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Ultrastructural and morphological changes in *Leishmania (Viannia) braziliensis* treated with synthetic chalcones

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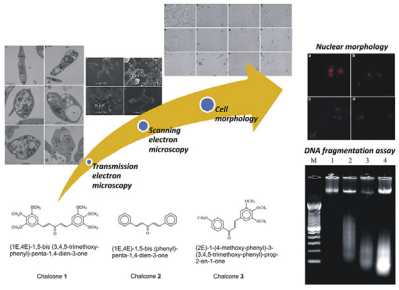
HIGHLIGHTS

- Chalcones 1–3 induced mitochondrial changes in promastigotes of *L. (V.) braziliensis*.
- Synthetic chalcones 1–3 induced rounded shape in *L. (V.) braziliensis*.
- Chalcones 1–3 showed cytoplasmic and nuclear shrinkage in *L. (V.) braziliensis*.
- Chalcones 1–3 exhibited nucleosome-sized DNA fragments in *L. (V.) braziliensis*.
- Chalcones induced changes in promastigotes suggesting apoptosis-like cell death.

GRAPHICAL ABSTRACT



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ABSTRACT

Cutaneous leishmaniasis has an estimated incidence of 1.5 million new cases per year and the treatment options available are old, expensive, toxic, and difficult to administer. Chalcones have shown good activity against several species of *Leishmania*. However few studies have discussed the mechanisms of action and drug target of this group of compounds in *Leishmania*. The synthetic chalcones that were evaluated in the present study were previously shown to exhibit activity against *Leishmania (Viannia) braziliensis*. The objective of the present study was to identify ultrastructural and morphological changes in *L. (V.) braziliensis* after treatment with three synthetic chalcones (1–3). Promastigotes were treated with chalcones 1–3 and evaluated by transmission and scanning electron microscopy. Cellular and nuclear morphology of the parasites, changes in membrane permeability, and DNA fragmentation in agarose electrophoresis gel were also investigated after exposure to synthetic chalcones. All three synthetic chalcones (1–3) induced ultrastructural alterations in mitochondria, intense vacuolization, two nuclei with rounding of parasites, and cellular and nuclear shrinkage. Chalcones 1–3 also induced no changes in membrane permeability, and presence of nucleosome-sized DNA fragments. Synthetic chalcones 1–3 induced ultrastructural and morphological changes, suggesting that chalcones 1–3 induce

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apoptosis-like cell death. Further studies should be conducted to elucidate other aspects of the action of these chalcones against *Leishmania* spp. and their use for the treatment of cutaneous leishmaniasis.

1. Introduction

Leishmaniasis is caused by bites from infected female phlebotomine sandflies with over 20 species of *Leishmania* involved as reported by World Health Organization (2014). Leishmaniasis has an estimated prevalence of 2 million new cases per year, with 1.5 million cases of the cutaneous form of the disease (WHO, 2010). Leishmaniasis has three forms: cutaneous, mucocutaneous, and visceral (WHO, 2014). The majority of reported cases of cutaneous leishmaniasis were in Afghanistan, Algeria, Brazil, Colombia, Iran, Pakistan, Peru, Saudi Arabia, and Syria (WHO, 2014). *Leishmania (Viannia) braziliensis* is distributed throughout most of Latin America and leads to both cutaneous and mucosal forms of the disease (WHO, 2010). Leishmaniasis is a neglected disease and has few investments in treatment. Currently, the options for the treatment of cutaneous leishmaniasis are pentavalent antimonials, amphotericin B, pentamidine and paromomycin (WHO, 2010). However, these drugs are toxic and have been used for a long time, thus enabling parasite resistance (Santos et al., 2008). Additionally, long treatment durations are required.

Chalcones have been studied in several species of *Leishmania* (Andrighetti-Fröhner et al., 2009; Chen et al., 1993; Piñero et al., 2006; Quintin et al., 2009). The structure of chalcones consists of two aromatic rings that are joined by a three-carbon unsaturated chain and a carbonyl group. Change in the substituents of the aromatic ring results in alterations in the activity of these compounds. It has been shown that some chalcones act on mitochondria of the parasite (Chen et al., 2001; Torres-Santos et al., 1999). Although several studies report the leishmanicidal activity of chalcones, few studies have demonstrated the likely mechanisms of action of these compounds against protozoa of the genus *Leishmania*.

Our group recently reported that three synthetic chalcones showed activity against promastigotes and amastigotes of *L. (V.) braziliensis*, and low toxicity for macrophages and red blood cells (de Mello et al., 2014). These chalcones stimulated nitric-oxide production by infected macrophages in the initial phase of infection and direct effect on protozoa can occur in the late phase of infection (de Mello et al., 2014). Knowledge about effects and mechanism of action of these three chalcones at *Leishmania* spp. can be useful for the development of new therapeutic strategies for leishmaniasis. The objective of the present study was to identify ultrastructural and morphological changes in *L. (V.) braziliensis* after treatment with these three synthetic chalcones (compounds 1–3).

2. Materials and methods

2.1. Chemicals

The chemical structures of the synthetic chalcones are shown in Fig. 1. Three synthetic chalcones (1–3) were synthesized in the laboratory of the Faculdade de Química, Universidade Federal do Pará, Belém, Brazil. The synthesis of chalcones 1 and 2 and chalcone 3 was previously described (Bitencourt et al., 2007; Franco et al., 2012). In all of the experiments, the chalcones were solubilized in dimethylsulfoxide (DMSO; Sigma Aldrich, St. Louis, MO, USA) to obtain a 1 mg/ml stock solution. The chalcones were then diluted in culture medium to a previously determined inhibitory concentration for 50% of promastigotes (IC_{50}) (de Mello et al., 2014). The DMSO concentration did not exceed 0.2% in the experiments.

2.2. Parasite cultures

L. (V.) braziliensis (MHOM/BR/1987/M11272) was maintained in hamsters by the inoculation of 10^7 parasites into a footpad. The animals were euthanized under deep anesthesia (xylazine [Rompum] and ketamine chlorohydrate [Ketamina]), and fragments of the popliteal lymph node were aseptically inoculated into culture medium 199 (Gibco, New York, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco, New York, NY, USA), 1% human urine, 2 mM L-glutamine (Sigma Aldrich Chemie, Steinheim, Germany), 100 UI/ml penicillin G (Sigma Aldrich Chemie, Steinheim, Germany), and 100 μg/ml streptomycin sulfate (Sigma Aldrich, St. Louis, MO, USA) at 25 °C (pH 7.2). Parasites were maintained by periodic subculture. To all experiments, promastigotes of *L. (V.) braziliensis* were treated with synthetic chalcones 1 (1.38 μM), 2 (5.88 μM), and 3 (5.69 μM).

2.3. Transmission electron microscopy

To assay ultrastructural changes induced by the synthetic chalcones, *L. (V.) braziliensis* (1×10^6 promastigotes/ml) were treated with chalcones for 24 h and fixed with 2.5% glutaraldehyde. The parasites were then post-fixed with 2% osmium tetroxide, dehydrated in acetone, embedded in Epon 812. Ultrathin sections were contrasted with 2% uranyl acetate and lead acetate. Transmission electron microscopy (TEM) was performed with a JEOL 1200EX II transmission electron microscope.

2.4. Scanning electron microscopy

To evaluate morphological changes, promastigotes of *L. (V.) braziliensis* were tested with chalcones for 24 h incubation. The parasites were fixed in 2.5% glutaraldehyde, dehydrated in a series of acetone, subjected to critical point drying in a Bal-Tec CPD-030, metallised in gold in a Balzers SCD-030. Scanning electron microscopy (SEM) was performed with a JEOL JSM-6360 LV scanning electron microscope.

2.5. Cell and nuclear morphology

To observe changes in the morphology of the parasites induced by the synthetic chalcones, tests were performed as previously described (Das et al., 2001). Briefly, *L. (V.) braziliensis* (1×10^7 promastigotes/ml) was incubated with the synthetic chalcones for 0, 4, 6, 8, 24, 48, 72, and 96 h. Observations were performed with a Floid Cell Imaging Station microscope (Molecular Probes Life Technology, Carlsbad, CA, USA), and at least 200 cells were observed in each condition.

The detection of nuclear morphological changes was based on a previous study (Das et al., 2001). Briefly, *L. (V.) braziliensis* (1×10^7 promastigotes/ml) was treated with the synthetic chalcones. After 24 h incubation, promastigotes were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100 for