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Original article

New phenalenone analogues with improved activity against *Leishmania* species

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ABSTRACT

The *in vitro* activity against *Leishmania* spp. of five novel designed compounds, phenalenone derivatives, is described in this study. Previous works have shown that some phenalenones present leishmanicidal activity, some of which could induce programmed cell death events in *L. amazonensis* parasites. In this research, we focused on the determination of the programmed cell death evidence by detecting the characteristic features of the apoptosis-like process, such as phosphatidylserine exposure and mitochondrial membrane potential, among others. The results showed that the new derivatives have comparable or better activity and selectivity than the commonly prescribed anti-leishmanial drug. This result was obtained by inducing stronger mitochondrial depolarization or more intense phosphatidylserine exposure than miltefosine, highlighting compound **8** with moreover 9-times better selectivity index. In addition, the new five molecules activated the apoptosis-like process in the parasite. All the signals observed were indicative of the death process that the parasites were undergoing.

1. INTRODUCTION

Leishmaniasis are vector-borne parasitic diseases caused by at least 20 species of the genus *Leishmania*, and are transmitted between mammalian hosts by female sandflies. The World Health Organization (WHO) considers Leishmaniasis a neglected tropical disease that affects mainly low-income people all around the world. From a quantitative point of view, its impact is estimated to be between 700,000 and 1 million infections worldwide, causing between 20,000 and 30,000 deaths per year (Global Health Observatory data from WHO, 2018).[1]

The distinct species of *Leishmania* cause different clinical manifestations, ranging in severity from self-curing cutaneous lesions to life-threatening visceral disease. The outcome is determined by the interplay of the following elements: parasite characteristics, vector biology, and host factors (with immune responses taking focus stage among the host factors).[2]

The combined problems of parenteral administration and the toxicity of anti-leishmanials precludes effective treatment programs for leishmaniasis. Furthermore, the selection of resistant parasites carrying genetic mutations that lessen the parasite's response to drugs, may emerge upon mass drug administration.[3]

Antimonials are the primary drugs employed against leishmaniasis,

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that have been in use since the 1920s. Nevertheless these toxic compounds have a narrow therapeutic window and their use has been largely superseded in some countries, where resistance has become widespread. However, they are still in use in other regions of the world.[3] Fifteen years ago miltefosine was adopted as the first-line regimen by the Asian elimination initiative. Notwithstanding, after a decade of use there is evidence of reduced effectiveness in both visceral leishmaniasis and post-kala-azar dermal leishmaniasis, while the existing dosing recommendations in children are likely to result in under-dosing and treatment failure. Indeed the research priorities for leishmaniasis treatment include the search for oral, safe, short course (<10 days) drugs for both visceral leishmaniasis and cutaneous leishmaniasis.[2]

Cutaneous leishmaniasis distribution in the Americas is complex, with diverse *Leishmania* species in the same geographical area, several reservoirs, hosts, and sand fly vectors, as well as variable clinical manifestations and responses to therapy.[2] Regarding American cutaneous leishmaniasis (ACL), *Leishmania braziliensis* and *Leishmania amazonensis* are the predominant pathogenic species. Besides, *L. amazonensis* can cause a broad spectra of the disease, including cutaneous, mucocutaneous, diffuse cutaneous and even visceral and post-kala-azar dermal leishmaniasis,[4] and is responsible for anergic diffuse cutaneous leishmaniasis (DCL), accounting for nearly 1% of all ACL cases each year in Brazil.[5]

Between 1994 and 2014 more than 400 families of molecules patents with leishmanicidal activity were found, but just 294 are still alive patents nowadays. Most of them have not been produced by the pharmaceutical industry, but rather by public research institutes or by universities, and the majority of the inventions disclosed are still in preclinical phase.[6]

Recent studies have focused the attention on naphtoquinones, as the lapachol derivatives, that are natural and synthetic compounds considered promising scaffolds for the development of new drugs, since they present many pharmacological activities including antileishmanial activities.[7] One example is the epoxymethoxy-lawsone, that has shown very promising results used as treatment of leishmaniasis on *in vivo* experiments, alone or in combination with reference drugs.[8]

During the last few years many other type of molecules have been patented, as is the case of bicycle carbohydrates, bisguanides, indazoles, selenophenes and thia-analogs, that have demonstrated promising results as good compounds for the future treatment of leishmaniasis.[9]

The term "apoptosis", coined by Kerr et al.[10], indicates by definition a type of cell death that is characterized by several morphological features among which cell rounding, retraction of pseudopods, cell shrinkage, chromatin condensation, nuclear fragmentation, few ultrastructural modifications of cytoplasmic organelles, plasma membrane modifications with maintenance of its integrity, membrane blebbing that culminates in the formation of apoptotic bodies and, in vivo, phagocytosis by nearby cells.[11] PCD in unicellular organisms has been reported so far in parasites like *Leishmania*, but in these organisms, this process occurs as a phenomenon that benefits the rest of the population. In any case, the evidence that apoptotic processes exist in protozoan parasites also has given new strategies for the researchers in the study of these diseases, comparing these processes to the one in human cells.[12]

Plants and fungi contain compounds like 1H-Phenalen-1-one, which play a crucial role in the defense of the organism. Natural and synthetic substituted 1H-phenalen-1-ones show many biological activities as antifungal, anti-malarial and cytotoxic activity against human cancer cells.[13] Previous studies from our group had evidenced that a set of 9-phenyl-1H-phenalen-1-one based anti-fungal phytoalexins, possessed modest leishmanicidal activity, pointing the disruption of the leishmanial respiratory chain system of the mitochondria, as the possible mechanism of action for these compounds.[14] In addition, in a posterior study we evidenced the property of inducing apoptotic-like cell death in *L. amazonensis* parasites of some active amino-phenalenones.[15] This conclusion highlights the importance of continuing to study mono-substituted phenalenone derivatives against *Leishmania* spp. parasites, emphasizing on *L. amazonensis* and to compare the obtained results with previous works. Further screening of synthetic 1H-phenalen-1-ones analogues lead to the discovery of significantly more potent derivatives.

2. Material and methods

2.1. General Methods

All reagents and solvents were obtained from Aldrich Chemical Co. and used without further purification. Reactions with sensitive reagents were performed under inert atmosphere (argon or nitrogen) and organic solvents were dried by standard methods. Unless otherwise stated, solvents were removed under reduced pressure using a rotary evaporator at 40-60 °C. All reactions were monitored by analytical thin layer chromatography (TLC) on POLIGRAM® SIL G/UV_{254} silica gel coated plates (0.20 mm) from MACHEREY-NAGEL. Column chromatography was performed on silica gel 60 (0.063-0.20 mm) from MERCK. Compounds were visualized by ultraviolet light (254 nm). The purity of the final compounds was determined to be ≥ 95% by high pressure liquid chromatography (HPLC) using a Jasco PU-2080 intelligent HPLC pump (Jasco MD 2020 Plus multiwavelength detector). ^{1}H and ^{13}C NMR spectra were recorded at room temperature (rt) on a Bruker Avance 400 or 500 MHz NMR spectrometer in the solvent indicated. Data for ^{1}H NMR are reported as follows: chemical shift (δ ppm), integration, multiplicity and coupling constant (Hz), whereas ^{13}C NMR analyses are reported in terms of chemical shift. IR and UV spectra were recorded on a BRUKER IFS 66 and a JASCO modelo V-560 spectrophotometer, respectively. Melting points were determined using a Stuart Scientific SMP11 instrument. Low resolution (EIMS) and high-resolution mass spectrometry (HRMS) were performed on a Micromass AutoSpec magnetic *tri-sector* (*EBE* geometry) mass spectromter. Microwave reactions were performed using a Biotage® Initiator (software version 2.5). Lyophilization was performed using a CHRIST ALPHA 2-4 lyophilizer.

2.2. Synthesis

2.2.1. Synthesis of 3-phenyl-9-hydroxy-1H-phenalen-1-one (3)

A solution of 2-methoxynaphthalene (**1**) (0.063 mol, 10 g) and (2*E*)-3-phenylacryloyl chloride (0.076 mol, 12.6 g) in anhydrous DCE (105 mL) was cooled in a water, ice and salt bath at −10 °C and $AlCl_3$ (0.069 mol, 9.2 g) was added. After one hour, the mixture was cooled again to -10 °C and more $AlCl_3$ (0.069 mol, 9.2 g) was added. The reaction mixture was kept under stirring at room temperature for 16 hours. At the end of that time frame, the reaction mixture was heated under reflux for 1 h with vigorous stirring. At the end of the reaction, the crude was cooled in an ice bath and a mixture of ice (120 g) and 1 N HCl (90 mL) was added. The precipitate was filtered and the aqueous phase was extracted with CH_2Cl_2 (3 x 25 mL). The combined organic phases were washed with water (3 x 20 mL), dried ($MgSO_4$), filtered, and the solvent was removed on the rotary evaporator, to obtain 9-hydroxy-1H-phenalen-1-one (**2**) (11 g, 89%) as an orange solid and 3-phenyl-9-hydroxy-1H-phenalen-1-one (**3**) (370 mg, 3%) as a yellow solid. Product **3**: ^{1}H RMN (400 MHz, $CDCl_3$) δ: 16.30 (1H, s, OH), 8.11 (1H, d, J =9.3 Hz, H-7), 8.02 (2H, d, J =7.6 Hz), 7.56-7.52 (6H, m, Ar), 7.19 (1H, d, J =9.3 Hz, H-8), 7.15 (1H, s, H-2). ^{13}C RMN (100 MHz, $CDCl_3$) δ: 179.5 (s, C-1), 178.1 (s), 153.9 (s, C-9), 141.4 (d), 138.4 (s), 133.2 (d), 132.4 (d), 129.8 (d, 2 x C), 128.8 (d), 128.6 (d, 2 x C), 127.5 (s), 125.9 (s), 125.2 (s), 123.9 (d), 123.8 (d), 123.7 (d), 110.9 (s). EIMS: m/z 272 (M^+, 100), 244 (24). HRMS: calcd. for $C_{19}H_{12}O_2$ (M^+) 272.0837, found 272.0829.

2.2.2. 9-(Propylamino)-1H-phenalen-1-one (4)

To a solution of **2** (0.153 mmol, 29.7 mg) in DMF (0.50 mL) was added 2-methyl-1-propanamine (5.59 mmol, 1.05 mL) and the mixture was heated at 180 °C for 90 min under argon using MW irradiation. After this time, 2-methyl-1-propanamine was removed under vacuum and the

crude was lyophilized to remove the DMF. Purification using column chromatography on alumina (toluene/CH_2Cl_2; 1:0 a 3:2) gave compound **4** (12.3 mg, 32%) as an orange solid. ^{1}H RMN (400 MHz, $CDCl_3$) δ: 12.4 (1H, brs, NH), 7.96 (1H, d, *J* =9.3 Hz, H-3), 7.87-7.82 (3H, m), 7.41 (1H, t, *J* =7.6 Hz, H-5), 7.20 (1H, d, *J* =9.4 Hz, H-8), 7.00 (1H, d, *J* =9.4 Hz, H-2), 3.38 (2H, t, *J* =6.2 Hz, H-1'), 2.14-2.08 (1H, m, H-2'), 1.11 (6H, d, *J* =6.6 Hz, H-3'). ^{13}C RMN (100 MHz, $CDCl_3$) δ: 184.2 (s, C-1), 156.5 (s, C-9), 138.5 (d), 138.1 (d), 131.8 (d), 131.4 (d), 129.1 (d), 128.5 (s), 125.2 (s), 124.3 (s), 121.7 (d), 114.6 (d), 108.2 (s), 50.6 (d, C-1'), 28.7 (t, C-2'), 20,6 (c, C-3'). EIMS: m/z 277 (47), 252 (M^++1, 100). HRMS: calcd. for $C_{17}H_{18}NO$ (M^++1) 252.1388, found 252.1384. UV-Vis (EtOH) λ_{max}: 474, 446, 421, 356, 342, 278, 252,205, 202 nm. IR ($CHCl_3$) ν_{max}: 3537, 1632, 1588, 1524, 1239, 844 cm-1. mp: 54-56 °C.

2.2.3. Synthesis of 2-phenyl-1H-phenalen-1-one derivatives

To a solution of 2-iodo-1H-phenalen-1-one (**5**) in a mixture of DME/H_2O 1:1, Na_2CO_3, boronic acid and 10% Pd/C were added. The reaction mixture was heated at 60 °C for 10 min. At the end of this period of time, EtOAc (10 mL) was added and filtered over Celite®. Then H_2O (20 mL) was added, extracted with EtOAc (3 x 8 mL) and dried ($MgSO_4$). After filtering and concentrating under vacuum, the crude was purified by column chromatography or on a silica gel preparative plate.

2.2.3.1. Synthesis of 2- (4-methoxyphenyl) -1H-phenalen-1-one (6). From 2-iodo-1*H*-phenalen-1-one (**5**) (0.228 mmol, 70.0 mg), DME/H_2O (1.4 mL), Na_2CO_3 (0.524 mmol, 56.0 mg), (4-methoxyphenyl) boronic acid (0.524 mmol, 80.0 mg) and 10% Pd/C (15.6 mg); heated for 10 min; column chromatography (hexane/EtOAc, 9.5: 0.5, 9: 1); product **6** (64.0 mg, 98%) was obtained as an orange solid. ^{1}H RMN (500 MHz, $CDCl_3$) δ: 8.67 (1H, dd, *J* = 1.1, 7.3 Hz, H-9), 8.16 (1H, dd, *J* = 0.9, 8.0 Hz, H-7), 7.96 (1H, d, *J* =7.9 Hz), 7.81 (1H, s, H-3), 7.76 (1H, dd, *J* = 7.5, 7.8 Hz), 7.73 (1H, d, *J* =6.8 Hz), 7.65 (2H, dd, *J* = 2.1, 6.7 Hz), 7.56 (2H, dd, *J* = 7.0, 8.2 Hz), 6.99 (1H, dd, *J* = 2.1, 6.7 Hz, H-4), 3.86 (3H, s, CH_3). ^{13}C RMN (125 MHz, $CDCl_3$) δ: 184.3 (s, C-1), 159.7 (s, C-2), 138.7 (s), 138.6 (d), 134.6 (d), 132.0 (s), 131.1 (d), 130.9 (d), 130.4 (d, 2 x C), 129.9 (s), 129.1 (s), 128.3 (s), 127.3 (d), 127.0 (s), 126.8 (d, 2 x C, C-2', C-6'), 113.5 (d, 2 x C, C-3', C-5'), 55.4 (c, CH_3). EIMS: m/z 286 (M^+, 97), 285 (51), 255 (100), 135 (13). HRMS: calcd. for $C_{20}H_{14}O_2$ (M^+) 286.0994, found 286.0980. UV-Vis (EtOH) λ_{max}: 421, 361, 347, 257, 245, 203 nm. IR ($CHCl_3$) ν_{max}: 2833, 1510, 1299, 1235, 1045, cm^{-1}. mp: 132-134 °C.

2.2.3.2. Synthesis of 2- (3-methoxyphenyl) -1H-phenalen-1-one (7). From 2-iodo-1*H*-phenalen-1-one (**5**) (0.228 mmol, 70.0 mg), DME/H_2O (1.4 mL), Na_2CO_3 (0.524 mmol, 56.0 mg), (3-methoxyphenyl) boronic acid (0.524 mmol, 80.0 mg) and 10% Pd/C (15.6 mg); heated for 1 hour; column chromatography (hexane/EtOAc, 9:1); product **7** (62.0 mg, 95%) was obtained as a yellow solid. ^{1}H RMN (500 MHz, $CDCl_3$) δ: 8.70 (1H, dd, *J* = 1.1, 7.3 Hz, H-9), 8.20 (1H, dd, *J* = 0.9, 8.0 Hz, H-7), 8.01 (1H, dd, *J* = 0.4, 8.2 Hz), 7.85 (1H, s, H-3), 7.80 (1H, dd, *J* = 7.4, 7.9 Hz), 7.79 (1H, d, *J* =7.4 Hz), 7.60 (1H, dd, *J* = 7.1, 7.0 Hz), 7.37 (1H, t, *J* =8.1 Hz, H-8), 7.26-7.24 (2H, m), 6.95 (1H, ddd, *J* = 0.9, 2.4, 8.3 Hz), 3.87 (3H, s, OCH_3). ^{13}C RMN (125 MHz, $CDCl_3$) δ: 184.0 (s, C-1), 159.5 (s, C-2), 139.8 (d), 139.3 (s), 138.1 (s), 134.8 (d), 132.1 (s), 131.6 (d), 131.5 (d), 131.1 (d), 130.0 (s), 129.3 (d), 128.2 (s), 127.4 (d), 127.3 (s), 127.0 (d), 121.6 (d), 114.7 (d), 114.7 (d), 55.5 (c, OCH_3). EIMS: m/z 286 (M^+, 58), 285 (28), 255 (21), 255 (100), 213 (11). HRMS: calcd. for $C_{20}H_{14}O_2$ (M^+) 286.0994, found 286.1008. UV-Vis (EtOH) λ_{max}: 405, 361, 280, 255, 209, 205, 201 nm. IR ($CHCl_3$) ν_{max}: 2838, 1637, 1242, 1029, 878 cm^{-1}. mp: 199-201 °C.

2.2.3.3. Synthesis of 2- (2-methoxyphenyl) -1H-phenalen-1-one (8). From 2-iodo-1*H*-phenalen-1-one (**5**) (0.228 mmol, 70.0 mg), DM/ H_2O (1.4 mL), Na_2CO_3 (0.524 mmol, 56.0 mg), (2-methoxyphenyl) boronic acid (0.524 mmol, 80.0 mg) and 10% Pd/C (15.6 mg); heated for 1 hour; column chromatography (hexane/EtOAc 7: 3); product **8** (59.0 mg, 90%) was obtained as a yellow solid. ^{1}H RMN (500 MHz, $CDCl_3$) δ: 8.69 (1H, d, *J* =7.3 Hz, H-9), 8.20 (1H, d, *J* =8.0 Hz, H-7), 8.25 (1H, d, *J* =8.2 Hz), 7.81-7.78 (2H, m), 7.76 (1H, d, *J* =6.9 Hz), 7.60 (1H, dd, *J* = 7.3, 8.0 Hz), 7.38 (1H, m), 7.35 (1H, dd, *J* = 1.4, 7.4 Hz), 7.05 (1H, t, *J* =7.4 Hz), 7.01 (1H, d, *J* =8.3 Hz), 3.80 (3H, s, OCH_3). ^{13}C RMN (125 MHz, $CDCl_3$) δ: 183.5 (s, C-1), 157.5 (s, C-2'), 140.7 (d), 138.3 (s), 134.4 (d), 132.1 (s), 132.2 (d), 131.1 (d), 131.0 (d), 130.8 (d), 129.8 (s), 129.5 (d), 128.2 (s), 127.4 (s), 127.2 (d), 126.7 (d), 126.3 (s), 120.5 (d), 111.3 (d, C-3), 55.8 (c, OCH_3). EIMS: m/z 286 (M^+, 50), 285 (18), 269 (24), 255 (100). HRMS: calcd. for $C_{20}H_{14}O_2$ (M^+) 286.0994, found 286.0996. UV-Vis (EtOH) λ_{max}: 396, 360, 344, 277, 251, 242, 202, 200 nm. IR ($CHCl_3$) ν_{max}: 2838, 1636, 1578, 1234, 1180 cm^{-1}. mp: 148-150 °C.

2.3. Biological analysis

2.3.1. Parasites

Promastigotes of *Leishmania amazonensis* (MHOM/BR/77/LTB0016) and *Leishmania donovani* (MHOM/IN/90/GE1F8R) were cultured in Schneider's medium (Sigma-Aldrich, Darmstadt, Germany) supplemented with 10% fetal bovine serum at 26 °C and were grown to the log phase. Parasites were also cultured in RPMI 1640 medium (Gibco, Massachusetts, USA), with or without phenol red.

2.3.2. In vitro anti-leishmanial activity

The five compounds were initially tested against *L. donovani* and *L. amazonensis* in their promastigote stages. Parasites were used to determine the antileishmanial activity by a colorimetric assay with alamarBlue, as previously described.[16]

Compounds were further tested against the intramacrophage stage of *L. amazonensis* using the assay developed by Jain et al.[17] Previously, cytotoxicity was also measured in a murine macrophage cell line, J774A.1, using the same colorimetric assay with alamarBlue, and then the selectivity index (SI) was calculated with the CC_{50} and the IC_{50} data for *L. amazonensis* amastigotes. Miltefosine was used as the positive control.

2.3.3. Analysis of ATP levels

Cell Titer-Glo® Luminescent Cell Viability Assay (Promega, Wisconsin, USA), which generates a proportional signal in relation to the amount of ATP, was used to measure changes in ATP levels after treatment. The IC_{90} of the phenalenones derivatives was utilized for 24 h over promastigotes. Protocol was followed on white plates by manufacturer's instructions for posterior measurement of luminescence on a spectrophotometer (PerkinElmer, Massachusetts, USA).[18]

2.3.4. Analysis of mitochondrial membrane potential

The JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical, Michigan, USA) was used to detect the collapse of an electrochemical gradient across the mitochondrial membrane during apoptosis. Parasites treated with IC_{90} of the phenalenones for 24 h were then centrifuged and resuspended in buffer. An aliquot of each treated culture was added to a black plate and the JC-1 was added. After 30 min of incubation at 26 °C, fluorescence intensity was measured for green and red spectra using an Enspire microplate reader (PerkinElmer).[19] Data are expressed as the ratio 595/535 (J-aggregates:J-monomers).

2.3.5. Phosphatidylserine externalization

A double-staining assay with annexin V/propidium Iodide (PI) was performed using the TALI apoptosis kit (Life Technologies), according to the manufacturer's instructions. Briefly, after treating parasites with the IC_{90} for 24 h, they were washed with the buffer and incubated with annexin V (alexa fluor 488) for 20 min. After that, promastigotes were centrifuged and resuspended in buffer containing PI and incubated again for 3 min. Finally, the stained cells were loaded into a slide and inserted in a TALI™ image-based cytometer.[20] Data was expressed in percentages of each sample, divided into three groups: apoptotic cells (green

fluorescence); dead cells (red or red and green fluorescence); and live cells (no fluorescence), provided by TALI™ data acquisition and analysis software (Life Technologies).

2.3.6. Statistical analysis

Data are presented as the mean ± standard error (SE), where all determinations were performed in duplicate and the data shown are the representative results from at least three independent experiments. Statistical differences between means were tested using a one-way analysis of variance (ANOVA; three or more samples), carried out using Tukey's test, using the SigmaPlot 12.0 software. A significance level of $P < 0.05$ was used.

3. RESULTS & DISCUSSION

3.1. Synthesis of 3-phenyl-9-hydroxy-1H-phenalen-1-one (3)

Compound **3** was obtained as a by-product during the synthesis 9-hydroxy-1*H*-phenalen-1-one (**2**) from 2-methoxynaphthalene (**1**), (2*E*)-3-phenylacrylyl chloride and $AlCl_3$ following known methodology (Scheme 1).[21–25]

3.2. Synthesis of 9-(propylamino)-1H-phenalen-1-one (4)

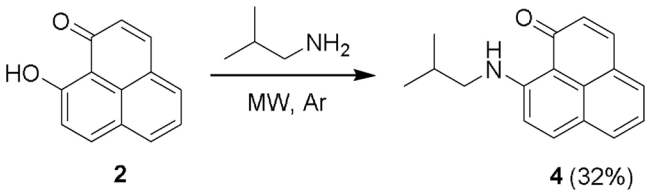
Compound **4** was synthesized following the method developed by Haddon,[26] in which the amine is heated, alone or with a polar solvent such as DMF, with 9-hydroxy-1*H*-phenalen-1-one (**2**) to temperatures generally above 120 °C. In our case, we performed the heating with microwaves at 180 °C for 90 minutes, obtaining **4** with a 32% yield (Scheme 2).

3.3. Synthesis of 2-phenyl-1H-fenalen-1-one derivatives (6, 7 and 8)

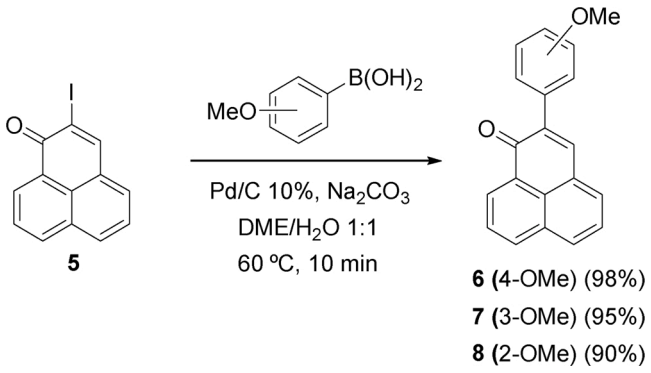
In order to prepare the 2-phenyl-1*H*-fenalen-1-one derivatives (**6-8**), we decided to use a Suzuki-Miyaura type coupling reaction[27] between 2-iodo-1*H*-phenalen-1-one (**5**) and various commercially available boronic acids. Traditionally, these types of reactions use homogeneous palladium catalysts such as $Pd(PPh_3)_4$, $Pd(OAc)_2$ or $Pd_2(dba)_3$ with different ligands. However, recently Francüois-Xavier has shown that the cheapest and most heterogeneous ligand-free catalyst, Pd/C, could be used as an effective catalyst in Suzuki-Miyaura coupling of 2-iodocycloenones with arylboronic acids.[28] Therefore, we decided to prepare the 2-phenyl-1*H*-fenalen-1-ones using 2-iodo-1*H*-phenalen-1-one (**5**) as a starting material and Pd/C as a catalyst for coupling with arylboronic acids, obtaining the products with excellent yields (Scheme 3).

3.4. Anti-leishmanial activity, cytotoxicity and selectivity

All compounds were tested against *L. donovani* and *L. amazonensis* in the promastigote stages on their exponential growth phase, to identify if the compounds have anti-leishmanial activity and if they were worthy for more studies. The prescribed anti-leishmanial drug miltefosine was used as the positive control. Results, shown on Table 1, demonstrate that the five compounds presented leishmanicidal activity against the promastigote stage of both strains with IC_{50} values ranging from 2.63 to 24.23 μM.



Scheme 2. Direct amination of **2**.



Scheme 3. Suzuki-Miyaura cross coupling of **5** with arylboronic acids catalyzed by Pd/C.

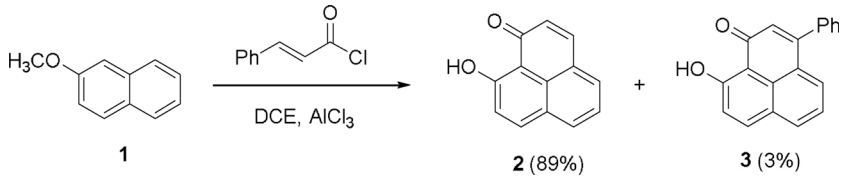
Table 1
Activity of phenalenones **3**, **4**, **6**, **7** and **8** against the promastigote stage of *Leishmania amazonensis* and *L. donovani*. Values correspond to the IC_{50} values in μM.

Compound	*L. amazonensis*	*L. donovani*
3	6.03 ± 0.17	24.23 ± 2.12
4	4.95 ± 0.32	7.16 ± 1.72
6	11.64 ± 1.27	4.99 ± 0.10
7	6.43 ± 0.98	2.63 ± 0.56
8	9.40 ± 0.76	8.83 ± 1.69
Miltefosine	6.48 ± 0.24	3.32 ± 0.27

To assess the selectivity of these compounds, their cytotoxicity was measured in a J774A.1 murine macrophage cell line, obtaining CC_{50} values that were between 7.91 and 31.19 μM. Moreover, the leishmanicidal activity of the phenalenones **3**, **6**, **7** and **8** were higher than miltefosine against the intracellular amastigote stage of *L. amazonensis* (Table 1), and compound **4** presented similar activity than the reference drug, showing IC_{50} values ranging from 0.15 to 3.40 μM (Table 2).

The selectivity index (SI) was calculated as the ratio of CC_{50} value for J774A.1 and the IC_{50} value for intracellular amastigotes of *L. amazonensis*, concluding that compound **8** is 9-fold more selective than miltefosine, followed by compounds **3** and **7**, that have similar selectivity than the reference drug.

Surprisingly, the same substituent 4-methoxyphenyl but in C3 had no activity. An interesting fact is the improvement in activity patent when the 4-Omethoxy (**6**) change to 3-Omethoxy (**7**) and to 2-Omethoxy (**8**), maintaining the leishmanicidal activity but decreasing its toxicity, resulting in a very selective compound.



Scheme 1. Synthesis of **3** under Friedel-Craft reaction conditions.

Table 2
CC_{50} corresponding to the cytotoxicity of phenalenones 3, 4, 6, 7 and 8 against eucariotic cells (murine macrophages), IC_{50} values against the amastigote stage of *Leishmania amazonensis*, both values are in μM. Selectivity Index was also calculated.

Compound	Murine macrophages CC_{50}	*L. amazonensis* amastigotes IC_{50}	SI
3	30.39 ± 0.24	1.82 ± 0.72	16.7
4	10.02 ± 0.73	3.40 ± 0.43	2.9
6	7.91 ± 0.24	1.17 ± 0.16	6.8
7	13. 74 ± 2.20	1.31 ± 0.06	10.5
8	31.19 ± 0.47	0.15 ± 0.03	207.9
Miltefosine	72.19 ± 3.06	3.12 ± 0.30	23.1

3.5. Mitochondrial damage

In our previous studies with 1H-phenalen-1-ones possessing antileishmanial activities, we suggested that they modulate the mitochondrial respiratory chain (decreasing the mitochondrial membrane potential and inducing apoptosis). Therefore, we investigated whether these new phenalenone derivatives also affected this important organelle. As the active compounds were generally more potent against *L. amazonensis*, we focused further investigations on this strain. The ΔΨm was measured using the mitochondrial membrane specific cationic dye JC-1, at the IC_{90} of each compound, and measuring the fluorescence in a spectrofluorometer EnSpire (Perkin Elmer). All compounds caused sizeable changes in decreasing the ΔΨm, showing statistically significant reductions (Fig. 1A). In particular, the reductions caused by compounds **7**, **8**, and **6** were quite strong, reaching just a 10% if compared to the negative control. This is an indicative of a strong depolarization of the leishmanial mitochondrial membrane by these derivatives.

The mitochondrial damage was as well confirmed by quantifying the ATP level after 24 h of incubation with their IC_{90}. All the compounds produced a strong decrease in the total ATP level in *L. amazonensis* parasites, confirming the damage in the mitochondrial membrane, been impossible to maintain the normal working of the ATP production machinery (Fig. 1B).

Altogether, these events strongly suggest that all the derivatives cause a dropped of the mitochondrial potential perpetrated by an intrinsic pathway of apoptosis induced in *L. amazonensis* promastigotes. The loss in membrane potential directly affects mitochondrial electron transport chain function and finally decreases ATP production, and consequently, parasites may undergo either necrosis or apoptosis, thus beeing necessary to perform subsequent assays.[29]

3.6. Cytoplasmic membrane alterations and apoptosis-like induction

Cell death by necrosis is characterized by cell swelling and posterior rupture of the plasma membrane. On the other hand, apoptosis is characterized by different events, as the phosphatidylserin exposure on the external part of the cytoplasmic membrane. After 24 hours of incubation, we could observe strong changes in the composition of the external part of the plasmatic membrane of the parasites, with a higher amount of bounded annexin V on *L. amazonensis* when incubated with all the studied phenalenones (Fig. 2). There were also strong statistically significant changes, not only in the bounded annexin V on promastigotes, but also for the propidium iodide stained population that represent the already dead parasites. Despite this strong disturbance in the plasma membrane, the morphology and shape of the promastigotes remained intact as observed under the microscope, excluding cell death by necrosis. Together, these data indicate that the treatment with these phenalenones induces distinct alterations associated with apoptosis-like cell death in *L. amazonensis* promastigotes.

Similar studies on apoptosis induction over *Leishmania* spp. perform only the annexin-propidium staining in order to determine whether the population is suffering apoptosis or necrosis, normally analysing by flow cytometry, resulting in similar staining results between the 24 and 48 h after the treatment of the parasites.[30]

In addition, annexin translocation is related with plasma membrane integrity and permeability, and is a remarkable indicative of an early stage of apoptosis, which is also confirmed by the mitochondrial membrane depolarization.[31]

The results showed that the new derivatives have comparable or better activity and selectivity than the commonly prescribed antileishmanial drug, by inducing stronger mitochondrial depolarization, deeper decrease of ATP levels and more intense phosphatidylserine exposure than miltefosine, highlighting compound **8** with 9-times better selectivity index than the mentioned reference drug, due to its low IC_{50} against intracellular amastigotes.

Taken together, phosphatidylserine exposure and mitochondrial membrane dysfunction, both hallmarks of classic apoptosis, confirm the apoptotic-like cell death in *L. amazonensis*promastigotes upon the five phenalenone derivatives treatments separately. There are many examples in the literature about different natural substances, or derivatives from natural sources, that are able to cause apoptosis in *Leishmania*

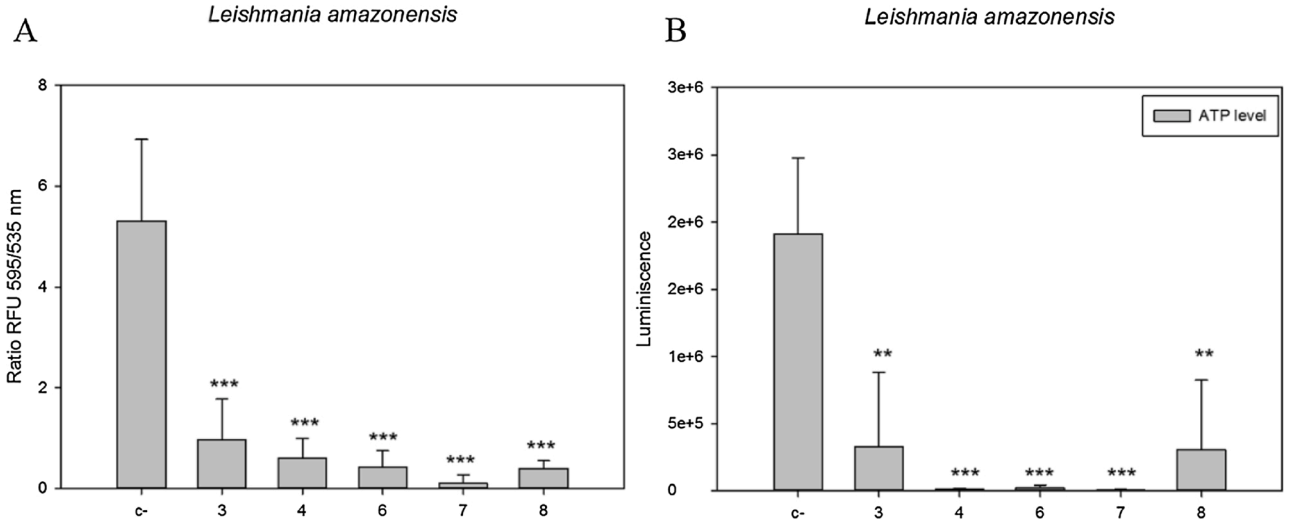


Fig. 1. *Leishmania amazonensis* (A) changes in the mitochondrial membrane potential (ΔΨm) and (B) ATP levels in relative luminescence units after 24 h of incubation with the IC_{90} of phenalenones. C-: Negative control (not treated parasites). Error bars represent the standard deviation (SD). Each data point indicates the mean of the results of three measurements (***) $p < 0.001$, (**) $p < 0.01$.

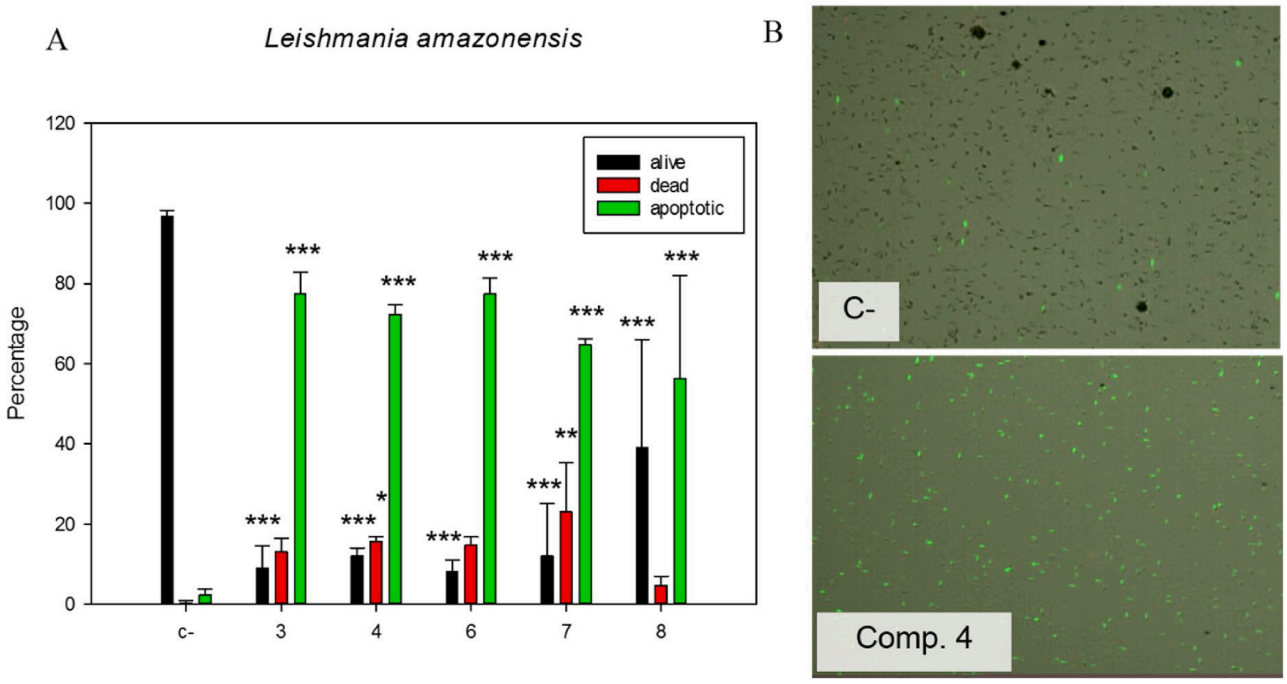


Fig. 2. *Leishmania amazonensis* changes in the phosphatidylserine exposure after 24 h of incubation with the IC_{90} of phenalenones. (A) Percentages of population: alive (without dye), dead (stained with PI) and apoptotic (stained with annexin V). (B) Parasites from negative control and parasites treated with compound 4 on the image-based cytometer Tali (Invitrogen). In green fluorescence, promastigotes stained with annexin V. C-: Negative control (not treated parasites). Error bars represent the standard deviation (SD). Each data point indicates the mean of the results of three measurements (***) $p < 0.001$, (**) $p < 0.01$, (*) $p < 0.05$.

parasites as the present phenalenones, including whitanolides,[32] oxasqualenoids[33] or lapachol.[34]

3.7. Structure-activity relationship

In a previous work from our group, we discovered the potential anti-leishmanial activity of the amino-phenalenones. In this research, two of the three better compounds resulted to be N,N-dimethylethylenediamine substituted in C2 and C3[6], with IC_{50} values between 2.1 and 9.6 μM for both strains (*L. amazonensis* and *L. donovani* respectively). In the present study, the position C9 resulted in good results of activity (4.95 μM for *L. amazonensis* and 7.16 μM for *L. donovani* promastigotes) although the case of compound **4** the substituent is missing one amine.

Regarding the phenil-phenalenones, Rosquete and col.[35] studied the anti-kinetoplastid activity of different phenil-phenalenones, resulting in two 4-methoxyphenyl similar to compound **6**, but instead to be in C2, they studied the activity in C9 and C4, which had IC_{50} values of 0.83 and 4.43 μg/ml (2.91 and 15.5 μM) against *L. amazonensis* promastigotes. But in our work compound **6** presented an intermediate IC_{50} of 11.64 μM against the same strain. However, the better value of IC_{50} resulted in the compound **8**, a 2-methoxyphenyl on C2, when incubated against the intracellular amastigotes of *L. amazonensis*.

4. Conclusions

The five synthetic phenalenones evaluated in this study presented strong anti-leishmanial activity against *L. donovani* promastigotes and against both stages of *L. amazonensis* parasites. Several ultrastructural and physiological changes like mitochondrial membrane potential changes, decrease in ATP levels and phosphatidylserine externalization were observed in *L. amazonensis* promastigotes after treating them with the mentioned phenalenones derivatives. The present results highlight the potential use of phenalenone derivatives as programmed cell death inducers on *L. amazonensis*, sharing several phenotypic characteristics with other cases of programmed cell death in metazoans. However, further studies are needed to unveil the specific target of these phenalenones in *Leishmania* spp. parasites that triggers this specific cell death. Furthermore, perform *in vivo* experiments could be necessary in order to establish them as novel and safe leishmanicidal therapeutic agents.

Declaration of Competing Interest

Proyects PI18/01380 and RD16/0027/0001(RICET), from the Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación, both cofounded by FEDER (Fondo Europeo de Desa

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