Amiodarone triggers induction of apoptosis in cutaneous leishmaniasis agents

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Leishmaniasis is a parasitic disease that is an important problem of public health worldwide. The aim of this study was to assess the cytotoxic effects of amiodarone (AMD) on *Leishmania tropica* (MHOM/AF/88/KK27) and *L. major* (MRHO/IR/75/ER) promastigotes and to observe the programmed cell death features. The colorimetric MTT assay was used to find *L. tropica* and *L. major* viability and the obtained results were expressed as 50% inhibitory concentration (IC₅₀). Annexin-V FLUOS staining was performed to study the cell death properties of AMD using fluorescence-activated cell-sorting analysis. Qualitative analysis of the total genomic DNA fragmentation was performed by agarose gel electrophoresis. Furthermore, to observe changes in cell morphology, promastigotes were examined using light microscopy. The IC₅₀ was achieved at 55 and 81 µM for *L. tropica* and *L. major* after 48 h of incubation, respectively. In both strains, AMD induced death with features of apoptosis, including externalization of phosphatidylserine, DNA laddering, and cell shrinkage. Our findings indicate that AMD may induce apoptosis on the causative agents of cutaneous leishmaniasis.

Keywords: Amiodarone, Apoptosis, *Leishmania major*, *Leishmania tropica*

Introduction

Chemotherapy is the most effective treatment against leishmaniasis, due to the lack of an effective vaccine.¹ The recommended first-line therapy for different forms of leishmaniasis includes pentavalent antimonials like glucantime and pentostam. But, overall treatment of leishmaniasis is often difficult due to expense, the necessity for intravenous administration, toxicity, and the increase in parasite drug resistance.² Also, these drugs have secondary effects on the renal, cardiac, and hepatic systems.³ Thus, the utilization of currently approved antimicrobials for use in leishmaniasis and the development of new chemotherapeutic agents are important for the control of disease. Apoptosis is a process of cell death in which the cells undergo nuclear and cytoplasmic shrinkage; the chromatin is condensed and partitioned into multiple fragments, and finally the cells are broken into multiple membrane-bound bodies.⁴ It was initially believed that apoptosis does not occur in unicellular organisms but, to date, there is enough evidence to confirm that this phenomenon also occurs in single-cell organisms.^{5,6} The mechanisms and pathways that lead to induction or inhibition of apoptosis in *Leishmania* spp. are of particular interests as they will be potential targets for development of anti-*Leishmania* medications.

Recently, the study of mitochondrial potential has become a focus of apoptosis regulation as many investigations demonstrate a major functional impact of mitochondrial alterations on apoptosis.^{7,8} Maintenance of proper mitochondrial transmembrane potential ($\Delta\Psi_m$) is essential for the survival of the cell as it drives the synthesis of ATP and maintains oxidative phosphorylation. Mitochondria are pivotal in controlling cell life and death and in a number of experimental systems; disruption of $\Delta\Psi_m$ constitutes a constant early event of the apoptotic process that precedes nuclear disintegration.⁹ It has recently been shown that the antiarrhythmic drug amiodarone (AMD) has selective activity against *Trypanosoma cruzi* and some species of *Leishmania* such as *L. mexicana* and *L. amazonensis*.^{10–12} The mechanisms of action of AMD reported in these different microorganisms involve disruption of $\Delta\Psi_m$ and Ca²⁺ homeostasis, as well as production of reactive oxygen species.^{11,13–15} Overall, this study was designed to investigate the possibility of AMD in apoptosis induction in cutaneous leishmaniasis agents.

Materials and methods

Culture of the parasites

The *Leishmania* strain used in this study was *L. tropica* (MHOM/AF/88/KK27) and *L. major* (MRHO/IR/75/ER). The promastigotes were grown at 26 °C in BHI medium plus 10% heat-inactivated fetal calf serum, pH 7.0 and 1% of Penicillin (50 u/ml) Streptomycin (50 µg/ml) solution

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(Sigma, St. Louis, Mo., U.S.A). AMD (Sigma, St. Louis, Mo., U.S.A) was added to the growth medium 24 h after starting the cultures with 10^6 parasites/ml. The parasites susceptibility to AMD was evaluated by following up the proliferation of parasites in the absence or presence of drug. AMD was added in triplicate at final dilutions ranging from 1 to 100 μ M. Stock solution of AMD was prepared in dimethyl sulfoxide (DMSO), with the final concentration of DMSO in the experiments never exceeding 0.05%. The plates were incubated at 25 °C for 48 h before MTT assay. All tests were performed in triplicates.

Cell proliferation measurements by colorimetric MTT assay

MTT [3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] colorimetric assay measures reduction of MTT dye (tetrazolium) into formazan by mitochondrial enzymes in viable cells. Relative numbers of live cells were determined based on the optical absorbance of the treated and untreated samples and blank wells.¹⁶

Results were expressed as the concentration that inhibited parasite growth by 50% (IC_{50}).

Flow cytometry analysis of cell death

Control and AMD-treated promastigotes of *L. major* and *L. tropica* (with IC_{50}) were evaluated for type of death. The Annexin-V FLUOS Staining Kit (Roche, Germany) was used for the detection of apoptotic and necrotic cells according to the manufacturer's protocol. Briefly, promastigotes were washed in cold phosphate-buffered saline (PBS) (twice) and centrifuged at 1400 g for 10 min. Then, they were incubated for 15 min in dark and at room temperature in 100 μ l of Annexin-V FLUOS in the presence of propidium iodide. Afterwards, the samples were analyzed with FACSCalibur flow cytometer (Becton Dickinson and Cell Quest software), and the percentage of positive cells was determined for each sample.

DNA ladder assay in the presence and absence of AMD

Qualitative analysis of total gDNA fragmentation was performed for control and AMD-treated promastigotes of *L. major* and *L. tropica* (with IC_{50}) by agarose gel

electrophoresis. Briefly, promastigotes (5×10^6 cells) were incubated and harvested in different time points. An apoptotic DNA ladder kit (DNA Laddering Assay Kit, Cayman Chemical, U.S.A) was used to extract DNA from apoptosis-induced and un-induced cells according to the manufacturer's instructions. DNA (10 μ g DNA samples) was electrophoresed in 1.5% agarose gels at 100 V for 2 h, visualized using an UV transilluminator and photographed.

Determination of promastigotes' morphology after treatment with AMD

To observe changes in cell morphology, promastigotes treated, with or without AMD (IC_{50}), were examined. Briefly, cells were centrifuged at low speed (1000 g) and the pellets suspended in PBS. Changes in morphology were observed under $\times 100$ objectives on a light microscope. Alteration of cellular morphology was studied in different time points, and for each sample, at least 10 microscopic fields were observed under $\times 100$ objectives.

Statistical analysis

In vitro anti-leishmanial activity, expressed as IC_{50} (50% inhibitory concentration), was determined by linear regression analysis.

Results

Cytotoxic effects of AMD on *L. tropica* and *L. major*

The effect of AMD on *L. tropica* and *L. major* promastigote viability was assessed by the quantitative colorimetric MTT assay after 2 days of incubation. Treatment with AMD resulted in a concentration-dependent inhibition of *L. tropica* and *L. major* promastigotes viability with an IC_{50} of 55 and 81 μ M, respectively.

Apoptosis results from AMD in promastigotes

Following treatment of *L. tropica* promastigotes with AMD at its IC_{50} value of 55 μ M for 24, 48 and 72 h, the percentage of annexin V-positive cells increased to 24.2, 36.2 and 69.2%, respectively. The percentage of PI-stained cells were 6.3% at 24 h, 7.3% at 48 h, and 4.8% at 72 h, and indicating that AMD exerts its anti-leishmanial activity primarily via apoptosis (Fig. 1). After 24, 48, and 72 h of *L.*

Figure 1 Flow cytometry analysis of *L. tropica* promastigotes following treatment with 55 μ M AMD at different time points. (A) *L. tropica* 24 h after treatment. (B) *L. tropica* 48 h after treatment. (C) *L. tropica* 72 h after treatment.

Notes: Lower right region (LR) belongs to apoptotic cells (annexin positive) and upper left region (UL) belongs to necrotic cells (PI positive), PI, Propidium iodide.

major promastigotes treatment with IC₅₀ of AMD (81 μ M), the percent of annexin-positive cells were 3.3, 14.3, and 56.4%, respectively. On the other hand, the percentage of PI-positive cells increased to 4.4% at 24 h, 5.6% at 48 h, and 13.1% at 72 h (Fig. 2). In untreated promastigotes of *L. tropica* and *L. major*, the degree of binding of annexin V at 72 h was 3.8 and 1.64% and the percentage of PI stained cells were 2.41 and 2.85%, respectively.

DNA fragmentation in *L. tropica* and *L. major* promastigotes

DNA fragmentation in promastigotes of *L. tropica* and *L. major* was confirmed by the presence of fragmented DNA in agarose gel electrophoresis. The fragments were in oligonucleosome size (in approximate multiples of 180–200 bp) in promastigotes treated with 55 μ M AMD for 24 h in *L. tropica*, whereas untreated cells did not show DNA fragmentation. No DNA fragmentation was observed in *L. major* promastigotes treated with 81 μ M concentrations of AMD post-24-h incubation. After 48 h, DNA fragmentation was observed in *L. major* promastigotes. Study of DNA fragmentation in promastigotes of *L. tropica* promastigotes did not show any difference in comparison with *L. major* after 48 h (Fig. 3).

Morphological changes of treated promastigotes with AMD

Microscopic examination of the treated cells showed that cell shrinkage 24 h after the drug treatment in both *L. tropica* and *L. major* promastigotes occurred. Most of the cells showed cytoplasmic condensation, shrinkage, and reduction in size in comparison to the control samples at the end of 72 h after treatment.

Discussion

Recently, different studies demonstrated that AMD, a well-known antiarrhythmic agent, also possesses intrinsic activity against parasites belonging to the family Trypanosomatidae. Veiga-Santos *et al.* confirmed that AMD has significant activity against the proliferation of *T. cruzi* epimastigote and amastigotes without affecting the viability of the host cell.¹² Macedo-Silva *et al.* also found that *L. amazonensis* promastigotes incubated with AMD exhibited large autophagosomes and alteration in the mitochondrion- kinetoplast complex.¹⁰ Serrano Martín *et al.*, study (2009), stated that AMD affected the biosynthesis of 5-dehydroepisterol in *L. mexicana* promastigotes incubated with amiodarone, which results in squalene accumulation. They suggested that AMD inhibits the squalene

Figure 2 Flow cytometry analysis of *L. major* promastigotes following treatment with 81 μ M AMD at different time points. (A) *L. major* 24 h after treatment. (B) *L. major* 48 h after treatment. (C) *L. major* 72 h after treatment.

Notes: Lower right region (LR) belongs to apoptotic cells (annexin positive) and upper left region (UL) belongs to necrotic cells (PI positive), PI, Propidium iodide.

Figure 3 DNA fragmentation detected with agarose gel electrophoresis. (1) Not treated promastigotes of *L. major*. (2) Not treated promastigotes of *L. tropica*. (3) *L. tropica* 72 h after treatment. (4) *L. major* 72 h after treatment. (5) *L. major* 24 h after treatment. (6) *L. major* 48 h after treatment. (7) *L. tropica* 24 h after treatment. (8) *L. tropica* 48 h after treatment.

epoxidase activity of the parasite.¹¹ Our results showed that AMD affects cutaneous leishmaniasis agent's proliferation and *L. tropica* promastigotes were more sensitive to AMD than *L. major* promastigotes (55 vs. 81 μ M).

Following an apoptotic stimulus, phosphatidylserine present in the inner leaflet of the plasma membrane flips out to the outer leaflet of the plasmalemma; thus, externalization of phosphatidylserine is considered to be a marker of apoptosis.¹⁷ The binding of annexin V, a Ca^{2+} -dependent phospholipid-binding protein known to have a strong affinity toward phosphatidylserine, is a proven measure of apoptosis. To distinguish apoptotic cell death from necrotic cell death, cells were counterstained with PI, a non-permeable stain with an affinity for nucleic acids, as it selectively enters necrotic cells. Therefore, co-staining of annexin V and PI can differentiate between cells undergoing early apoptosis (annexin V-positive, PI-negative) or necrosis (PI-positive, annexin V-negative), and live cells (PI-and annexin V-negative).¹⁸ Following treatment of *L. tropica* promastigotes with AMD at its IC_{50} value of 55 μ M for 24, 48, and 72 h, the percentage of annexin V-positive cells increased to 24.2, 36.2, and 69.2%, respectively. After 24, 48, and 72 h of *L. major* promastigotes treatment with IC_{50} of AMD (81 μ M), the percent of annexin-positive cells were 3.3, 14.3, and 56.4%, respectively.

Apoptotic DNA fragmentation is a key feature of apoptosis. Apoptosis is characterized by the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into internucleosomal fragments of roughly 180 base pairs (bp) and multiples thereof (360, 540 etc.).¹⁹ In the present study, DNA fragmentation was occurred in treated promastigotes of *L. tropica* after 24 h, while this happened for treated promastigotes of *L. major* after 48 h. Study of DNA fragmentation in promastigotes of *L. tropica* promastigotes did not show any difference in comparison with *L. major* after 48 h. Microscopic examination of treated cells showed cytoplasmic condensation, shrinkage, and reduction in size in comparison to the control samples at the end of 72 h after treatment. Based on the findings, AMD can induce apoptosis in both *L. major* and *L. tropica*. Different studies have found that treatment with AMD leads to a collapse of the mitochondrial membrane potential.^{10,11} These alterations in the mitochondrial metabolism trigger a sequence of cellular events leading to apoptosis. Apoptosis results from the action of a genetically encoded suicide program that leads to series of characteristic morphological and biochemical changes.²⁰ These changes include activation of caspase, cell shrinkage, chromatin condensation, and nucleosomal degradation.²¹ Different studies suggest that the type of programmed cell death (PCD) pathway observed in *Leishmania* may have some similarities to the PCD processes in metacellular organisms. However, one has to be cautious in generalizing the similarities. It is possible that there are separate PCD pathways in unicellular versus multicellular organisms. The most significant event in apoptosis is mitochondrial

dysfunction, which was shown to be involved in an early phase of apoptosis.²²

Kinetoplastids are single-celled eukaryotes that belong to one of the most ancient diverging branches of the eukaryote phylogenetic trees and are among the first mitochondrial eukaryotes containing only one giant mitochondrion.²³ Therefore, the importance of functioning of this organelle in *Leishmania* spp. is very vital as compared to organisms with numerous mitochondria because the presence of multiple mitochondria ensures compensation for the injured ones but for organisms with a single mitochondrion, no such choice exists and survival depends on proper functioning of a single organelle. Mitochondrion acts by releasing apoptogenic factors, such as cytochrome c, from the intermembrane space into the cytoplasm, which activates the downstream execution phase of apoptosis.²⁴ Apaf-1 is a protein contained in the cytosol, and cytochrome c binds and induces it to oligomerize. This then recruits an initiator caspase, procaspase-9. The apoptosome now recruits procaspase-3, which is cleaved and activated by the active caspase-9 and released to mediate apoptosis.²⁵ Recently, Serrano-Martín *et al.*¹¹ demonstrated that AMD increases the cytoplasmic Ca^{2+} concentration as a result of the release of the cation from intracellular compartment. Calcium has been implicated in diverse cellular actions, and the intracellular concentration of free Ca^{2+} is tightly controlled.¹¹ The main organelles that regulate Ca^{2+} homeostasis are also the main sites of apoptotic and autophagic regulation.²⁶ Up to now, cellular calcium overload has been considered as a final common pathway of cell death. Recent findings suggest that alterations in calcium could act as a relevant amplification loop of the apoptosis signal.^{27,28}

Finally, it seems that two possible explanations for AMD apoptosis induction can be: (1) Sterol biosynthesis inhibition, leading to alteration in the lipid composition of the mitochondrial membranes that modify their biophysical properties and loss of mitochondrial function and (2) Calcium release from a direct action of AMD on the mitochondria can cause apoptosis.

In conclusion, these data indicate that AMD has promising anti-leishmanial activity that is mediated by PCD and, accordingly, merits consideration and further investigation as a therapeutic option for the treatment of leishmaniasis.

Conflict of interest

The authors declare no conflict of interest.

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