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Methyl jasmonate induces cell death and loss of hydrogenosomal membrane potential in *Trichomonas vaginalis*

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ABSTRACT

Trichomonas vaginalis is an important human parasite of the urogenital tract. Jasmonates are a group of small lipids that are produced in plants and function as stress hormones. Naturally occurring methyl jasmonate (MJ) has been used to treat several types of cancer cells and it is cytotoxic to protistan parasites. It has been suggested that mitochondria are the target organelles of jasmonates. Here, we tested this drug against *T. vaginalis*. Although metronidazole has been the drug of choice for trichomoniasis, side effects from this treatment are common, and nausea and dizziness have been reported in up to 12% of patients. In addition, there has been increased recognition of resistance to metronidazole. We demonstrate here using flow cytometry, JC-1 and scanning and transmission electron microscopy that MJ induced the cell death of *T. vaginalis* parasites. Our results are discussed with previous findings published by others.

1. Introduction

Trichomonas vaginalis, an extracellular aerotolerant protozoan parasite, is the cause of trichomoniasis, the most prevalent non-viral sexually transmitted infection in the world [1]. Over 170 million people are infected with *T. vaginalis* annually worldwide [1]. Infection by *T. vaginalis* is associated with serious adverse health consequences in women, including infertility [2], atypical pelvic inflammatory disease [3], preterm birth and low birth weight infants [4] and predisposition to cervical neoplasia [5]. Trichomoniasis among men can cause non-chlamydial, non-gonococcal urethritis [6,7] and *T. vaginalis* was recently found to be associated to prostate cancer [8].

Metronidazole has been the drug of choice for *T. vaginalis* treatment, but side effects from this treatment are common, and nausea and dizziness have been reported in up to 12% of patients [9]. In addition, resistance to metronidazole is increasingly observed [10].

Jasmonates are a group of small lipids that are produced in plants and function as stress hormones. Recently, researchers found that jasmonates are directly cytotoxic to mitochondria of several types of cancer cells [11] and also affect protozoan parasites [12].

A recent study used methyl jasmonate (MJ) against *T. vaginalis* [13]. The authors showed that this drug could induce both G2/M cell cycle arrest and cell death in this parasite. However, the authors did not demonstrate the effect of this drug on hydrogenosomes, which are ATP-producing, mitochondrion-like organelles present in this organism. In addition, no morphological data were obtained after the drug treatment. In the present study, we employed transmission electron microscopy and flow cytometric analysis to verify that the amitochondrial parasite trichomonas was affected by MJ. We followed the effects of MJ not only in the ultrastructure of this parasite, but also in its hydrogenosomes.

2. Material and methods

2.1. Chemicals

All reagents were purchased from Sigma Chemicals (St. Louis, MO, USA) unless otherwise stated.

2.2. Microorganisms

The JT strain of *T. vaginalis* was isolated at the Hospital Universitário at Universidade Federal do Rio de Janeiro, Brazil, and has been maintained in culture since the 1980s. Parasites were cultivated in trypticase–yeast extract–maltose (TYM) medium [14] supplemented with 10% fetal calf serum. The cells were grown for 36–48 h at 37 °C, which corresponds to the logarithmic growth phase.

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2.3. Effect of MJ on the growth of T. vaginalis

To test the effect of MJ, 0.75 mM, 1.5 mM, 3 mM or 6 mM of the drug was added to the *T. vaginalis* cultures at time 0 h and maintained for periods ranging from 1 min to 30 h at 37 °C. The drug was added directly in parasite's media without anterior dilutions with diluents. At different time points, samples were recovered and analyzed for viability and ultrastructural studies, as described below.

2.4. Scanning electron microscopy (SEM)

Before and after parasite MJ treatment, the cultures were fixed in 2.5% (v/v) glutaraldehyde (in 0.1 M sodium cacodylate buffer, pH 7.2), post-fixed for 15 min in 1% OsO_4, dehydrated in ethanol, critical point dried with CO_2 and sputter coated with gold–palladium. The samples were examined with a JEOL 5800 scanning electron microscope.

2.5. Transmission electron microscopy (TEM)

Cells were fixed in 2.5% (v/v) glutaraldehyde, post-fixed for 15 min in 1% OsO_4, dehydrated in acetone and embedded in Epon. The samples were examined with a JEOL 1210 transmission electron microscope.

2.6. Flow cytometry analysis

To test if MJ inhibited cell division, carboxyfluorescein diacetate succinimidyl ester (CFSE) was used according to the manufacturer's instructions (Molecular Probes, USA). Control parasites and parasites treated with 0.75 and 1.5 mM MJ were incubated with 10 μM CFSE for 15 min. Cell kinetics were then evaluated at different times of drug incubation (0, 6, 24 and 30 h). Data acquisition and analysis were performed using a FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). A total of 10,000 events were acquired in the regions previously established as those corresponding to *T. vaginalis*. Mean value comparisons were performed using the Mann–Whitney test, and $P \leq 0.04$ was considered significant.

2.7. Viability stains

The viability of the cells was assessed using fluorescein diacetate (FDA) (Sigma, USA), propidium iodide (PI) (Sigma, USA) or JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanineiodide; Molecular Probes, USA). At different time and drug concentration points, the cells were stained with 10 μg/ml of FDA and 10 μg/ml of PI for 20 min for fluorescence analysis. Viable cells were seen in green color with FDA, whereas dead cells fluoresced with an orange color with PI.

2.8. Mitochondrial/hydrogenosomal membrane potential

T. vaginalis with or without MJ treatment were treated with 5 μg/ml JC-1 dye at 37 °C for 15 min. JC-1 has the ability to selectively enter into mitochondria or hydrogenosomes and, according to the magnitude of the mitochondrial/hydrogenosomal membrane potential, change its oligomeric state, thereby allowing it to fluoresce. The ratio of red:green fluorescence intensity for JC-1 acts as an index of the hydrogenosomal membrane potential, where a higher ratio indicates a higher hydrogenosomal membrane potential. CCCP (carbonyl cyanide 3-chlorophenylhydrazone), a mitochondrial membrane potential disrupter, was used as positive control to confirm the JC-1 response. A 1 μL aliquot of a 50 mM CCCP solution was added to the cells at 37 °C for 5 min in a final volume of 1 mL. Cytometric dot plots were acquired using the flow cytometer.

2.9. Host-cell interactions

Madin Darby Canine Kidney (MDCK) cells were seeded onto 24-well tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA) and allowed to form a confluent monolayer (1×10^5 cells). These cultures were then washed and co-incubated with either MJ-treated and untreated parasites in 2:1 DMEM: TYM medium for different periods of time at 37 °C. A cell ratio of five parasites to one host cell was used. As a control, parasites were not added to monolayers. At the end of the incubation periods, the wells were gently washed twice with PBS and the remaining host-cells that were still attached to the wells were fixed with 1% formaldehyde and submitted to a crystal violet test for cell viability as described below.

The cells were analyzed after exposure to 0, 2, 4 or 6 h of treatment at 37 °C. After fixation, the wells were washed with PBS and stained with 0.13% crystal violet dissolved in a 5:2 (vol/vol) ethanol-formaldehyde solution. The stained cells were subsequently washed twice with distilled water, air dried and solubilized in 1% (wt/vol) SDS in 50% (vol/vol) ethanol. The staining intensity was determined using ELISA equipment (EL×800 Biotek, USA) at a wavelength of 570 nm. Each experiment was performed in triplicate, and the mean of the data is presented. Cytotoxicity was calculated as $1-(E/C)$. All measurements of experimental (E) samples (A_{570}) were indexed to those of control (C) samples (E/C), which showed no loss of cells from the well [15].

3. Results

3.1. Antiproliferative effect of MJ on T. vaginalis (CFSE)

MJ is a plant stress hormone and according previous articles we choose this concentration based on plasma concentrations achieved upon administration of another stress hormone, salicylic acid [16]. While most of anti-inflammatory drugs act in micromolar range, it has been described that salicylates act in low millimolar range [11,13]. CFSE is a fluorescent stain that allows the identification of a certain number of cell division cycles using flow cytometry. Thus, when CFSE is used prior to MJ incubation (Fig. 1), it is possible to detect whether the cells are dividing or are arrested and then compare this with control cells. When a CFSE-stained cell divides, the peaks tend to dislocate concomitantly with the decrease of fluorescence intensity. During each time-step, it is possible to wait for one of the following situations: (1) the cells divide normally, (2) the cells survive, but without division or (3) the cells die. In the present study, we followed *T. vaginalis* cell division for 4 time periods (0, 6, 24 and 30 h), and untreated and MJ-treated cells were compared. We decided to reduce the MJ concentration (0.75 and 1.5 mM) to follow the kinetics for a longer time because we observed that the drug concentration normally used was damaging the cells in a short period of time (6 h). Fig. 1 shows histograms of cell counts versus fluorescence activity. These histograms clearly demonstrate the dose-dependent effect of MJ on *T. vaginalis* cell division. In Fig. 1a, the histogram shows the normal kinetics of untreated parasites. In contrast, the histograms in Fig. 1b and c (0.75 mM and 1.5 mM MJ, respectively) show dose-dependent fluorescence arrest, indicating a blockage of cell division.

3.2. Scanning electron microscopy

To evaluate the morphological effects of MJ on *T. vaginalis*, both the control and MJ-treated cells were analyzed by SEM, as shown in Fig. 2. A general view revealed an abnormal morphology in MJ-treated parasites, with reduced cell size and internalized flagella (Fig. 2b) relative to untreated control cells (Fig. 2a), which presented the typical tear-drop morphology and externalized flagella.