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Microbes and Infection 13 (2011) 1018–1024

www.elsevier.com/locate/micinf

Original article

Trypanocidal action of eupomatenoid-5 is related to mitochondrion dysfunction and oxidative damage in *Trypanosoma cruzi*

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Received 27 November 2010; accepted 24 May 2011
Available online 12 June 2011

Abstract

Because of its severe side effects and variable efficacy, the current treatment for Chagas disease is unsatisfactory. Natural compounds are good alternative chemotherapeutic agents for the treatment of this infection. Recently, our group reported the antiproliferative activity and morphological alterations in epimastigotes and intracellular amastigotes of *Trypanosoma cruzi* treated with eupomatenoid-5, a neolignan isolated from leaves of *Piper regnellii* var. *pallescens*. Here, we demonstrate that eupomatenoid-5 exhibited activity against trypomastigotes, the infective form of *T. cruzi* (EC_{50} 40.5 μM), leading to ultrastructural alteration and lipoperoxidation in the cell membrane. Additionally, eupomatenoid-5 induced depolarization of the mitochondrial membrane, lipoperoxidation and increased G6PD activity in epimastigotes of *T. cruzi*. These findings support the possibility that different mechanisms may be targeted, according to the form of the parasite, and that the plasma membrane and mitochondria are the structures that are most affected in trypomastigotes and epimastigotes, respectively. Thus, the trypanocidal action of eupomatenoid-5 may be associated with mitochondrial dysfunction and oxidative damage, which can trigger destructive effects on biological molecules of *T. cruzi*, leading to parasite death.

Keywords: Trypanosoma cruzi; Eupomatenoid-5; Antioxidant system

1. Introduction

Since its discovery by Carlos Chagas more than a hundred years ago, Chagas disease, caused by *Trypanosoma cruzi*, still poses many challenges, including the need for new chemotherapeutic agents, as the available drugs nifurtimox and benznidazole cause severe toxic side effects and have limited efficacy, especially in the chronic phase of the disease. Today, Chagas disease affects 12 million people around the world, particularly in Latin America [1] and 28 million are at risk of acquiring this infection. One promising line of investigation is the discovery of new antiparasitic agents of natural origin (reviewed in [2]). Intense research has led to extensive and well-documented literature regarding the effects of natural products on *T. cruzi* [3–7]. Not only the effects of these compounds, but also their mechanisms of action have been described [8,9]. Although *T. cruzi* offers several possible targets for drugs, the single mitochondrion and the parasite redox metabolism (most of them based on the trypanothione pathway) are widely studied [10–12].

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doi:10.1016/j.micinf.2011.05.011

Electron microscopy has been used to evaluate morphological alterations and target organelles in *T. cruzi*. For example, our group has demonstrated important morphological and ultrastructural alterations, such as mitochondrion swelling, intense cytoplasm vacuolization, and an apparent increase in the endoplasmatic reticulum of epimastigote and intracellular amastigote forms of *T. cruzi* caused by eupomatenoid-5 (Fig. 1), a neolignan isolated from leaves of *Piper regnellii* var. *pallescens* [13,14]. As a consequence of these alterations, eupomatenoid-5 inhibited the growth of epimastigotes and intracellular amastigotes.

Although the activity of eupomatenoid-5 against epimastigotes has been reported previously [13,14], the mechanism involved and the effect on the infective trypomastigote form of *T. cruzi* are still unknown. The present study was undertaken to investigate these issues. The results presented herein show that eupomatenoid-5 acts also on the infective form of *T. cruzi,* and this effect might be associated with mitochondrion dysfunction and oxidative damage according to the form of the parasite. We also discuss the involvement of the trypanothione system.

2. Materials and methods

2.1. Isolation of eupomatenoid-5 from leaves of Piper regnellii *var.* pallescens

Eupomatenoid-5 was isolated from leaves of *P. regnellii* collected in the Prof. Irenice Silva Garden of Medicinal Plants on the campus of the State University of Maringá (UEM) in Paraná. A voucher specimen (No. HUM 8392) was deposited at the UEM Herbarium. The dry plant material was extracted by exhaustive maceration at room temperature in the dark in ethanol:water (90:10). The fractionation was performed from the ethyl-acetate crude extract to obtain the hexane fraction, and a dihydrobenzofuran neolignan, eupomatenoid-5, was isolated from this fraction, as described previously [13]. The compound was purified by absorption-chromatographic methods and identified by spectral analyses of UV, IR, ^{1}H NMR and ^{13}C NMR, DEPT, COSY, HETCOR, gNOESY, HMBC, HETRL, and GC/MS. The data were compared with the literature [15]. Stock solutions of eupomatenoid-5 were prepared aseptically in dimethyl sulfoxide (DMSO − Sigma Chemical Co., St. Louis, MO, USA) and diluted in culture medium so that the DMSO concentration did not exceed 1% in the experiments.

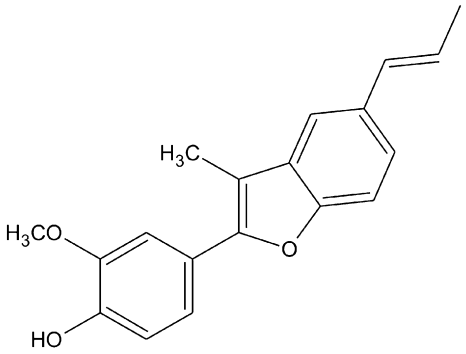

Fig. 1. Chemical structure of eupomatenoid-5, the neolignan isolated from *Piper regnellii* var. *palescens.*

2.2. Parasites and cell cultures

All experiments were performed with the Y strain of *T. cruzi*. Epimastigote forms were maintained axenically at 28 °C with weekly transfers in liver infusion tryptose (LIT) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) at pH 7.4 [16]. Trypomastigote forms were obtained from the supernatant of an infected $LLCMK_2$ cell monolayer (epithelial cells of monkey kidney, *Macaca mulatta*) in DMEM medium in a 5% CO_2 air mixture at 37 °C. $LLCMK_2$ cells were maintained in DMEM supplemented with 2 mM L-glutamine, 10% FBS, and 50 mg/l gentamicin, and buffered with sodium bicarbonate.

2.3. Viability and ultrastructure of trypomastigote forms

Trypomastigote forms (1×10^7 cells/ml) were treated with eupomatenoid-5 in a range of 3.4–170.0 μM for 2 h at 37 °C. The number of viable cells was determined by direct counting using the dye exclusion method (erythrosine B) [17], and the results were expressed in the compound concentration that lyses 50% of the parasites (EC_{50}). Parasites treated with 34.0 μM of eupomatenoid-5 for 2 h were processed for electron microscopy. For this, treated and untreated parasites were harvested and washed twice with phosphate-buffered saline (PBS), and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4 °C. For transmission electron microscopy (TEM), cells were post-fixed in a solution containing 1% OsO_4, 0.8% potassium ferrocyanide, and 10 mM $CaCl_2$ in 0.1 M cacodylate buffer, dehydrated in an increasing acetone gradient, and embedded in Epon® resin. Next, ultrathin sections were stained with uranyl acetate and lead citrate, and images were obtained on an FEI TECNAI 12 TEM. For scanning electron microscopy (SEM), parasite cells were fixed as before. Next, small drops of the sample were placed on a specimen support with poly-L-lysine. The samples were dehydrated in graded ethanol, critical-point dried in CO_2, coated with gold, and observed on a Shimadzu SS-550 SEM.

2.4. Lipid peroxidation assay

Epimastigotes (2×10^6 cells/ml) were incubated in the LIT medium supplemented with 10% FBS, and eupomatenoid-5 was added in concentrations of 34.0, 85.0, or 170.0 μM. Cells were incubated at 28 °C for 24 h. When trypomastigote forms were used in the assay, the same treatment was performed, but the incubation was at 37 °C for 2 h. The extent of lipid peroxidation was determined as the amount of thiobarbituric acid-reactive substances (TBARS) in terms of malondialdehyde (MDA). After incubation, samples (0.2–0.5 mg protein) were heated in a solution containing 0.37% thiobarbituric acid, 15% trichloroacetic acid, and 0.25 N HCl at 95 °C for 45 min. After cooling, the absorbance was read at 532 nm and the concentration of TBARS was calculated based on a ϵ value of

153,000 M^{-1} cm^{-1} [18]. Each experiment was conducted in duplicate and repeated at least three times.

2.5. Flow cytometry

Epimastigote forms (2×10^6 cells/ml), treated or untreated with 34 and 51 μM at 28 °C for 96 h and trypomastigote forms (2×10^6 cells/ml), treated or untreated with 34 and 68 μM at 37 °C for 2 h, were washed and incubated with 5 μg/ml rhodamine 123 (Rh123) for 15 min at 37 °C to evaluate the mitochondrial membrane potential ($\Delta\Psi m$). All the material was kept on ice until analysis. Data acquisition and analysis were performed using a FACSCalibur flow cytometer (Becton–Dickinson, Rutherford, NJ, USA) equipped with the CellQuest software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA). A total of 10,000 events were acquired in the region previously established as that corresponding to the parasites. Alterations in the fluorescence of Rh123 were quantified as the percent of reduction of the fluorescence compared with the control (untreated parasites).

2.6. Glucose-6-phosphate (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) assay

After 5 days (early stationary phase), epimastigotes (2×10^6 cells/ml) were collected by centrifugation, washed with PBS, and the treatment was performed in the presence or absence of eupomatenoid-5 (34.0, 85.0, and 170.0 μM) for 24 h, or with 34.0 and 51 μM for 96 h at 28 °C. Then, cells were pelleted and resuspended in PBS in the presence of protease inhibitors (1 mM PMSF and 13 μM Leupeptin). The combined activities of G6PD and 6PGD were measured by the addition of 5×10^6 cells/ml to the reaction mixture (50 mM Tris–HCl, pH 7.6, 50 mM KCl, 0.1% Triton X-100, 250 μM NADP+, 2 mM $MgCl_2$, 1 mM 6-phosphogluconate, and 1 mM glucose-6-phosphate), and the course of NADP+ reduction was followed at 340 nm [19]. G6PD activity was obtained by subtracting the measured activity of 6PGD from the combined activities of 6PGD and G6PD. Each experiment was conducted in duplicate and repeated at least three times.

2.7. Hydrogen peroxide consumption

Hydrogen peroxide consumption was measured using phenol red and horseradish peroxidase (HRP). Epimastigote forms were treated as described above, in G6PD and 6PGD activity. Then, the parasites were harvested by centrifugation, washed twice with incubation buffer (IB) (5 mM KCl, 80 mM NaCl, 2 mM $MgCl_2$, 16.2 mM Na_2HPO_4, 3.8 mM NaH_2PO_4, 50 mM Glucose, pH 7.4, and 0.15% bovine albumin) containing 0.05 mg/ml phenol red, and resuspended in the same buffer at a concentration of 10^7 cells/ml. Then, epimastigotes were exposed to oxidative stress by the addition of H_2O_2 in a final concentration of 20 μM. After 1 h, the concentration of H_2O_2 in the medium, and its decay were measured. Aliquots of 1000 μl of the cell suspension were taken, and 10 μl of horseradish peroxidase (1 mg/ml) was added, the mixture was incubated for 5 min at room temperature, and the samples were centrifuged at 7000×*g* for 2 min. Twenty microliters of 2 N NaOH was added to 1000 μl of the supernatant, and the absorbance at 610 nm was measured. Values were assessed according to a calibration curve developed between 0.05 and 25 μM H_2O_2 in IB buffer. Data were expressed as percent of H_2O_2 consumed relative to the control. Each experiment was conducted in duplicate and repeated at least three times.

2.8. Statistical analyses

Statistical analyses were performed with the GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). ANOVA followed by Tukey's test was used, and *p*-values less than 0.05 were regarded as significant. Results were expressed as mean ± standard deviation.

3. Results

3.1. Effect of eupomatenoid-5 on viability and ultrastructure of trypomastigote forms

For trypomastigotes, treatment with eupomatenoid-5 for 2 h caused a dose-dependent decrease in the number of viable cells (Fig. 2A), giving an EC_{50} value of 40.5 μM. Additionally, cultures of $LLCMK_2$ cells were treated with eupomatenoid-5 to investigate the cytotoxicity of this compound to mammalian cells. The viability was checked by the sulforhodamine B assay [20], and the result indicated a CC_{50} (concentration cytotoxic to 50% of the cells) of 122.45 μM after 96 h of treatment. The toxicity to $LLCMK_2$ cells and the antitrypanosomal activity were compared using the selective index (SI) ratio: CC_{50} $LLCMK_2$ cells/EC_{50} trypomastigote forms. The SI of eupomatenoid-5 on trypomastigotes was three times more selective against the protozoa and was less toxic to the cells.

The morphological analyses by SEM of trypomastigotes treated with eupomatenoid-5 (34 μM) for 2 h showed changes in the integrity of the cell membrane, with apparent leakage of cytoplasmic contents (Fig. 2C, D), compared to untreated cells (Fig. 2B). TEM ultrastructural analyses of similarly treated trypomastigotes demonstrated striking alterations in the treated cells, such as plasma membrane detachment and intense cytoplasmic vacuolization (Fig. 2F, G). These morphological alterations contrasted strongly with the control cells, in which the plasma membrane and organelles showed normal morphology (Fig. 2E).

3.2. Effect of eupomatenoid-5 on lipid peroxidation of trypomastigote and epimastigote forms

To further confirm the alteration of cell membrane integrity shown by SEM and TEM, the putative oxidative damage in trypomastigotes and also in epimastigotes treated with eupomatenoid-5 was assessed by measuring the production of TBARS, which is frequently used to quantify lipoperoxidation of the cell membrane and is expressed by the production of

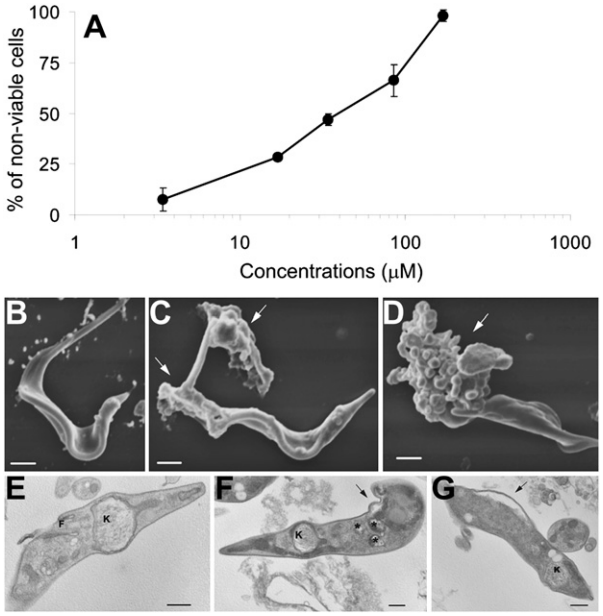

Fig. 2. Effects of eupomatenoid-5 on the viability, morphology, and ultrastructure of trypomastigote forms of *Trypanosoma cruzi*. (A) The protozoa were cultured for 2 h in the presence or absence of eupomatenoid-5 in concentrations of 3.4, 17.0, 34.0, 85.0, and 170.0 μM. Each experiment was conducted in duplicate and repeated at least three times. The results were analyzed as percentages of non-viable cells in relation to untreated parasites. Bars represent standard errors. All results were significant at $p \leq 0.05$ compared to the control group. (B) SEM image of an untreated parasite, showing the typical elongated morphology. (C, D) SEM images of parasites treated with 34.0 μM of eupomatenoid-5 for 2 h reveal apparent leakage of cytoplasmic contents (white arrow). (E) TEM image of an untreated parasite, showing the typical elongated morphology with normal kinetoplast (K), flagellum (F), and nucleus (N). (F, G) TEM images of parasites treated with 34.0 μM of eupomatenoid-5 for 2 h reveal separation between the membrane and cytoplasm (arrow) and vacuoles (asterisks). Bars = 1 μm.

MDA. The measurement of TBARS revealed a dose-dependent effect of eupomatenoid-5 on trypomastigotes. Treatment with 34.0, 85.0, or 170.0 μM for 2 h induced 2-, 3.2-, and 6-fold increases in lipoperoxidation compared to the control (Table 1). The same treatment in epimastigotes for 24 h, in the exponential growth phase, resulted in an increase of about 1.32-, 1.35-, and 2-fold of lipoperoxidation (Table 1).

Table 1
Effect of eupomatenoid-5 on lipid peroxidation (production of MDA) of trypomastigote and epimastigote forms of *Trypanosoma cruzi*.

Treatment concentrations (μM)	MDA (nmol/mg protein)	
	Trypomastigotes	Epimastigotes
Control	13.9 ± 4.3	9.6 ± 0.5
34.0	28.1 ± 8.2	12.7 ± 1.0 d
85.0	44.4 ± 11.5 a	12.9 ± 0.5 d
170.0	83.5 ± 31.3 b,c	19.3 ± 2.7 e

Data are presented as mean ± SD from three independent experiments. a, $p < 0.05$ compared to control; b, $p < 0.001$ compared to control and μM; c, $p < 0.01$ compared to 85.0 μM; d, $p < 0.01$ compared to control; e, $p < 0.001$ compared to control, 34.0 and 85.0 μM.

3.3. Effect of eupomatenoid-5 on mitochondrial membrane potential of trypomastigote and epimastigote forms

Based on our previous work that indicated the effect of eupomatenoid-5 on *T. cruzi* mitochondria [10], we decided to evaluate the mitochondrial membrane potential in eupomatenoid-5-treated epimastigotes by flow cytometry. Histograms of total Rh123 fluorescence showed a marked decrease in fluorescence intensity, indicating that the mitochondria were depolarized in cells treated with 34.0 and 51.0 μM of eupomatenoid-5 for 96 h, where ΔΨm reductions of 29.0 and 62.8% were observed, respectively (Fig. 3A). Additionally, in order to perform a head-to-head comparison between the potential different mechanisms of action between epimastigotes and trypomastigotes, we also evaluated the mitochondrial membrane potential in eupomatenoid-5-treated trypomastigotes. The mitochondrial membrane despolarization was observed in trypomastigotes treated with 34.0 and 68.0 for 2 h, where ΔΨm reductions of 45.0 and 55.2% were observed, respectively (Fig. 3B).

3.4. Effect of eupomatenoid-5 on glucose-6-phosphate and 6-phosphogluconate dehydrogenase activity of epimastigote forms

Another widely studied drug target is the *T. cruzi* trypanothione-dependent system. This system plays a key role in the antioxidant activity of trypanosomatids, where an enzymatic cascade is activated to promote hydroperoxide detoxification. To work properly, the trypanothione-dependent system must have a current supply of NADPH, provided by glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD), enzymes of the pentose phosphate pathway (PPP) [21]. For that reason, we performed additional experiments to evaluate the effect of eupomatenoid-5 and, more importantly, its effects in association with H_2O_2 on *T. cruzi* G6PD and 6PGD activity. As seen in Fig 4A, NADPH production by G6PD measured in treated (34.0, 85.0, and 170.0 μM of eupomatenoid-5) or untreated epimastigotes after 24 h induced an increase of 265, 1403, and 3601% in NADPH total production. However, when epimastigotes were treated with 34.0 and 51.0 μM of eupomatenoid-5 for 96 h, relative decreases in the NADPH production were observed (39.5 and 50.5% less than the control) (Fig 4B). With respect to the enzyme 6PGD, an increase in NADPH production (787%) was found only at a concentration of 170.0 μM of eupomatenoid-5 after 24 h (Fig. 4A).

To further confirm if NADPH produced by G6PD is related to the detoxification of H_2O_2, experiments were performed to assess the ability of epimastigotes to metabolize low concentrations of exogenous H_2O_2 (20 μM) [22]. H_2O_2 consumption by epimastigotes was more efficient when they were treated with eupomatenoid-5 for 24 h than for 96 h (Fig. 4A, B). At concentrations of 34.0, 85.0, or 170.0 μM, the consumption of H_2O_2 was 14, 28, and 34% higher than the control, respectively, at 24 h (Fig. 4A). After 96 h of incubation with 34.0 and

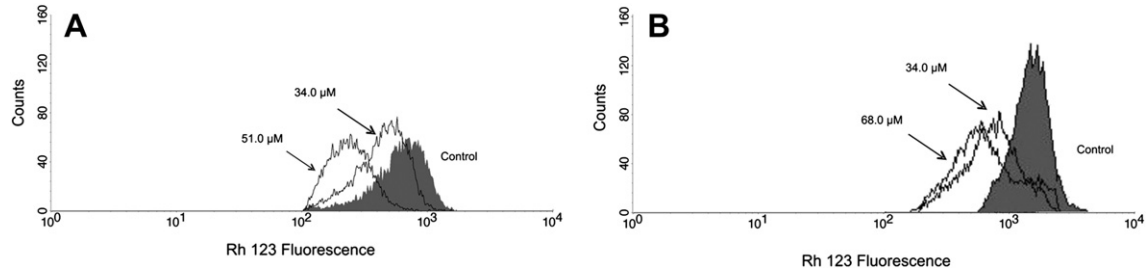

Fig. 3. Flow cytometry analysis of Rh 123-labeled epimastigotes treated with 34.0 and 51.0 μM of eupomatenoid-5 for 96 h (A) and trypomastigotes treated with 34.0 and 68.0 μM of eupomatenoid-5 for 2 h (B). Arrows correspond to doses tested. Control group is also shown. Typical histograms of at least three independent experiments are depicted.

51.0 μM, the increases in H_2O_2 consumption were 4.9 and 8.5% higher, respectively (Fig. 4B).

4. Discussion

This study describes the effect of eupomatenoid-5 on the viability and ultrastructural alterations of trypomastigotes, the infective form of *T. cruzi*. This compound has previously been reported to have a variety of other microbicidal activities including insecticidal, antibacterial, antifungal, as well as leishmanicidal activity [15,23–25].

We found that eupomatenoid-5 induced oxidative damage to epimastigotes and trypomastigotes, as evidenced by lipid peroxidation. The literature reports that lipid peroxidation may cause damage to cells by alterations in cell membranes and in mitochondrial function [26]. As shown here, trypomastigotes were more susceptible to lipoperoxidation than were epimastigotes. This may indicate that the latter form is somehow more efficient than trypomastigotes at detoxifying reactive oxygen species (ROS), perhaps because the levels of trypanothione, the main dithiol antioxidant system of *T. cruzi*, are higher in epimastigotes [11]. These data may explain why eupomatenoid-5 caused no ultrastructural alterations in the cell membrane of epimastigotes [14]. Taken together, these facts suggest two hypothetical conditions: (i) eupomatenoid-5 induces an increase of ROS production or (ii) eupomatenoid-5 decreases antioxidant enzymes activity. Both situations would make this parasite, especially trypomastigotes, sensitive to oxidative stress conditions.

We therefore focused our studies on unraveling the role of eupomatenoid-5 as an inhibitor of the trypanothione system. Based on measurements of G6PD activity, we suggest that the increased G6PD activity obtained when epimastigotes were incubated with eupomatenoid-5, may be a strategy of the parasite to survive in a new oxidizing intracellular environment. Supporting this proposal, G6PD activity is increased in the presence of methylene blue, which oxidizes NADPH, a situation that mimics oxidative stress [27]. Hence, the increased G6PD activity obtained in the lag phase (1 day) may be related to an attempt by the cell to resist the stress caused by the presence of eupomatenoid-5. In the log phase, the decrease observed in G6PD activity may be a result of the continuous oxidative stress induced by eupomatenoid-5 over time. Different patterns of G6PD and 6PGD activities among different strains were also observed along the growth curve when cells were treated with H_2O_2 [22]. Finally, the different

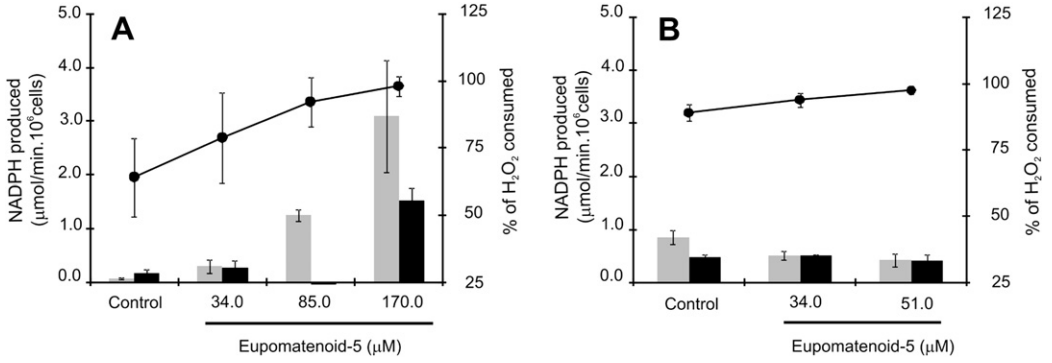

Fig. 4. G6PD, 6PGD activity, and H_2O_2 consumption by epimastigote forms of *Trypanosoma cruzi* treated with eupomatenoid-5. Cells were incubated in LIT medium with different concentrations of eupomatenoid-5 for 24 h (A) or 96 h (B), G6PD (gray bars), 6PGD (black bars), and H_2O_2 consumption (line). Statistical analyses for enzyme activity: G6PD activity in the treatment with 170.0 μM of eupomatenoid-5 compared to the control and 34.0 μM, $p < 0.05$. 6PGD activity in the treatment with 170.0 μM of eupamatenoid-5 compared to the control, 34.0 μM, and 85.0 μM, $p < 0.01$ at 24 h. Statistical analyses for H_2O_2 consumption: at 24 h, 85.0 μM was significantly different from the control, $p < 0.001$, while 170.0 μM was significantly different from the control and 34.0 μM. At 96 h, 34.0 and 51.0 μM was significantly different from the control, $p < 0.01$ and $p < 0.001$, respectively. Each experiment was conducted in duplicate and repeated at least three times.

patterns of G6PD activity along the growth curve resulted in different rates of H_2O_2 detoxification, as we found in the H_2O_2 consumption assay.

We also found that eupomatenoid-5 altered the mitochondrial membrane potential of epimastigotes and trypomastigotes, as evidenced by a decrease in Rh123 fluorescence intensity. These data concord with epimastigotes SEM and TEM observations by Luize et al. [14]. On the other hand our presently trypomastigotes SEM and TEM data reveal exclusively plasma membrane rupture. This fact is reasonable to understand considering the higher susceptible of trypomastigotes to lipoperoxidation and the higher sensibility of rhodamine assay when compared to microscopy assays where only a group of cells is analyzed. Additionally, the mitochondrial destabilization and disorganization have previously been described for other trypanocidal drugs [9,28–31].

In conclusion, our results indicate that eupomatenoid-5 is active against trypomastigote forms, in addition to previously reported effects on epimastigotes and intracellular amastigotes of *T. cruzi*. Our findings support the possibility that different mechanisms, according to the form of the parasite, may be targeted, and that the plasma membrane and mitochondria are the structures that are most affected in trypomastigotes and epimastigotes, respectively. Although many of the specific targets of drugs used to treat *T. cruzi* are known, it is reasonable to suppose that one compound could act on more than one target with independent or combined action, as well in a single preferred pathway. From this perspective we can form a picture of the action of eupomatenoid-5 on *T. cruzi,* independent of the parasite forms, involving alteration of the mitochondrial membrane potential or oxidative damage in different patterns, or in a sequence such as the recently described process "Reactive Oxygen Species (ROS)-induced ROS-release" (RIRR) [32]. This phenomenon involves the increase of ROS within the mitochondria, followed by a collapse of the mitochondrial membrane potential and a transient increase in ROS generation by the electron transfer chain, leading to potentially significant mitochondrial and oxidative cellular damage and cell death.

Acknowledgements

We thank Erika Izumi and Marco Antonio Costa for excellent technical assistance. This study was supported through grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico — CNPq, Capacitação de Aperfeiçoamento de Pessoal de Nível Superior — CAPES, Financiadora de Estudos e Projetos — FINEP, PRONEX/ Fundação Araucária, and Programa de Pós-graduação em Microbiologia da Universidade Estadual de Londrina.

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