Free Radical Biology and Medicine 60 (2013) 17–28

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

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

Original contribution

Further evidence of the trypanocidal action of eupomatenoid-5: Confirmation of involvement of reactive oxygen species and mitochondria owing to a reduction in trypanothione reductase activity

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ARTICLE INFO

Article history:
Received 5 November 2012
Accepted 11 January 2013
Available online 31 January 2013

Keywords:
Trypanosoma cruzi
Eupomatenoid-5
Trypanothione reductase
ROS
Mitochondria
Cell death
Free radicals

ABSTRACT

Our group assays natural products that are less toxic and more effective than available nitroheterocycles as promising therapeutic options for patients with Chagas disease. Our previous study reported the trypanocidal activity of eupomatenoid-5, a neolignan isolated from the leaves of *Piper regnellii* var. *pallescens,* against the three main parasitic forms of *Trypanosoma cruzi.* The present study further characterizes the biochemical and morphological alterations induced by this compound to elucidate the mechanisms of action involved in the cell death of *T. cruzi.* We show that eupomatenoid-5 induced oxidative imbalance in the three parasitic forms, especially trypomastigotes, reflected by a decrease in the activity of trypanothione reductase and increase in the formation of reactive oxygen species (ROS). A reduction of mitochondrial membrane potential was then triggered, further impairing the cell redox system through the production of more ROS and reactive nitrogen species. Altogether, these effects led to oxidative stress, reflected by lipid peroxidation and DNA fragmentation. These alterations are key events in the induction of parasite death through various pathways, including apoptosis, necrosis, and autophagy.

Introduction

Chagas disease, caused by the protozoan *Trypanosoma cruzi,* is considered a serious public health problem that affects approximately 10 million people in Latin America. The incidence of this disease has been estimated to include 300,000 new cases per year, and approximately 10,000 people die from this infection annually [1–3]. Since its discovery in 1909 [4], the treatment of this infection is still challenging because it is restricted to only two nitroderivative compounds, benznidazole and nifurtimox, that have limited efficacy, especially in the chronic phase of the disease, and serious side effects [5].

An urgent need exists for new active compounds that are less toxic and more effective for the treatment of patients with Chagas disease. Numerous studies have reported natural compounds with selective trypanocidal action [6]. Natural products are promising for the treatment of both infectious and noninfectious diseases because of the diversity of their molecular structures [6]. However, few studies have reported the efficacy of trypanocidal compounds against the three forms of *T. cruzi* [7,8]. The scarcity of such studies is reasonable because of the complex life cycle and distinct morphological and functional forms of *T. cruzi* [9,10]. Another important issue is that few studies have demonstrated the likely mechanisms of action of these trypanocidal compounds.

Our group recently reported the trypanocidal activity of eupomatenoid-5, a neolignan isolated from the leaves of *Piper regnellii* var. *pallescens,* against epimastigote, trypomastigote, and amastigote forms of *T. cruzi* [11–13]. This compound induced ultrastructural alterations and was shown to be more selective for parasitic cells than for mammalian cells [12,13].

Considering the trypanocidal activity of eupomatenoid-5, this study sought to better characterize the biochemical alterations induced by this compound in the three parasitic forms of *T. cruzi.* Our goal was to elucidate the mechanisms of action of eupomatenoid-5 involved in the cell death of *T. cruzi.* Based on our previous work [13], we focused our study on mitochondrial dysfunction and plasma membrane disruption induced by eupomatenoid-5. Our results provide further insights into the mechanisms of action of eupomatenoid-5 and strongly suggest that eupomatenoid-5 effectively treats Chagas disease with remarkable trypanocidal action against both forms of *T. cruzi* (i.e., trypomastigotes and amastigotes) that are relevant for mammalian infection. We suggest that the primary target for eupomatenoid-5 may be the trypanothione system, a pathway

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http://dx.doi.org/10.1016/j.freeradbiomed.2013.01.008

that is unique to the parasite and absent in the mammalian host. This system is highly dependent on trypanothione reductase (TR) and plays a key role in the antioxidant activity of trypanosomatids [14].

Materials and methods

Chemicals

Actinomycin D, antimycin A (AA), bovine serum albumin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), digitonin, dihydrorhodamine 123 (DHR), dimethyl sulfoxide (DMSO), monodansylcadaverine (MDC), rhodamine 123 (Rh123), thiobarbituric acid, wortmannin, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen (Grand Island, NY, USA). Annexin-V FITC, the 3,8-phenanthridinediamine-5-(6-triphenylphosphoniumhexyl)-5,6-dihydro-6-phenyl (MitoSOX) kit, propidium iodide (PI), and the terminal deoxynucleotide transferase dUTP nick-end labeling (TUNEL) kit were obtained from Invitrogen (Eugene, OR, USA). The protein assay kit was obtained from Bio-Rad (Hercules, CA, USA). All of the other reagents were of analytical grade.

Isolation of eupomatenoid-5 from leaves of P. regnellii var. pallescens

Eupomatenoid-5 (Fig. 1) was isolated from the leaves of *P. regnellii* collected in the Prof. Irenice Silva Garden of Medicinal Plants on the campus of the State University of Maringa (UEM) in Parana, Brasil. 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). 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 [11]. The compound was purified using absorption–chromatographic methods and identified by analyzing the ultraviolet, infrared, ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, distortionless enhancement polarization transfer, correlated spectroscopy, heteronuclear correlation, nuclear Overhauser effect spectroscopy, heteronuclear multiple bond correlation, and gas chromatography/mass spectrometry spectra. The data were compared with the literature [15].

Stock solutions of eupomatenoid-5 were prepared aseptically in DMSO and diluted in culture medium so that the DMSO concentration did not exceed 1% in the experiments. The concentrations of eupomatenoid-5 used in the assays were based on the IC_{50} and IC_{90} values [11].

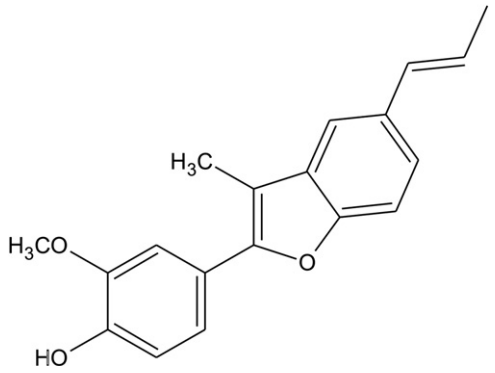

Fig. 1. Structure of eupomatenoid-5, the neolignan isolated from the leaves of *P. regnellii* var. *pallescens*.

Parasites and cell cultures

All of the experiments were performed using the Y strain of *T. cruzi*. Epimastigote forms were maintained axenically at 28 °C with weekly transfers in liver infusion tryptose medium supplemented with 10% heat-inactivated FBS at pH 7.4 [16]. Trypomastigote and amastigote forms were obtained from the supernatants of previously infected monolayers of $LLCMK_2$ cells (i.e., epithelial cells of monkey kidney (*Macaca mulatta*); CCL-7; American Type Culture Collection, Rockville, MD, USA) in DMEM supplemented with 2 mM L-glutamine, 10% FBS, and 50 mg/L gentamicin and buffered with sodium bicarbonate in a 5% CO_2 air mixture at 37 °C.

Mitochondrial membrane potential assay

Mitochondrial membrane potential ($\Delta\Psi m$) was evaluated during exposure of epimastigote forms (1×10^7 cells/ml) to 23.8, 51.0, and 170.0 μM eupomatenoid-5 for 3 h at 28 °C and exposure of trypomastigote and amastigote forms (1×10^7 cells/ml) to 34.0, 68.0, and 170.0 μM eupomatenoid-5 for 3 h at 37 °C using the fluorescent probe Rh123, which accumulates within mitochondria. Afterward, the parasites were washed and incubated with 5 μg/ml Rh123 for 15 min to verify $\Delta\Psi m$. CCCP (100.0 μM) was used as a positive control. The data acquisition and analysis were performed using a FACSCalibur flow cytometer (Becton–Dickinson, Rutherford, NJ, USA) equipped with CellQuest software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA, USA). A total of 10,000 events were acquired in the region that was previously established as the one that corresponded to the parasites. Alterations in Rh123 fluorescence were quantified using an index of variation (IV) obtained from the equation $(M_T - M_C)/M_C$, in which M_T is the median fluorescence for the treated parasites and M_C is the median fluorescence for the control parasites. Negative IV values correspond to depolarization of the mitochondrial membrane.

Cell membrane integrity assay

Cell membrane integrity was evaluated during exposure of epimastigote forms (1×10^7 cells/ml) to 23.8, 51.0, and 170.0 μM eupomatenoid-5 for 3 h at 28 °C and exposure of trypomastigote and amastigote forms (1×10^7 cells/ml) to 34.0, 68.0, and 170.0 μM eupomatenoid-5 for 3 h at 37 °C using PI, a probe that binds to DNA in ruptured membrane cells. Afterward, the parasites were washed and incubated with 0.2 μg/ml PI for 10 min to verify cell membrane integrity. Digitonin (40.0 μM) was used as a positive control. Data acquisition and analysis were performed using a FACSCalibur flow cytometer equipped with CellQuest software. A total of 10,000 events were acquired in the region that was previously established as the one that corresponded to the parasites. Alterations in the fluorescence of PI were quantified as the percentage of increase in the fluorescence compared with the control (untreated parasites).

Fluorimetric detection of mitochondrial-derived superoxide anion ($O_2^{\bullet -}$)

The mitochondrial production of $O_2^{\bullet -}$ was evaluated during exposure of epimastigote forms to 23.8, 51.0, and 170.0 μM eupomatenoid-5 and exposure of trypomastigote and amastigote forms to 34.0, 68.0, and 170.0 μM eupomatenoid-5 using the fluorescent $O_2^{\bullet -}$-sensitive, mitochondrial-targeted probe MitoSOX.