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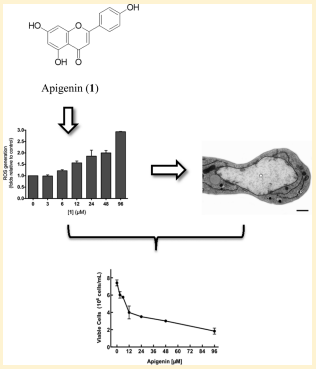
Effect of Apigenin on *Leishmania amazonensis* Is Associated with Reactive Oxygen Species Production Followed by Mitochondrial Dysfunction

Fernanda Fonseca-Silva,[†] Marilene M. Canto-Cavalheiro,[†] Rubem F. S. Menna-Barreto,[‡] and Elmo E. Almeida-Amaral*,[†]

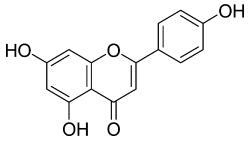
[†]Laboratório de Bioquímica de Tripanosomatídeos and [‡]Laboratório de Biologia Celular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Av. Brasil 4365, Manguinhos, Rio de Janeiro, Brazil

ABSTRACT: Leishmaniasis is an important neglected disease caused by protozoa of the genus *Leishmania* that affects more than 12 million people worldwide. Leishmaniasis treatment requires the administration of toxic and poorly tolerated drugs, and parasite resistance greatly reduces the efficacy of conventional medications. Apigenin (**1**), a naturally occurring plant flavone, has a wide range of reported biological effects. In this study, antileishmanial activity of **1** in vitro was investigated, and its mechanism of action against *Leishmania amazonensis* promastigotes was described. Treatment with **1** for 24 h resulted in concentration-dependent inhibition of cellular proliferation (IC_{50} = 23.7 μM) and increased reactive oxygen species (ROS) generation. Glutathione and *N*-acetyl-L-cysteine protected *L. amazonensis* from the effects of **1** and reduced ROS levels after the treatment. By contrast, oxidized glutathione did not reduce the levels of ROS caused by **1** by not preventing the proliferation inhibition. Apigenin **1** also induced an extensive swelling in parasite mitochondria, leading to an alteration of the mitochondrial membrane potential, rupture of the *trans*-Golgi network, and cytoplasmic vacuolization. These results demonstrate the leishmanicidal effect of **1** and suggest the involvement of ROS leading to mitochondrial collapse as part of the mechanism of action.



Leishmaniasis is an important neglected disease caused by protozoa of the genus *Leishmania* that affects more than 12 million people worldwide; 350 million people are considered at risk of contracting leishmaniasis, and some 2 million new cases occur yearly.[1,2] The severity of disease caused by various *Leishmania* species varies widely, ranging from cutaneous and/or mucosal to visceral infection. Leishmaniasis treatment requires the administration of toxic and poorly tolerated drugs, such as pentavalent antimonials, the first-line compounds used to treat leishmaniasis, pentamidine, amphotericin B, and miltefosine. However, the parasite resistance greatly reduces the efficacy of conventional medications.[3,4]

Natural sources such as plants are a major resource for the discovery of new lead molecules for neglected diseases.[5] Isolated compounds, including some flavonoids, have been reported to present significant antiprotozoal activities.[6−12] Apigenin (**1**), a naturally occurring plant flavone that is abundantly distributed in common fruits and vegetables and is recognized as a bioactive flavonoid, has a wide range of reported biological effects, including antioxidant, antihypertensive, anti-inflammatory, antimicrobial, and antiprotozoal activities.[13,14]



In this study, antileishmanial activity of **1** in vitro and its mechanism of action against *Leishmania amazonensis* promastigotes are described. Compound **1** inhibited promastigote proliferation in a concentration-dependent manner. This leishmanicidal activity was reactive oxygen species (ROS)-dependent and promoted mitochondrial dysfunction, culminating in parasite death.

RESULTS AND DISCUSSION

Antiparasitic activity of **1** against *L. donovani*, *Trypanosoma brucei rhodesiense*, *T. cruzi*, *Encephalitozoon intestinalis*, and *Cryptosporidium parvum* has been described.[15,16] To determine the effect of **1** on the proliferation of *L. amazonensis*, promastigotes were incubated with different concentrations of **1** (3−96 μM) for 24 h. Concentration-dependent inhibition of cellular proliferation was observed ($p < 0.001$), with an IC_{50} of 23.7 μM (Figure 1A). Compound **1** (96 μM) inhibited 75% of the proliferation of *L. amazonensis* after 24 h.

To confirm the inhibition of the cellular proliferation of *L. amazonensis* by **1**, the stable intracytoplasmic dye CFSE (5,6-carboxyfluorescein succinimidyl ester) was used to evaluate cellular proliferation by measuring the relative fluorescence intensity by flow cytometry.[17,18] The CFSE stain forms stable conjugates with aliphatic amines in the cytoplasm.[19] A remarkable decrease in the CFSE fluorescence of untreated promastigotes was observed after 24 h (Figure 1B), indicating

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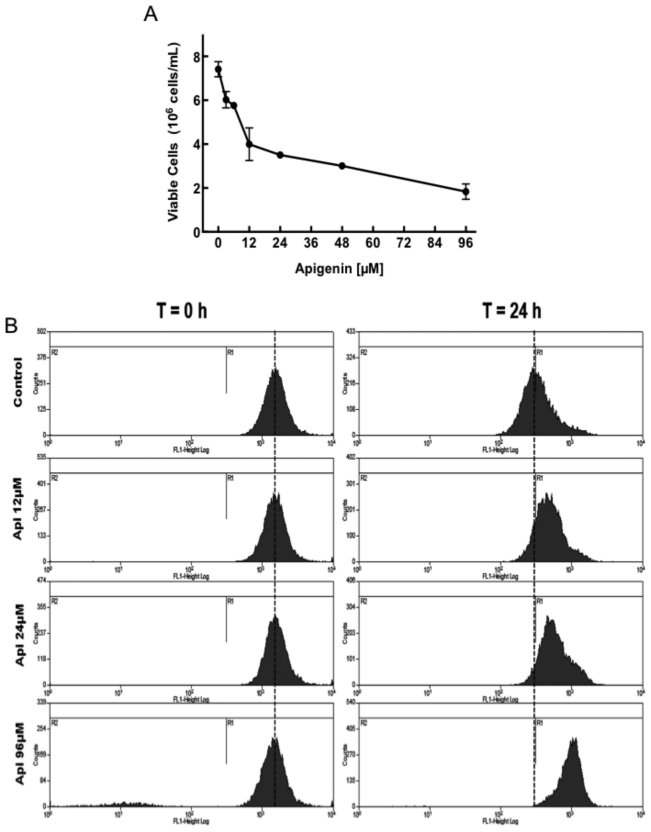

Figure 1. Effect of apigenin (**1**) on the cellular proliferation of *L. amazonensis*. Promastigotes were cultivated in Schneider's *Drosophila* medium at 26 °C for 24 h in the absence or presence of **1** (3−96 μM). Cellular proliferation was determined by direct counting of the number of parasites using a Neubauer chamber (A) or using the stable intracytoplasmic dye CFSE (B). In the control (absence of **1**), the same volume of dimethylsulfoxide (solvent of **1**) was added to the growth medium. The values are presented as the mean ± standard error of three different experiments. The histograms are representative of three independent experiments.

cell division. This decrease in CFSE fluorescence was inhibited in a concentration-dependent manner by **1** (12 , 24 , and 96 μM), clearly indicating inhibition of cell division. These results demonstrate the antileishmanial activity of **1** against promastigote forms. Similar effects have been observed for others flavonoids, such as quercetin and (−)-epigallocatechin 3-*O*-gallate, which inhibit the cellular proliferation of *L. amazonensis* in a concentration-dependent manner.[9,10] However, compound **1** was more potent than quercetin (−)-epigallocatechin 3-*O*-gallate, which demonstrated an IC_{50} of 31.4 μM at 48 h and an IC_{50} of 63 μM at 120 h.

Although flavonoids are known to exhibit antioxidant properties, some studies have demonstrated pro-oxidant activities in vitro.[10−12,20] Treatment with **1** for 24 h increased ROS generation in *L. amazonensis* in a concentration-dependent manner ($p < 0.01$) (Figure 2). ROS levels in *L. amazonensis* treated with **1** (96 μM) were 2.9-fold higher than those of the control. ROS production in a concentration-dependent manner has also been reported for the exposure of *L. amazonensis* and *L. braziliensis* promastigotes to quercetin[10] and (−)-epigallocatechin 3-*O*-gallate,[12] respectively. This ROS production induced a severe reduction in the number of parasites. ROS levels were 4.4-fold higher with 96 μM quercetin at 48 h and 2.9-fold higher with 500 μM (−)-epigallocatechin 3-*O*-gallate at 72 h.

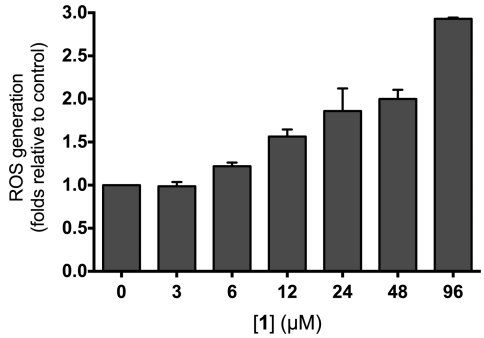

Figure 2. ROS production in *L. amazonensis* treated with **1**. Promastigotes cultivated in Schneider's *Drosophila* medium at 26 °C for 24 h in the absence or presence of **1** (3−96 μM). ROS was measured using the fluorescent dye H_2DCFDA as described in the Experimental Section. The data are expressed as the fold increase in ROS production relative to the control. The values presented are the mean ± standard error of three different experiments.

In *L. amazonensis*, reduced glutathione (GSH) is a component of trypanothione, a major antioxidant of this parasite,[21] and intracellular GSH levels can be increased by treatment with the antioxidant *N*-acetyl-L-cysteine (NAC).[22] To confirm that the inhibitory effects of **1** are mediated by ROS production, *L. amazonensis* promastigotes were preincubated with GSH or NAC (300 μM). GSH and NAC protected *L. amazonensis* from the effects of **1** (Figure 3A) and reduced ROS levels in cells treated with **1** (Figure 3B). By contrast, oxidized glutathione (GSSG) did not reduce ROS levels and did not protect against the proliferation inhibition caused by **1**, suggesting ROS production as a possible mechanism for the induction of *L. amazonensis* promastigote death. This mechanism is the basis of various antiprotozoal medications used to combat parasites in infected cells.[23]

Damage to the mitochondrial membrane potential ($\Delta\Psi_m$) can be induced by the direct addition of ROS in vitro or the induction of ROS production by chemical agents.[24,25] Transmission electron microscopy images of untreated promastigotes (panels A and B) and promastigotes treated with **1** for 24 h (12 μM, panels C and D; 24 μM, panels E and G; and 96 μM, panel H) are shown in Figure 4. The treatment induced extensive swelling of the parasite mitochondria (Figure 4C−G; white stars), and the inner mitochondrial membrane was severely damaged, with a washed-out matrix appearance and decreased matrix electron density (Figure 4C−G). Concentric membrane structures were also observed inside the organelle (Figure 4C,E,F; black arrow). Treatment with 12 μM **1** (Figure 4D) also led to the appearance of endoplasmic reticulum (ER) profiles surrounding the cellular structures and cytosolic vacuolization (V). The highest concentration employed (96 μM) strongly affected the mitochondrial ultrastructure (white star), resulting in a washed-out appearance of this organelle matrix and loss of cristae (Figure 4H). In addition, rupture of the *trans*-Golgi network (Figure 4E,G; white asterisk) and cytoplasmic vacuolization (Figure 4D) were also observed.

To confirm that **1** induces an alteration in the mitochondria, the parasite $\Delta\Psi_m$ was measured using JC-1, a cationic mitochondrial vital dye. This dye is lipophilic and concentrates in mitochondria in proportion to the membrane potential; increased dye accumulation is observed in mitochondria with greater $\Delta\Psi_m$. The spectrofluorometric data presented in

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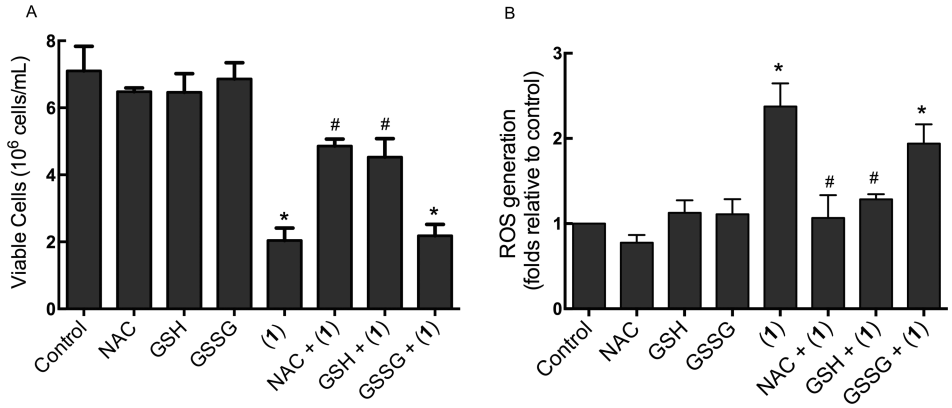

Figure 3. Effect of thiol antioxidants on apigenin-induced inhibition of cellular proliferation (A) and ROS generation (B). *L. amazonensis* was cultivated in Schneider's *Drosophila* medium at 26 °C as described in the Experimental Section for 24 h in the presence of *N*-acetyl-L-cysteine (NAC), reduced glutathione (GSH), or oxidized glutathione (GSSG) in the absence or presence of **1**. NAC, GSH, or GSSG was solubilized in phosphate buffered saline and added to the culture at a final concentration of 300 μM. Compound **1** was solubilized in dimethylsulfoxide (DMSO) and added to the culture at a final concentration of 96 μM. The values shown are the mean ± standard error of three different experiments. In the control (absence of **1**), the same volume of vehicle (0.2% DMSO) was added to the growth medium. ROS generation was measured using the fluorescent dye H_2DCFDA as described in Experimental Section. The data are expressed as the fold increase in ROS production relative to the control. The values shown are the mean ± standard error of three different experiments. CTRL, control; *indicates significant difference relative to the control group ($p < 0.05$); #indicates significant difference relative to the **1**-treated group ($p < 0.05$).

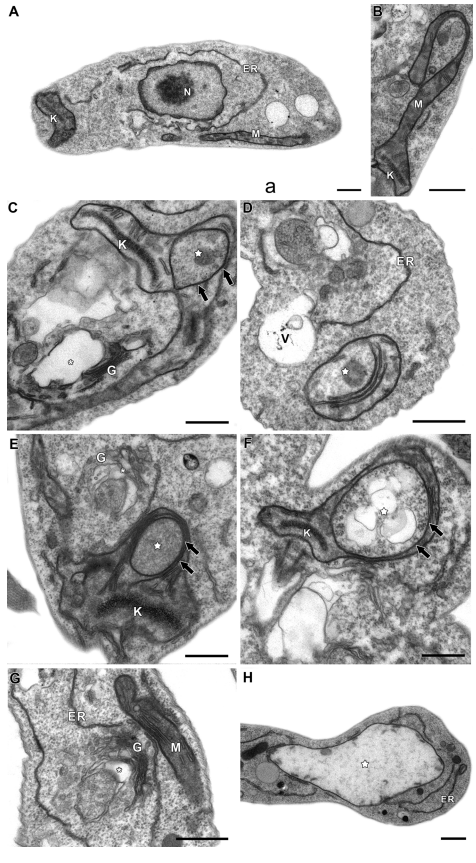

Figure 4. Ultrastructural alterations in *L. amazonensis* promastigotes treated with compound **1**. Untreated parasites (A,B) presented typical elongated morphology with normal kinetoplast (K), mitochondria (M), endoplasmatic reticulum (ER), and nucleus (N). Promastigotes treated with 12 μM (C,D), 24 μM (E–G), or 96 μM (H) **1** exhibited extensive swelling of the parasite mitochondria, with a washed-out matrix appearance (white stars) and the presence of concentric membrane structures inside the organelle (black arrow). Rupture of the *trans*-Golgi network (white asterisk) and cytoplasmic vacuolization (V) were observed. All scale bars correspond to 0.5 μm.

Figure 5A indicate a marked concentration-dependent decrease in the relative fluorescence intensity ($\Delta\Psi_m$ values) ($p < 0.001$). These results indicate membrane potential depolarization in cells upon treatment with **1** (3–96 μM). $\Delta\Psi_m$ was reduced by 78.3% upon treatment with 96 μM compound **1**. Decreased relative fluorescence intensity values were also observed following treatment with 20 μM FCCP (79.8% reduction). A linear correlation (R^2 = 0.9325) was observed between the alteration of $\Delta\Psi_m$ and inhibition of *L. amazonensis* proliferation by **1** (Figure 5B). Therefore, we suggest that **1** exerts its antileishmanial effect on *L. amazonensis* via ROS production followed by a loss of $\Delta\Psi_m$.

The mitochondria of *Leishmania* spp. are an exceptional chemotherapeutic target due to their unique structure, which is functionally distinct from that of mammalian mitochondria. Ultrastructural disorganization of the mitochondria was observed in promastigotes treated with **1**, confirmed by the decrease in $\Delta\Psi_m$.

Mitochondria are responsible for respiration and oxidative phosphorylation in eukaryotic cells, including trypanosomatides, and they provide ATP via respiratory-coupled oxidative phosphorylation. During oxidative phosphorylation, electrons are transported through the mitochondrial respiratory chain, and a proton gradient is established across the inner mitochondrial membrane as the energy source for ATP production.[26] The observed decrease in $\Delta\Psi_m$ suggests increased proton permeability across the inner mitochondrial membrane, which would lead to a decrease in ATP synthesis and, ultimately, lead to parasite death. Ultrastructural alterations and collapse of $\Delta\Psi_m$ have been observed in *L. amazonensis*,[10,27] *L. donovani*,[28−30] and *T. cruzi*[31−33] after treatment with various drugs.

In conclusion, these results demonstrate the leishmanicidal effect of apigenin (**1**) and suggest the involvement of ROS leading to mitochondrial collapse as part of the mechanism of action. These results support **1** as a possible candidate for leishmaniasis chemotherapy. Further studies are needed to confirm this effect in a murine experimental model of leishmaniasis infection.

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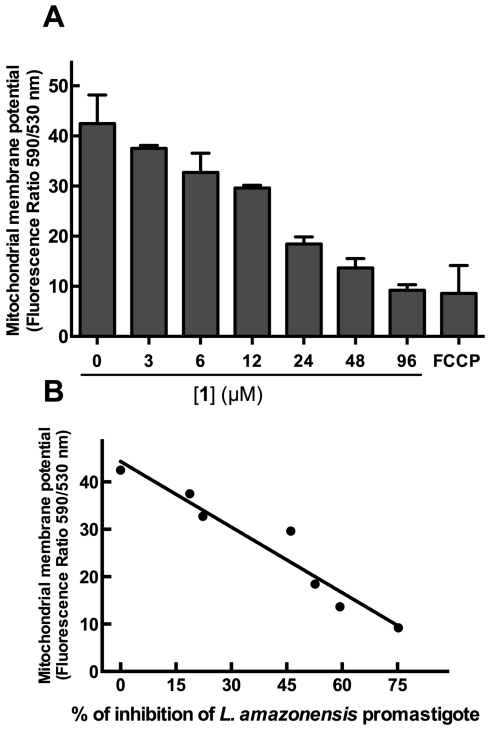

Figure 5. Evaluation of the mitochondrial membrane potential of *Leishmania amazonensis* promastigotes treated with **1**. *Leishmania amazonensis* was cultivated in Schneider's *Drosophila* medium at 26 °C for 24 h in the absence or presence of **1** (3−96 μM). Promastigotes were labeled with the potentiometric probe JC-1 (10 μg/mL) (A). The positive control was treated with FCCP (20 μM) for 20 min. In the control (absence of **1**), the same volume of vehicle (0.2% DMSO) was added to the growth medium. Concentration-dependent alterations in relative $\Delta\Psi_m$ values are expressed as the ratio of the fluorescence measurements at 590 nm (for J-aggregate) versus 530 nm (for J-monomer). The data are expressed as the means ± standard errors of three different experiments. (B) Correlation between the alteration of $\Delta\Psi_m$ and inhibition of *L. amazonensis* proliferation by **1** (R^2 = 0.9325).

EXPERIMENTAL SECTION

Test Compound and Reagents. Apigenin (**1**) (100% purity; lot 081M1457 V), Schneider's *Drosophila* medium, fetal calf serum, RPMI-1640 medium, penicillin, streptomycin, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylimidacarbocyanine iodide), *N*-acetyl-L-cysteine, reduced glutathione, oxidized glutathione, glutaraldehyde, sodium cacodylate, osmium tetroxide, potassium ferricyanide, uranyl acetate, and FCCP [carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone] were obtained from Sigma-Aldrich (St. Louis, MO, USA). H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) and 5,6-carboxyfluorescein succinimidyl ester were obtained from Invitrogen Molecular Probes (Leiden, The Netherlands). All other reagents were purchased from Merck (São Paulo, Brazil). Deionized distilled water obtained using a Milli-Q system (Millipore Corp., Bedford, MA, USA) was used to prepare all solutions. Apigenin **1** was prepared in DMSO and diluted in culture medium such that the solvent concentration did not exceed 0.2% in the final solution.

Parasites. The MHOM/BR/75/LTB0016 strain of *L. amazonensis* was used throughout this study. This strain was isolated from a human case of cutaneous leishmaniasis in Brazil. Promastigotes were cultivated at 26 °C in Schneider medium (pH 7.2) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% (v/v) heat-inactivated fetal calf serum.

Evaluation of Cellular Proliferation Using a Neubauer Chamber. Promastigotes were seeded into fresh medium (1.0 mL final volume) either in the absence (2 μL of DMSO) or the presence of various concentrations of **1** (2 μL; 3−96 μM). The cells were maintained for 24 h at 26 °C. Cell density was estimated using a Neubauer chamber. The proliferation curve was initiated with 1.0×10^6 cells/mL. The 50% inhibitory concentration (IC_{50}) was determined by logarithmic regression analysis using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

Evaluation of Cellular Proliferation Using CFSE. Promastigotes (2.5×10^7 cells/mL) were washed three times with phosphate buffered saline (PBS) and resuspended in PBS with albumin (0.1%) and CFSE (10 μM). The parasites were incubated at 26 °C for 15 min, during which they were carefully mixed three times. Staining was stopped by the addition of ice-cold medium. Stained promastigotes were centrifuged at 1500*g* for 15 min at 4 °C and resuspended in fresh medium (1.0×10^6 cells/mL) either in the absence (0.2% DMSO) or the presence of various concentrations of **1** (3−96 μM). The cells were maintained for 24 h at 26 °C. Data acquisition and analysis were performed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with Cell Quest software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA). A total of 10 000 events were acquired in the region previously established as corresponding to the parasites.

Measurement of ROS Levels. Intracellular ROS levels in promastigotes treated with **1** or untreated were measured using the cell-permeable dye H_2DCFDA. Promastigotes (5×10^6 cells/mL) were cultured for 24 h in the absence or presence of **1** (3−96 μM), harvested, and resuspended in Hanks' balance salt solution (HBSS), and 1×10^7 cells/mL were incubated with H_2DCFDA (20 μM) for 20 min at 26 °C. Data acquisition and analysis were performed by flow cytometry as described above.

Transmission Electron Microscopy Analysis. Promastigotes (5×10^6 cells/mL) were treated with **1** (12−96 μM) for 24 h in Schneider medium at 26 °C. After being washed with PBS, the parasites were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature for 40 min and postfixed in a solution of 1% osmium tetroxide, 0.8% potassium ferricyanide, and 2.5 mM $CaCl_2$ for 20 min. The cells were dehydrated in an acetone series and embedded in a PolyBed 812 resin.[34] Ultrathin sections were stained with uranyl acetate and lead citrate and examined using a JEOL 1011 transmission electron microscope (Tokyo, Japan) in the Plataforma de Microscopia Eletrônica, IOC, Fiocruz.

Determination of $\Delta\Psi_m$. The cationic probe JC-1 was used to determine the $\Delta\Psi_m$ as described.[10] Promastigotes (5×10^6 cells/mL) were cultured for 24 h in the absence or presence of **1** (3−96 μM). Cells were harvested and resuspended in HBSS. The cell number was obtained via counting in a Neubauer chamber. Promastigotes (1×10^7 cells/mL) were incubated with JC-1 (10 μg/mL) for 10 min at 26 °C. After being washed twice with HBSS, fluorescence was measured spectrofluorometrically at 530 and 590 nm using an excitation wavelength of 480 nm. The ratio of values obtained at 590 and 530 nm was plotted as the relative $\Delta\Psi_m$. The mitochondrial uncoupling agent carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (20 μM) was used as a positive control.

Statistical Analysis. All experiments were performed in three independent trials. The data were analyzed using Student's *t* test or analysis of variance (ANOVA) followed by Bonferroni's post-test in GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). The results were considered significant when $p \leq 0.05$. The data are expressed as the mean ± standard error.

AUTHOR INFORMATION

Corresponding Author

*E-mail: elmo@ioc.fiocruz.br. Tel: +55(21)3865-8220. Fax: +55(21)3865-8200.

Notes

The authors declare no competing financial interest.

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