Chemico-Biological Interactions 256 (2016) 16–24

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

C5 induces different cell death pathways in promastigotes of *Leishmania amazonensis*

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ARTICLE INFO

Article history:
Received 20 January 2016
Received in revised form
16 May 2016
Accepted 14 June 2016
Available online 16 June 2016

Keywords:
Leishmania amazonensis
β-carboline
Oxidative damage
Cell death

ABSTRACT

Leishmaniasis is a neglected infection that is caused by *Leishmania* protozoa, affecting millions of people worldwide, mainly in tropical and subtropical regions. This disease has different clinical forms: cutaneous, mucocutaneous, and visceral. The drugs that are currently available for the treatment of this infection have limitations, such as high toxicity, long-term treatment, and leads to drug-resistant strains. Numerous studies, in various experimental models, have sought to develop more effective and less toxic chemotherapeutic agents against leishmaniasis. In the present study, we evaluated the mechanism of cell death that is induced by *n*-benzyl 1-(4-methoxy)phenyl-9H-β-carboline-3-carboxamide (**C5**) against *Leishmania amazonensis*. **C5** increased reactive oxygen species production, depolarization of the mitochondrial membrane, DNA fragmentation, decrease of cell volume, lipoperoxidation, the accumulation of lipid bodies, and acidic vesicular organelles (AVOs) and caused the intense formation of autophagic compartments in *L. amazonensis* promastigotes. The results indicate that **C5** causes *L. amazonensis* death through different pathways.

1. Introduction

Leishmaniasis is an infection that is caused by *Leishmania* protozoa, which are transmitted by bites from phlebotomine sandflies [1]. This disease has different clinical forms: cutaneous, mucocutaneous, and visceral [2]. Leishmaniasis has an annual incidence of 1.6 million new cases, and this infection has been reported in 88 countries, mainly in tropical and subtropical regions [3].

Current chemotherapies for leishmaniasis are based on the administration of pentavalent antimonials as first-line drugs, and amphotericin B, pentamidine, paromomycin, and miltefosine as second-line drugs [4]. These drugs, however, have limitations, such as high toxicity and long-term treatment [5]. No vaccines against *Leishmania* infections are available [6]. This reveals an urgent need to develop new therapeutic agents for the treatment of this pathology.

New treatments approaches planned are under development to promote cell death in protozoa with less collateral effects on host cells [7,8]. The literature presents studies of various compounds, both synthetic and natural, that may have potential for the treatment of this pathology [9–11], including β-carboline alkaloids [12–14]. These β-carboline compounds induced several cells alterations leading to cell death (e.g., apoptosis). These alterations are involved with some chemotherapeutic agents that act against leishmaniasis. One example of an antileishmanial compound is *n*-benzyl 1-(4-methoxy)phenyl-9H-β-carboline-3-carboxamide (**C5**; Fig. 1), which presented activity against promastigote forms of *Leishmania amazonensis* and a selectivity index that was 930.2-times for the protozoan [15]. This study also reported electron microscopy data on the effects of **C5** on the nuclear membrane, cell division, and exocytic activity in the flagellar pocket region. Although this study detailed the leishmanicidal activity of this β-carboline alkaloid, it did not report its likely mechanism of action.

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http://dx.doi.org/10.1016/j.cbi.2016.06.018
0009-2797/

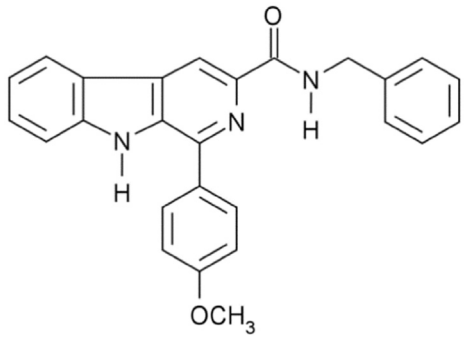

Fig. 1. Chemical structure of *n*-benzyl 1-(4-methoxy)phenyl-9H-*β*-carboline-3-carboxamide (**C5**).

These compounds also have been shown to exert multiple pharmacological effects, including antitumor effects [16], psychoactive effects [17], antiviral effects [18], antimicrobial effects [19], and antiprotozoal effects [20].

Considering previous studies on the effects of *β*-carboline on *L. amazonensis*, we performed further biochemical studies to better characterize the alterations that are induced by **C5** in *L. amazonensis* promastigotes to elucidate the mechanism of cell death that is induced by **C5**. Interestingly, we found that **C5** caused promastigote death through different cell death pathways.

2. Materials and methods

2.1. Chemicals and materials

Actinomycin D, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), digitonin, dimethylsulfoxide (DMSO), rhodamine 123, monodansylcadaverine (MDC), acridine orange (3,6-Bis(dimethylamino)acridine hydrochloride), 9-diethylamino-5H-benzo[alpha] phenoxazine-5-one (Nile red), 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), and wortmannin (WTM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Invitrogen (Grand Island, NY, USA), 1 Kb plus DNA ladder, proteinase K, and RNAse were obtained from Invitrogen (Eugene, OR, USA). All other reagents were of analytical grade.

2.2. Parasites

Leishmania amazonensis promastigotes (MHOM/BR/Josefa) were maintained at 25 °C in Warren's medium (brain-heart infusion plus hemin and folic acid, pH 7.2) supplemented with 10% fetal bovine serum (FBS).

2.3. Synthesis of *C5*

The compound **C5** was synthesized as previously described by Ref. [15]. The concentrations of **C5** that were used in the assays were based on the concentrations that inhibited 50% (IC_{50}: 2.6 μM) and 90% (IC_{90}: 11.2 μM) of the parasites. We also tested one higher concentration (20 μM).

2.4. Fluorescence detection of reactive oxygen species

Promastigotes (2×10^7 cells/ml) in the exponential phase were treated with 2.6, 11.2, and 20 μM **C5** for 3 and 24 h at 25 °C, centrifuged, washed, and resuspended in phosphate-buffered saline (PBS; pH 7.4). The parasites were then loaded with 10 μM of the permeant probe H_2DCFDA in the dark for 45 min. Reactive oxygen species (ROS) were measured as an increase in fluorescence that is caused by the conversion of nonfluorescent dye to highly fluorescent 2′,7′-dichlorofluorescein. Fluorescence was read with a microplate reader (VICTOR X3, PerkinElmer, Turku, Finland) at an excitation wavelength of 488 nm and emission wavelength of 530 nm.

2.5. Determination of mitochondrial membrane potential

Promastigotes (5×10^6 cells/ml) in the exponential phase were treated with 2.6, 11.2, and 20 μM **C5** for 24 h at 25 °C, harvested, and washed with PBS. The parasites were then incubated at 37 °C with rhodamine 123 (Rh123; 5 μg/ml), which accumulates within mitochondria, for 15 min, resuspended in 0.5 ml of PBS, and incubated for an additional 30 min. The parasites were analyzed in a BD FACSCalibur flow cytometer (Becton–Dickinson, Rutherford, NJ, USA) equipped with and Cell-Quest Pro software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA, USA). A total of 10,000 events were acquired in the region that corresponded to the parasites. CCCP (100 μM) was used as a positive control. Alterations in Rh123 fluorescence were quantified using an index of variation (IV) that was obtained from the equation *(MT−MC)/MC*, in which *MT* is the median fluorescence for the treated parasites, and *MC* is the median fluorescence for the control parasites. Negative IV values correspond to depolarization of the mitochondrial membrane.

2.6. Evaluation of DNA fragmentation

Promastigotes (5×10^6 cells/ml) in the exponential phase were treated with 2.6, 11.2, and 20 μM **C5** for 72 h at 25 °C, harvested, and resuspended in TELT digestion buffer (500 μl; 10 mM ethylenediaminetetraacetic acid [EDTA], 62.5 mM Tris-HCl [pH 8.0], 4% Triton-X, and 2.5 M LiCl), to which 0.5 mg/ml proteinase K was added. The mixture was incubated for 3 h in an ice bath and again for 1 h at 55 °C. The DNA material was extracted by phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated by adding 3 M sodium acetate and ice-cold ethanol (100%). Following overnight incubation at −20 °C, the material was centrifuged at 14,000× *g*, and the pellet was collected, air-dried, and resuspended in Tris-EDTA buffer (30 μl, pH 8.0) in the presence of 0.1 mg/ml RNase A for 1 h at 37 °C. Camptothecin (25 μM) was used as a positive control. DNA aliquots (10 μl) were electrophoresed in 1.5% agarose gel, stained with 1 μl Gel Red (Biotium) at 80 V for 1.5 h, visualized under an ultraviolet transilluminator, and photographed by Lpix Molecular Imaging (Loccus Biotecnologia).

We analyzed DNA double-strand ruptures *in situ* by TUNEL staining. Promastigotes (1×10^6 cells/ml) in the exponential phase were treated with 2.6, 11.2, and 20 μM **C5** for 72 h at 25 °C, and the cells were subjected to the TUNEL assay according to the manufacturer's instructions. Actinomycin D (10 μg/ml) was used as a positive control. Fluorescence was observed using an Olympus BX51 fluorescence microscope, and images were captured with an Olympus UC30 camera.

2.7. Determination of cell volume

Promastigotes (5×10^6 cells/ml) in the exponential phase were treated with 2.6, 11.2, and 20 μM **C5** for 24 h at 25 °C, harvested, and washed with PBS. The parasites were analyzed in a BD FACSCalibur flow cytometer (Becton–Dickinson, Rutherford, NJ, USA) equipped with and Cell-Quest Pro software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA, USA). Histograms and analysis were performed, FSC-H which represents the cell volume. A total of 10,000 events were acquired in the region that corresponded to the