JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

Resveratrol analogues present effective antileishmanial activity against promastigotes and amastigotes from distinct *Leishmania* species by multitarget action in the parasites

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Keywords
Leishmania; mechanism of action; N-aryl hydrazones; N-aryl imines; resveratrol

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Received July 16, 2019
Accepted September 14, 2019

doi: 10.1111/jphp.13177

Abstract

Objectives The *in vitro* antileishmanial effect of analogues of resveratrol (**AR**) present in the N-aryl imines and N-aryl hydrazones series was investigated. In addition, possible parasite targets were evaluated.

Methods Antipromastigote activity of *Leishmania amazonensis*, *L. braziliensis* and *L. infantum*, as well as the cytotoxicity on macrophages was determined by MTT assay and *L. braziliensis*-infected macrophages effect by Giemsa stain. After staining, effects on the parasite targets were analysed by flow cytometry or by fluorescence microscopy.

Key-findings Among the tested compounds, the derivative **AR26** showed the best effect against promastigotes of all *Leishmania* species (IC_{50} < 3.0 μg/ml), being more active than miltefosine, the control drug. **AR26** was also effective against amastigotes of *L. braziliensis* (IC_{50} = 15.9 μg/ml), with low toxicity to mammalian cells. The evaluation of mechanism of action of **AR26** on *L. braziliensis* promastigotes indicates mitochondrial potential depolarization, plasma membrane permeabilization, interference in the progression of the cell cycle and accumulation of autophagic vacuoles. In addition, any increase of the reactive oxygen species levels was detected in the treated *L. braziliensis*-macrophages.

Conclusions Data indicate that the antileishmanial activity of **AR26** is related to multitarget action, and the resveratrol analogues could be used in future studies as antileishmanial agent.

Introduction

Leishmaniasis is a disease complex caused by species of flagellate protozoa of the genus *Leishmania*. It represents a serious global health problem, which can affect 350 million people in more than 98 countries, with an estimated incidence of 20,000–30,000 deaths annually.[1] The infection by the *Leishmania* parasite manifests in humans with multiple clinical symptoms, affecting the skin, mucosal and/or visceral organs of the patients. The main determining factors for the diversity of the clinical manifestations are associated with the immunological status of the hosts, as well as by etiologic agent and insect vector involved in the transmission cycle.[2,3]

The tegumentary leishmaniasis (TL) of the diseases can manifest as cutaneous leishmaniasis, the main clinical form characterized by unique lesions with favourable response to treatment, showing high frequency of spontaneous healing.[4,5] The mucosal leishmaniasis (ML) is a debilitating clinical form of TL characterized by the presence of destructive and metastatic lesions in the oronasal mucosa and respiratory tract, resulting from the lymphatic or haematogenous dissemination of the parasites.[6] The cutaneous-diffuse leishmaniasis is a severe form of TL, which

manifests in patients with deficiency in the immune cellular response against antigens of the parasite. This infection begins with a single lesion that is not responsive to treatment and evolves with the formation of multiple non-ulcerated lesions, which are characterized by abundance of parasites.[4,7] Visceral leishmaniasis (VL) is the most serious clinical syndrome of leishmaniasis, resulting from haematogenous dissemination of parasites to internal organs. The disease can manifest as persistent fever, splenomegaly, hepatomegaly and severe anaemia with risk of fatal progression, mainly in cases of secondary infections, malnutrition and organ failure.[8,9]

The current treatment against leishmaniasis is considered unsatisfactory, mainly due to the severe toxicity, high cost and/or emergence of the parasite resistance.[10] The pentavalent antimonials are the first-line drugs, although they present limitations, such as the prolonged treatment by parenteral route and side effects, including cardiotoxicity, hepatotoxicity and pancreatitis. [9,11] Amphotericin B is a therapeutic option applied in cases of antimonials treatment failure, presenting higher cure rate against VL and TL. However, the toxicity of this drug causing renal insufficiency and hepatotoxicity, along with the long treatment course and the emergence of treatment failure, represents complications of this therapeutic process.[12] Pentamidine has been presented as an option in areas where the treatment failure occurs; however, its clinical efficacy is unsatisfactory, with its use currently being restricted to the treatment of infections caused by *L. guyanensis* species in the South America.[11] Miltefosine is the only therapeutic option recommended for oral use, although its employ is restricted in some countries. The main inconveniences are the teratogenic potential and therapeutic failure to treat against TL.[13,14] In this context, and due to the distinct problems related to the current scenario of the treatment against leishmaniasis, additional efforts need to be performed aiming to identify new therapeutic antileishmanial agents.

Resveratrol and its derivates represent a chemical diversity of substances with pharmacological relevance, presenting activities such as antioxidant, anticancer, cardioprotective, anti-inflammatory, immunomodulatory, antibacterial and neuroprotective.[15–17] Moreover, resveratrol presents efficacy against chronic chagasic cardiomyopathy in experimental models, promoting significant reduction of parasite burden and improvement of heart function, which are associated with antioxidative activity, reduction in the reactive oxygen species (ROS) levels and induction of lipid peroxidation.[18]

The antileishmanial effect of resveratrol and its analogues against different *Leishmania* species have been reported in the literature.[19–25] However, in this study, we extended the investigation of effect of a new class of resveratrol analogues against promastigotes and intramacrophage amastigotes of different *Leishmania* species, aiming to evaluate their action against parasites able to cause TL and VL in the world. Also, the mechanism of action of the best analogue was evaluated in *Leishmania braziliensis*, the main etiological agent of localized and mucosal forms of TL in the Americas.[26]

Materials and Methods

Synthesis of the compounds

Imine resveratrol analogues (**AR26** and **AR27**) and hydrazone resveratrol analogues (**AR29**, **AR30** and **AR32**) were obtained by condensation of 2-hydroxyaniline, aniline, phenylhydrazine or 2,4-dinitrophenylhydrazine with the equimolar amount aromatic aldehydes in ethanol as solids after being filtered, washed with ethanol and dried in an oven (yield 65–82%) (Scheme 1). All the compounds were characterized via one-dimensional nuclear magnetic resonance (1D-NMR) and melting point (Appendix S1).[27,28]

Mice

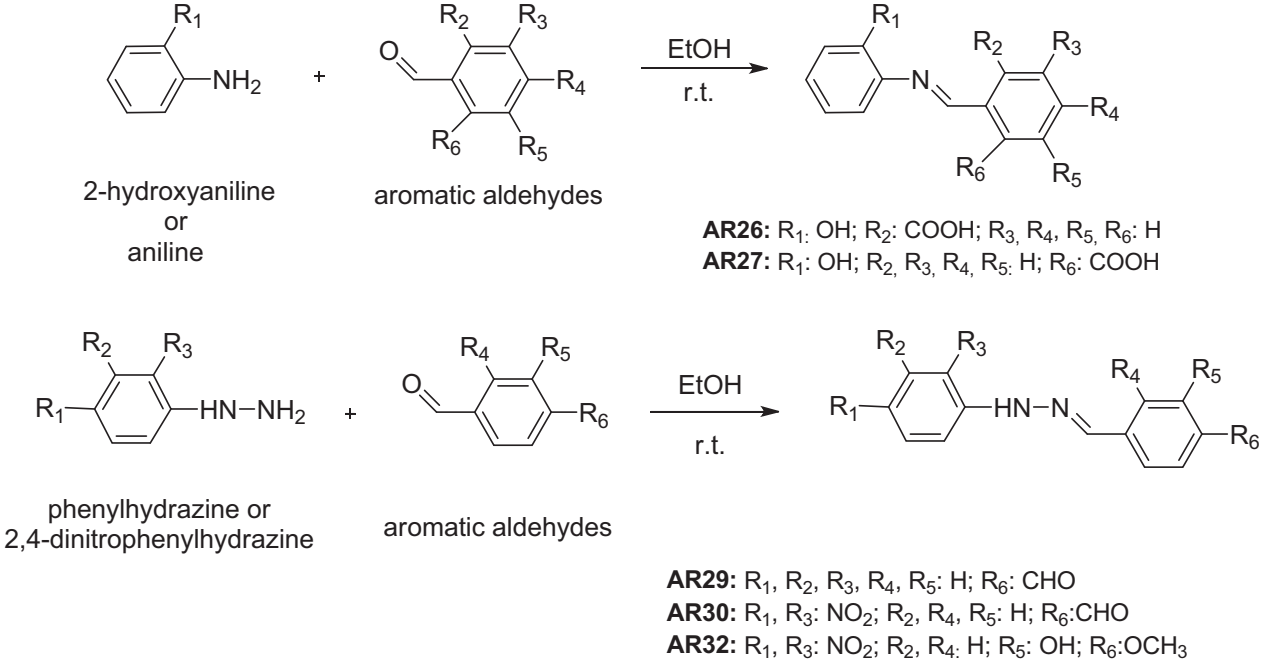
BALB/c mice (female, 8 weeks old, weighing 20–25 g) were purchased from the Reproductive Biology Center of Federal University of Juiz de Fora (UFJF), and they were maintained under specific pathogen-free conditions. The study was approved by the Committee of Ethical Handling of Research Animals of UFJF (protocols number 007/2018 and 008/2018).

Parasites

Leishmania amazonensis (IFLA/BR/1967/PH8) was cultured in Warren's medium; *L. braziliensis* (MHOM/Br/75/M2903) was cultured in BHI medium supplemented with 20 mM L-glutamine, and *L. infantum* (MHOM/Br/74/PP75) was cultured in LIT medium, all supplemented with 10% Fetal Bovine Serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin and 0.1 mg/ml of streptomycin, and they were maintained at 25 °C in BOD incubator.[29]

In vitro antipromastigote activity

Leishmania braziliensis and *L. infantum* (3×10^6 cells) or *L. amazonensis* promastigotes (2×10^6 cells) in logarithmic-phase were platted in 96-well plates and exposed to the resveratrol analogues (concentrations ranging from 0.47 to 30.0 μg/ml), for 72 h at 25 °C. The parasite viability was determined using the colorimetric reagent 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich). The optical density values were read in a microplate spectrophotometer (Multiskan MS



Scheme 1 Synthesis of resveratrol analogues **AR26**, **AR27**, **AR29**, **AR30** and **AR32**.

Microplate Reader; LabSystems Oy, Helsink, Finland), at 570 nm. AmpB (0.03–1.0 μg/ml, Cristália, São Paulo, Brazil) and miltefosine (0.63–40.0 μg/ml) were used as drug controls.[29]

Cytotoxicity

The cytotoxicity assay was assessed in murine macrophages collected from peritoneal cavity of BALB/c mice, which were previously stimulated with 2 ml thioglycolate. Then, macrophages (2×10^5 cells) were prepared in an RPMI-1640 medium supplemented with 10% FBS and incubated in 96-well plates at 37 °C in 5% CO_2. After 24 h, the non-adherent cells were removed and the others were incubated with serial dilutions of the compounds (2.3–150.0 μg/ml), for 72 h at 37 °C in a humidified atmosphere containing 5% CO_2. Miltefosine (3.8–30.0 μg/ml) and AmpB (4.3–138.6 μg/ml) were used as control. The cell viability was analysed by the MTT method.[30]

Treatment of *L. braziliensis*-infected macrophages

Macrophages (6×10^5 cells) were plated on round glass coverslips in 24-well plates in RPMI 1640 medium added with 10% FBS and incubated for 24 h at 37 °C in 5% CO_2. Afterwards, *L. braziliensis* stationary-phase promastigotes (6×10^6 cells; at a ratio of 10 parasites per one macrophage) were added into the wells, and cultures were incubated for 4 h at 33 °C in 5% CO_2. Free parasites were removed by extensive washing with PBS (0.1 M, pH 7.4), and infected macrophages were treated with the compounds (3.1–50.0 μg/ml), miltefosine (0.63–40.0 μg/ml) or AmpB (0.06–1.0 μg/ml), for 72 h at 33 °C in 5% CO_2. After fixation, cells were stained with Giemsa solution, and 200 macrophages were counted in a light microscope.[29] The infection index was calculated by: number of amastigotes per macrophage × percentage of infected macrophages/total number of macrophages.

Evaluation of mitochondrial dysfunction

The mitochondrial membrane potential (ΔΨm) was evaluated in *L. braziliensis* promastigotes in the log phase (10^7 cells), which were incubated for 24 h in the absence or presence of the best resveratrol-analogue (**AR26**; 1.0 and 2.0 μg/ml, corresponding to one and two times the IC_{50} values) at 25 °C. Afterwards, parasites (10^7 cells) were labelled with Mitotracker (500 nM; Invitrogen, Eugene, OR, USA) for 40 min at 37 °C. After washing twice with PBS (0.1 M, pH 7.4), samples were added into a black 96-well plate, and fluorescence intensity was measured in a spectrofluorometer (FLx800; BioTek Instruments, Inc., Winooski, VT, USA), at both 540 and 600 nm wavelengths. Parasites incubated with 2.0 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; Sigma-Aldrich) for 10 min were used as control.[30] For measurement of ROS levels, parasites (2×10^7 cells) were marked using the cell-permeable probe

H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate, Molecular Probes, Eugene, OR, USA), for 30 min in the dark at room temperature. ROS levels were measured in a fluorimetric microplate reader (FLx800, BioTek Instruments), with excitation filter of 485 nm and emission filter of 528 nm.[30] Parasites incubated with miltefosine (10.4 μg/ml) for 24 h were used as positive control.

Evaluation of the cell membrane integrity

Leishmania braziliensis promastigotes in the log phase (10^7 cells) were either untreated or treated with **AR26** (1.0 and 2.0 μg/ml) or miltefosine (10.4 μg/ml), for 24 h at 25 °C. Afterwards, parasites were washed with PBS (0.1 M, pH 7.4) and stained with 1.0 μg/ml propidium iodide (PI; Sigma-Aldrich) for 15 min and in the dark. PI-stained parasites were analysed in a fluorimetry microplate reader (FLx800; BioTek Instruments), with excitation wavelength of 540 nm and emission wavelength of 600 nm.[31]

Analysis of the cell cycle

Leishmania braziliensis promastigotes in the log phase (10^7 cells) were treated with **AR26** (1.0 and 2.0 μg/ml) for 24 h at 25 °C. After treatment, parasites were permeabilized with 70% ethanol for 1 h at 4 °C. Then, cells were incubated with 200 μg/ml Ribonuclease A (Sigma-Aldrich) for 1 h at 37 °C and subsequently stained with 7.0 μg/ml PI (Sigma-Aldrich), for 20 min in the dark at room temperature. For each sample, 10,000 events were acquired using the FACsCanto II flow cytometer (Becton Dickinson, Rutherford, NJ, USA), and obtained data were analysed in a FlowJo VX software.[32]

Evaluation of the accumulation of autophagic vacuoles

Leishmania braziliensis promastigotes in the log phase (10^7 cells) were untreated or treated with **AR26** (1.0 and 2.0 μg/ml) for 24 h. Then, parasites were washed with PBS (0.1 M, pH 7.4), and parasites were labelled with 100 μM monodansylcadaverin (Sigma-Aldrich) for 1 h in the dark at 25°C. Afterwards, the fluorescence intensity was analysed in a spectrofluorometer (Varioskan® Flash, Thermo Scientific, Waltham, MA, USA), using 335 nm for excitation and 460 nm for emission, respectively. Untreated stationary promastigotes were used as control.[33]

Measurement of ROS levels in *Leishmania*-infected macrophages

Macrophages were infected with *L. braziliensis* species and untreated or treated with **AR26** (15.88 and 31.76 μg/ml) or miltefosine (1.3 μg/ml) for 24 h. Then, cells were washed with PBS (0.1 M, pH 7.4), and incubated with 20 μM H_2DCFDA for 30 min in the dark at 33 °C, 5% CO_2. The fluorescence intensity was measured in a fluorimeter (FLx800; BioTek Instruments), with 485 nm excitation and 528 nm emission.[30] Infected macrophages were treated with zymosan derived from *Saccharomyces cerevisiae* (250 μg/ml, Sigma-Aldrich) and used as positive control.

Statistical analysis

The compound concentrations necessary to inhibit 50% of *Leishmania* viability (IC_{50} values) and of the macrophages (CC_{50} values) were calculated by the Probit program. Data were analysed by One-way ANOVA followed by Dunnett's post-test to compare the groups. GraphPad Prism 5 was also used (GraphPad Software, San Diego, CA, USA). Results were expressed as the mean ± standard error of the mean of three independent experiments, which presented similar results. Differences were considered significant with $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

Results

Firstly, the antileishmanial activity of a series of resveratrol analogues was investigated against promastigotes of *L. amazonensis, L. braziliensis* and *L. infantum* species and the cytotoxic effect on murine macrophages. The effect of compounds was assessed using the MTT method, based on the reduction of dehydrogenase system of metabolically active/live cells.[34] Although disadvantages, it is easy to use, has a high reproducibility, and considered as one the most widely assay for evaluation of cell viability and cytotoxicity for screening the drugs.[34] In addition to MTT assay, parasites and macrophages were also observed under the light microscope. Results showed that all molecules exhibited inhibitory effect against *L. braziliensis* promastigotes, whereas the analogues **AR26**, **AR30** and **AR32** showed better activity against *L. infantum*, and only **AR26** was effective against *L. amazonensis* (Table 1). Comparing all analogues, **AR26** was this presenting better action against the three parasite species, with IC_{50} values lower than miltefosine, used as a control. The cytotoxicity on murine macrophages showed that these analogues were not toxic for these cells in the highest concentration tested ($CC_{50} > 30.0$ μg/ml) (Table 1). Since the compounds were more effective (lower IC_{50} values) against *L. braziliensis* species and that **AR26** was active against all parasite species, it was thus tested to treat macrophages that were infected with *L. braziliensis*, and results showed an IC_{50} value of 15.9 μg/ml against the intramacrophage amastigotes (Table 1). In the evaluation of

Table 1 Antileishmanial activity and cytotoxicity in murine macrophages of resveratrol analogues

Compounds	Promastigotes *L. amazonensis* IC_{50}[a] μg/ml	Promastigotes *L. braziliensis* IC_{50}[a] μg/ml	Promastigotes *L. infantum* IC_{50}[a] μg/ml	Macrophages CC_{50}[b] μg/ml	Amastigotes *L. braziliensis* IC_{50}[a] μg/ml
AR26	2.7 (2.3–2.9)	1.0 (0.8–1.2)	3.0 (4.1–4.7)	>150.0	15.9 (13.9–18.1)
AR27	2.6 (2.1–3.0)	0.7 (0.6–0.9)	13.1 (9.7–17.6)	>30.0	>30.0
AR29	>30.0	2.7 (2.1–3.3)	>30.0	7.3 (6.0–8.9)	>5.0
AR30	>30.0	15.4 (13.6–17.4)	11.4 (8.2–15.9)	>30.0	>30.0
AR32	>30.0	26.6 (23.4–30.2)	12.7 (8.3–19.6)	>30.0	>30.0
Amphotericin B	0.11 (0.09–0.12)	0.12 (0.09–0.14)	0.05 (0.04–0.06)	10.1 (7.8–13.1)	0.069 (0.057–0.085)
Miltefosine	9.0 (8.5–9.5)	10.4 (9.0–12.1)	11.8 (10.0–14.0)	>30.0	1.3 (1.16–1.47)

Results correspond to the mean of three independent experiments performed in duplicate. Miltefosine and amphotericin B were used as drug controls. [a]IC_{50}, Inhibitory concentration of 50% of parasites. [b]CC_{50}, Cytotoxic concentration of 50% of macrophages.

the chemical composition between the analogues, the imine analogues (**AR26** and **AR27**) exhibited better activity than their respective hydrazones counterparts (**AR 29**, **AR30** and **AR32**). For imine group, **AR26** presenting the hydroxy group in the ortho position was more active than **AR27** against *Leishmania* parasites, indicating that the hydroxy substitution was critical for biological activity.

The treatment of *L. braziliensis*-infected macrophages using **AR26** showed reductions in the order of 96.95%, 63.71%, 31.75%, 25.34% and 19.30%, when 50.0, 25.0, 12.5, 6.25 and 3.12 μg/ml of the compound were used, respectively (Figure 1). No toxic effect was observed in the host cell; thus, confirming results obtained previously by cytotoxicity assay (CC_{50} > 150 μg/ml), demonstrating a satisfactory selectivity index against intracellular amastigotes of *L. braziliensis*.

Due to the promising antileishmanial effect presented by **AR26**, more detailed studies were conducted to investigate the mechanism of action of this compound in *Leishmania* parasites. The mitochondrial function was evaluated based on (i) determination of the mitochondrial transmembrane electric potential (ΔΨm) and (ii) by detection of ROS production in **AR26**-treated parasites. The effect of this molecule on the ΔΨm depolarization was detected using the cationic probe MitoTracker® Red. Results showed that the treatment with **AR26** caused a decrease in ΔΨm in the order of 20.66% and 22.68%, respectively, when 1.0 and 2.0 μg/ml of the compound (corresponding to one and two times the IC_{50} values, respectively) were used, after 24 h of incubation (Figure 2). The reduction in the ΔΨm in FCCP-treated parasites, which was used as a control, showed reduction in the order of 14.24%.

To investigate whether the effect of **AR26** on ΔΨm depolarization is associated to the induction of oxidative stress, ROS levels were evaluated using the sensitive cell permeating probe, H_2DCFDA. Results showed any increase in the ROS production after 24 h of incubation, when compared to the untreated parasites (Figure 3). On the other hand, miltefosine induced an increase in the ROS levels in the order of 63.33%.

The plasma membrane integrity of **AR26**-treated parasites was evaluated following the incubation with propidium iodide cell probe (PI). Results showed that the compound induced permeabilization of plasma membrane of the promastigote forms, at a dose of 2.0 μg/ml, when compared with the control group (Figure 4). In addition, an increase in the order of 107.60% was observed in the percentage of PI-positive promastigotes that were treated with miltefosine, indicating also the disrupting of plasma membrane integrity.

To evaluate the effect of **AR26** in the parasite's cell cycle, promastigotes were labelled with PI, and the DNA content was evaluated by flow cytometry. Results showed the deleterious effect of treatment with **AR26** at 1.0 or 2.0 μg/ml on the parasite´ cell cycle, with increase of $subG_0/G_1$ population (2.7- and 2.5-fold greater than untreated parasites, respectively), as well as a significant reduction of G_0/G_1 (32.09% and 36.12%, respectively) and G_2 (47.17% and 53.91%, respectively) populations (Figure 5).

The study assessed also whether the treatment with **AR26** induces the activation of autophagic cell death. To quantify the accumulation of autophagic vacuoles, a staining using the MDC marker was performed. Results showed that the treatment of promastigotes with **AR26** at concentrations of 1.0 and 2.0 μg/ml induced increase in the order of 30.81% and 21.78%, respectively, in the formation of autophagic vacuoles, when compared with untreated parasites (Figure 6).

To investigate whether the effect of **AR26** against intramacrophage amastigotes was related with the activation of oxidative mechanisms by host cell, the ROS production was evaluated in infected macrophages that were thus treated with this molecule. Results showed that any increase in the ROS levels was detected in the infected macrophages after treatment with **AR26**. As expected, both uninfected and infected macrophages respond to zymosan stimulation with increase in the ROS production (Figure 7).

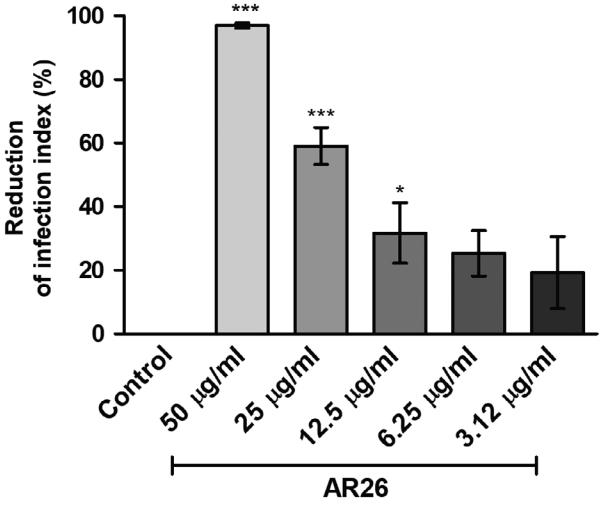


Figure 1 Effect of **AR26** in the treatment of macrophages infected by *L. braziliensis*. Murine macrophages were infected with *L. braziliensis* promastigotes (in a ratio of 10 parasites per cell) and treated with **AR26** (3.12–50.0 μg/ml) for 72 h at 33 C in 5% CO_2. Cells were stained with Giemsa, and the percentage of reduction of infection was determined by counting of 200 cells in duplicate in a light microscope. $P < 0.001$ (***) and $P < 0.01$ (*) showed significant difference when compared with the control group.

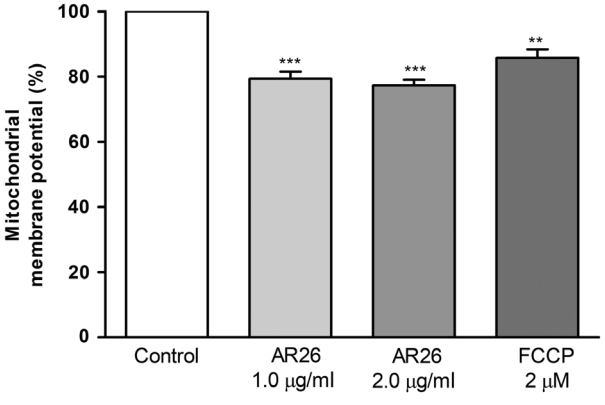


Figure 2 Effects of **AR26** on the mitochondrial membrane potential ($\Delta\Psi m$) of *L. braziliensis* promastigotes. Promastigotes were untreated (control group) or treated with **AR26** (1.0 and 2.0 μg/ml) for 24 h, when they were probed with fluorescent probe Mitotracker® Red. Results were expressed as the percentage of $\Delta\Psi m$ reduction in comparison with untreated parasites. $P < 0.001$ (***) and $P < 0.01$ (**) showed significant difference compared with the control group.

Discussion

In the search for new potential drug targets to be employed in the treatment against leishmaniasis, in this study, the antileishmanial action of a series of **AR**-analogues, a class of bioactive compounds that has been already showed presenting distinct pharmacological activities, was exploited.[17,35] Among the tested compounds, the most prominent molecule was **AR26**, which exhibited a selective action against distinct *Leishmania* species, presenting IC_{50} values below of 3.0 μg/ml, and an activity in the treatment of *L. braziliensis*-infected macrophages, when IC_{50} value of 15.9 μg/ml against the intramacrophage amastigotes was obtained. Interestingly, **AR26** was more effective than resveratrol, which exhibited IC_{50} values of 75.13 μM (17.15 μg/ml) and 124.48 μM (28.41 μg/ml) against *L. braziliensis* promastigotes and intramacrophage amastigotes, respectively, after 72 h of treatment.[24] Trans-

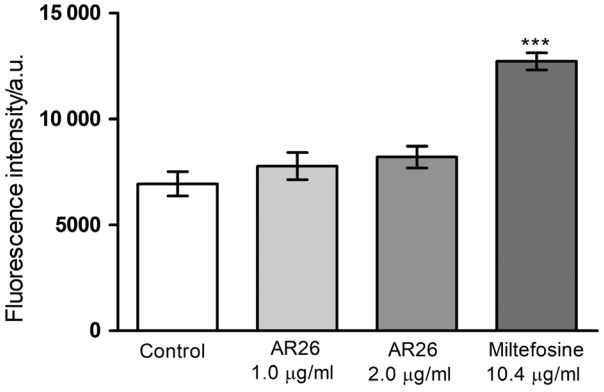


Figure 3 Reactive oxygen species (ROS) levels in **AR26**-treated *L. braziliensis* promastigotes. Parasites were untreated (control group) or treated with 1.0 or 2.0 μg/ml of **AR26** or miltefosine (10.4 μg/ml) for 24 h, when they were probed with H_2DCFDA. The fluorescence intensity was measured in a fluorimeter, and results were expressed in arbitrary units (A.U.) of the mean of three independent experiments. $P < 0.001$ (***) showed significant difference compared with the control group.

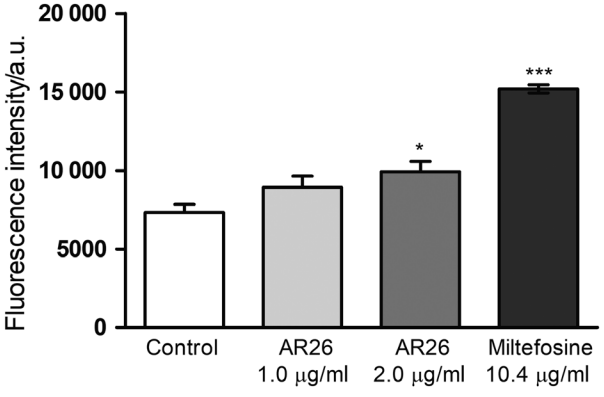


Figure 4 Evaluation of cell membrane integrity of **AR26**-treated *L. braziliensis* promastigotes. Parasites were untreated (control group) or treated with 1.0 or 2.0 μg/ml of **AR26** or miltefosine (10.4 μg/ml) for 24 h. Then, cells were loaded with propidium iodide (PI) and the fluorescence intensity was evaluated using a fluorimeter. $P < 0.001$ (***) and $P < 0.01$ (*) indicate statistically significant difference compared with untreated parasites.

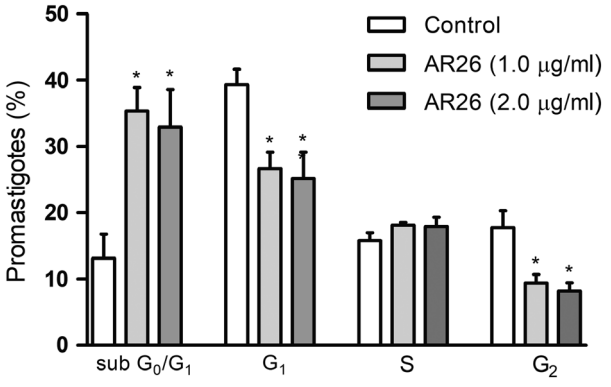


Figure 5 Analysis of the cell cycle progression of **AR26**-treated *L. braziliensis* promastigotes. Parasites were untreated or treated with **AR26** (1.0 or 2.0 μg/ml) for 24 h, when they were loaded with propidium iodide (PI), and the DNA content was evaluated by flow cytometry. The percentage of promastigotes in each phase of cell cycle was determined. $P < 0.01$ (*) indicates significant statistical difference in comparison to the control group.

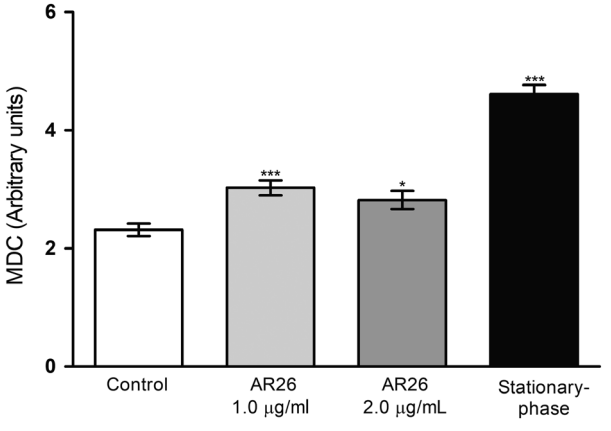


Figure 6 Detection of autophagic vacuole accumulation in **AR26**-treated *L. braziliensis* promastigotes. The formation of autophagic vacuoles in the treated promastigotes was evaluated by fluorimetry, after 24 h of incubation with **AR26** (1.0 or 2.0 μg/ml). Stationary promastigotes were used as positive control. $P < 0.001$ (***) and $P < 0.01$ (*) indicate significant statistical difference in comparison to the control group.

resveratrol analogues such as pterostilbene, piceatannol, polydatin and oxyresveratrol were assayed against *L. amazonensis* amastigotes, with IC_{50} values ranging from 6 μg/ml to 10 μg/ml (29–45 μM), below than that obtained with **AR26**.[22] As with these trans-resveratrol analogues, **AR26** did not show toxicity against murine macrophages, highlighting the selective effect of these resveratrol analogues. Resveratrol-treated *L. major* amastigotes revealed antileishmanial effect, but the authors point out that this biological activity and toxicity on mammalian cells are closed related to the host cell, and therefore, the results must be carefully analysed.[25] In addition to the antileishmanial activity, a recent study showed that **AR26** exhibited also promising *in vitro* anti-inflammatory, antioxidant and immunomodulatory properties, thus demonstrating the pharmacological potential of this molecule.[35,36]

In our study, we also advanced towards a better understanding regarding the distinct pathways of cell death, such as apoptosis-*like*, autophagy or even necrosis involved in the mode of action of this class of resveratrol analogues in *Leishmania*, and we evaluated the action of **AR26** against *L. braziliensis* promastigotes. Investigations into the mechanism of antileishmanial effect of resveratrol suggest disorders in the cell cycle of the parasite, reduction of the mitochondrial membrane potential and distinct morphological changes revealed by an irregular number of flagella and nuclei and alteration in cell shape. Moreover, its leishmanicidal effect is also dependent on the inhibition of proinflammatory cytokines and ROS production by murine-infected macrophages.[21] Also, compounds structurally related to resveratrol, such as stilbene-based compounds induced similar disorders, altering the cell cycle of the parasite and inducing depolarization of mitochondrial membrane potential in *L. amazonensis* promastigote forms and decreased ROS levels in *L. amazonensis*-infected macrophages.[22]

The single mitochondria of trypanosomatids parasites presents a peculiar ultrastructure and functional plasticity that makes this organelle an excellent indicator of cell irreversible dysfunction and, consequently, an attractive therapeutic target for new therapeutic strategies.[37,38] A critical event for the monitoring of the proper functioning of the mitochondrial respiratory chain of parasite is the determination of the mitochondrial membrane potential ($\Delta\Psi m$).[39,40] Corroborating with this hypothesis, a significant reduction in $\Delta\Psi m$ was detected to parasites exposed to **AR26**, suggesting that this molecule directly acted causing mitochondrial dysfunction.

The drastic alterations in the bioenergetics metabolism of parasites and the resulting impairment in the functioning of respiratory chain as a consequence of drug treatment could strongly contribute to the excessive generation of intramitochondrial ROS levels. In turn, such toxic products, when in high levels, present a potent ability to promote multifactorial cellular responses, causing cell damage in proteins, DNA and lipids, thus culminating in the activation of cell death by distinct pathways.[41,42] However, in this study, no increase in the ROS production was detected in **AR26**-treated parasites, suggesting the regulation of ROS levels by the antioxidant metabolism of the parasites.

To investigate whether the **AR26** caused damage to the integrity of the plasma membrane, an event clearly associated with cell death by necrosis, a staining with PI was performed. Results showed that the treatment of

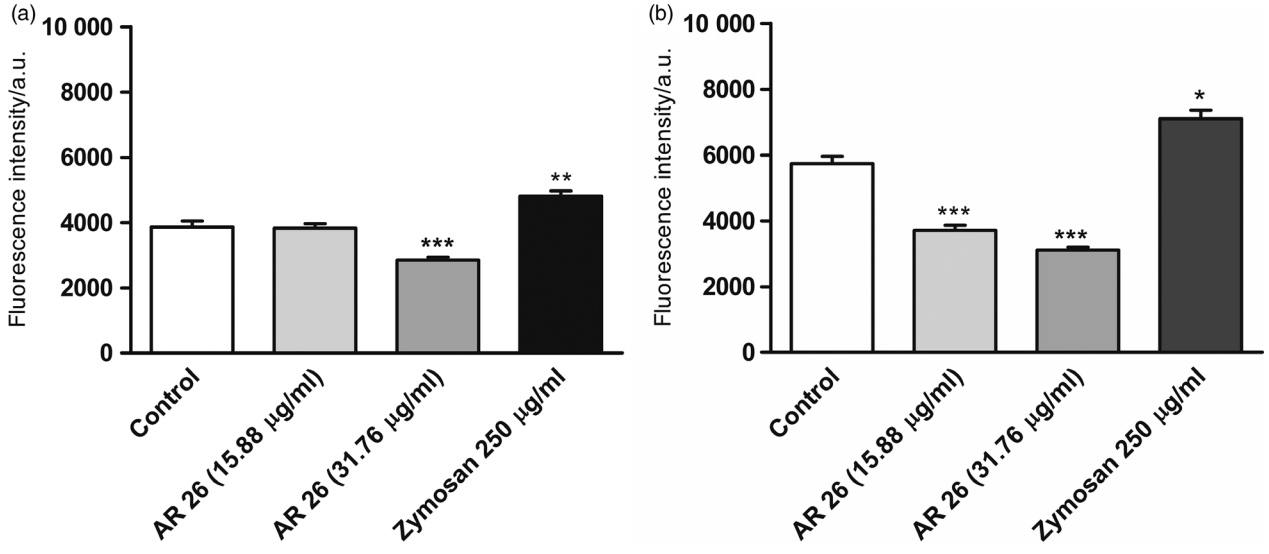


Figure 7 Production of reactive oxygen species in *L. braziliensis*-infected macrophages and treated with **AR26**. Macrophages that were infected (a) or not (b) with *L. braziliensis* were treated with **AR26** at 15.88 or 31.76 μg/ml, or with amphotericin B (AmB, 0.069 μg/ml) or miltefosine (1.3 μg/ml), used as control. After 24 h, ROS levels were evaluated by incubation with H_2DCFDA (20 μM) for 30 min in the dark. The fluorescence intensity was measured in arbitrary units (a.u.). Zymosan (250.0 μg/ml) was used as a positive control. $P < 0.001$ (***), $P < 0.01$ (**), and $P < 0.05$ (*) indicate statistical difference in comparison to the control group.

promastigotes with **AR26** resulted in simultaneous impairment of plasma membrane integrity. Such alterations directly affected the pathway of biosynthesis of sterols in the parasites, transport of nutrients and ions as well as their lipid and protein composition; culminating to the deleterious effects in the growth and viability of these cells.[43,44]

An intense dysregulation in the cell cycle progression was also evidenced in **AR26**-treated parasites, since a marked increase in the level of subG_0/G_1 phase cells with concomitant decrease of the G_0/G_1 and G_2/M phases´ cell population were encountered. The cell cycle arrest in the subG_0/G_1 peak is suggestive of parasite population with low DNA content, thus indicating the occurrence of apoptotic-like death.[45] In accordance with our findings, previous reports using resveratrol and their analogues have also showed increase in the promastigotes population in the sub-G_0/G_1 phase of their cell cycle, culminating with collapse of the mitochondrial membrane potential.[21,22,46] Moreover, studies confirmed that resveratrol displays its antiproliferative action in tumour cell lines by activating cell cycle arrest, reinforcing that this molecule class has common targets in different biological targets.[16,47]

Autophagy has been also showed as a mechanism of action of molecule classes presenting antileishmanial action.[48] Autophagy is a catabolic process of degradation and recycling of injured organelles and cytosol components, where cellular homoeostasis is ruptured in stress conditions, such as nutrient deprivation, infection, exposition to drugs and toxins, among others.[49–51] In trypanosomatids, autophagy is a biological process required for the maintenance of parasite life cycle, contributing to the regulation of cell density in the vectors and mammalian hosts, thus responding to the cell differentiation and modulation of host immunity.[51] Despite its protective role, the exacerbation of this autophagic pathway can also lead to regulated cell death.[37,52] To investigate the activation of this pathway of cell death caused by **AR26**, the accumulation of autophagic vacuoles was evaluated by labelling with MDC. Evidences of autophagic death were observed in the *L. braziliensis* promastigotes that were exposed to **AR26**, thus indicating that the autophagic responses could be an attempt to recover the cell homoeostasis, resulting from extensive damage in the parasite cellular processes. Corroborating with this hypothesis, studies have pointed to the action of resveratrol in the cell death by autophagy also in tumour cell lines.[16,53,54]

The action of antileishmanial molecules against parasites inside the host cell can occur as a direct effect of drug about the parasites or as a consequence of the activation of microbicidal mechanisms of host cell.[55,56] To explore the mode of action of **AR26** against intracellular amastigotes, the ROS production in treated and infected macrophages was analysed and, interestingly, the ROS production was decreased in macrophages that were infected with *L. braziliensis* promastigotes and treated with the molecule, thus suggesting its antioxidant action. Corroborating our

results, studies also showed that resveratrol and its analogues exert their antileishmanial action by suppressing the NO and ROS production in murine macrophages.[21,22] Our findings are also consistent with such works, which corroborated with the results described here.

Conclusions

In summary, this study presents a new class of resveratrol analogues based on N-aryl imines, which showed selective antileishmanial action against distinct *Leishmania* species. The evaluation of the mechanism of action of the most effective compound, **AR26**, suggested that this molecule is involved in multiple biochemical and cellular alterations in the parasites, including disruption of mitochondrial function causing $\Delta\Psi m$ depolarization, plasma membrane permeabilization and interference in the progression of the cell cycle. Therefore, data showed here encourages the development of future studies to evaluate **AR26** as an antileishmanial agent against *Leishmania* infection in mammalian hosts.

Declarations

Conflicts of interest

The authors confirm that they have no conflicts of interest in relation to this work.

Acknowledgements

The authors would like to acknowledge the 'Programa de Pós-Graduação em Ciências Biológicas (Universidade Federal de Juiz de Fora)' for allowing the use of the FACsCanto II flow cytometer multiuser equipment facility. This work received financial support from FAPEMIG and CNPq. LMRA, ADS and EAFC, and ESC receive grants from CNPq.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Spectrometric data.