high values, 6.23 and 8.48, respectively; conferring them high lipophilicity, which may explain the low leishmanicidal efficiency observed ($EC_{50ama} > 40$ μM). With greater lipophilicity, transposition through the plasma membrane increases, as well as its retention, which reduces the contact of the compound with intracellular amastigotes. Furthermore, compounds with high logP value are more prone to toxicity, since they are more difficult to be excreted and consequently less suitable to use orally.[38] On the other hand, compounds DL01 and DL03 had logP values lower than 5, 4.36 and 4.94, respectively, which does not violate Lipinski's rule, therefore being good candidates for oral use.[39] However, their low SI confers them less selectivity to the parasite. In regard to DL21, its logP value was close to 6 (5.54), which may have conferred its high efficiency against intracellular amastigotes, and high SI, indicating that the insertion of an aldehyde in R1 and a halide group in R2 resulted in better leishmanicidal activity against both forms of leishmania. The aldehyde group is also capable of carrying out hydrogen interactions, which play fundamental roles in many physiological processes, and thus contributes to the stability of the drug-receptor complex. According to the results attained in this work, it is possible to notice that DL01 and DL21 showed better

results against amastigotes, which justifies the need to understand this compound's mechanism of action.

The mitochondria, besides being principal the main site for generation of reactive oxygen species (ROS) and adenosine triphosphate (ATP), is also extensively involved in different events in cells, which means their functionality is crucial for the maintenance of cell viability.[40] In leishmania, this organelle is one of the primary targets that are studied, since it is involved in the mechanism of action of many drugs, like amphotericin B, miltefosine, antimonials and various leishmanicidal drug candidates.[41-45] Mitochondrial functionality is directly related to the maintenance of $\Delta\Psi$, which is a parameter that is deeply involved in oxidative phosphorylation, ATP synthesis, control of ROS production and intracellular Ca^{2+} homeostasis.[46] Thus, mitochondrial functionality was assessed through the analysis of $\Delta\Psi$ in *L. amazonensis* promastigotes, treated or not (control) with EC_{50} values of compounds DL01 and DL21. Treatment with DL01 promoted a 13.35% reduction in $\Delta\Psi$, while with DL21 there was no significant change, both in relation to the control (Figure 4). Henceforth, in order to establish a mechanism of action for DL21, further experiments will be done, such as the evaluation of ATP and ROS production.

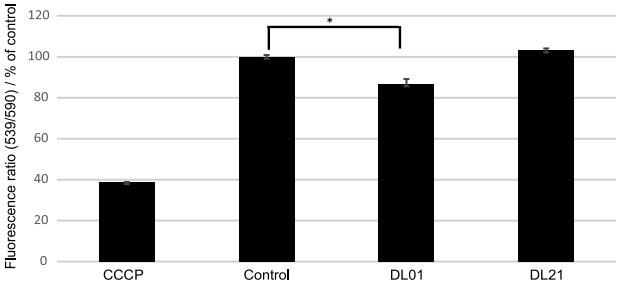

Figure 4. Evaluation of the mitochondrial membrane potential ($\Delta\Psi$). *L. amazonensis* promastigotes (1×10^7 cells mL^{-1}) in the log phase of the proliferation curve were treated or not (control) with EC_{50} values of compounds DL01 (licarin) and DL21. Carbonyl cyanide chlorophenylhydrazone (CCCP) was used as uncoupler. The values are expressed as the ratio between the fluorescence measurements at 530 nm over 590 nm. Three independent experiments were performed in duplicate. *Significantly different ($p < 0.01$).

The alteration of $\Delta\Psi$ observed with DL01 corroborates with the results of action of licarin A in lung tumor, which may be associated with apoptosis.[47] Morais *et al.*[9] evaluated licarin A derivatives with activity against *T. cruzi* trypomastigotes, where an increase in ROS production and ATP consumption was noticed, as well as mitochondrial hyperpolarization. Small changes in $\Delta\Psi$ could straight up interfere with the production of ROS, such as hydrogen peroxide affecting the cell redox state, and/or interfering with proliferation rates.[48-50] According to Dagnino *et al.*,[51] in leishmania promastigotes, both hyperpolarization and depolarization can result in cell death by apoptosis.

Conclusions

The complexity of developing effective compounds in the treatment of leishmaniasis is directly related to the lack of specificity of the drugs used so far, as well as the persistence of side effects. Therefore, with significant EC_{50pro} and EC_{50ama} values, cytotoxicity and mainly SI for amastigotes, DL21 stands out among the compounds studied here. As a result, it is justifiable that further studies are needed to elucidate its mechanism of action, in addition to *in vivo* experiments, in order to further validate this compound as a promising drug candidate in the treatment of leishmaniasis.

Supplementary Information

Supplementary data (^{1}H NMR, ^{13}C NMR, DEPT-135 NMR subspectra, mass and IR spectra) is available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Marcilene A. Alves was responsible for data curation, investigation; Patrícia F. Espuri for data curation, investigation; Dalila J. Alvarenga for resources (compounds); Thalles H. F. de Souza for writing-review and editing; Matheus F. Alves for data curation, investigation; Diogo T. Carvalho for resources (compounds); Marcos J. Marques for data curation; Eduardo de F. Peloso for conceptualization, formal analysis funding acquisition, project administration, validation, writing original draft.

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