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Andrographolide induces oxidative stress-dependent cell death in unicellular protozoan parasite *Trypanosoma brucei*

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ABSTRACT

African sleeping sickness is a parasitic disease in humans and livestock caused by *Trypanosoma brucei* throughout sub-Saharan Africa. Absence of appropriate vaccines and prevalence of drug resistance proclaim that a new way of therapeutic interventions is essential against African trypanosomiasis. In the present study, we have looked into the effect of andrographolide (andro), a diterpenoid lactone from *Andrographis paiculata* on *Trypanosoma brucei* PRA 380. Although andro has been recognized as a promosing anti-cancer drug, its usefulness against *Trypanosoma* spp remained unexplored. Andro showed promising anti-trypanosomal activity with an IC_{50} value of 8.3 μM assessed through SYBR Green cell viability assay and also showed no cytotoxicity towards normal murine macrophages. Cell cycle analysis revealed that andro could induce sub-G_0/G_1 phase arrest. Flow cytometric analysis also revealed that incubation with andro caused exposure of phosphatidyl serine to the outer leaflet of plasma membrane in *T. brucei* PCF. This event was preceded by andro-induced depolarization of mitochondrial membrane potential (Δym) and elevation of cytosolic calcium. Andro also caused elevation of intracellular reactive oxygen species (ROS) as well as lipid peroxidation level, and depletion in reduced thiol levels. Taken together, these data indicate that andro has promising antitrypanosomal activity mediated by promoting oxidative stress and depolarizing the mitochondrial membrane potential and thereby triggering an apoptosis-like programmed cell death. Therefore, this study merits further investigation to the therapeutic possibility of using andro for the treatment of African trypanosomiasis.

1. Introduction

African trypanosomiasis is a vector-transmitted tropical disease responsible for death and morbidity in humans and domesticated animals in sub-Saharan Africa. Tsetse fly-transmitted trypanosome infections are caused by three subspecies of protozoan parasite *Trypanosoma brucei*, viz. *T. b. brucei, T. b. gambiense* and *T. b. rhodesiense*. The subspecies *T. brucei brucei*, the causative agent of the disease Nagana in cattle is genotypically very similar to human infective subspecies which makes it a good experimental model (Crompton et al., 2010). This unicellular protozoan parasite evades the host immune defence by altering surface antigens (Borst et al., 1996) as well as by suppressing the immune response (Sternberg and Mabbott 1996) attributing the infection as invariably fatal if left untreated. While no available vaccine is in use, very few drugs are effective to treat human African trypanosomiasis (HAT) (La Greca and Magez 2011). Suramin and pentamidine were reported to be effective only against the early stage of the disease and both of these treatments have significant side effects (Feuillan et al., 1987; Nok 2003; Fèvre et al., 2008). Melarsoprol, currently, is the most frequently used drug for the treatment at the second stage of the disease, however, chance of manifestation of an encephalopathic syndrome cannot be ignored (Chappuis et al., 2005). Nifurtimox-eflornithine combination therapy (NECT) has been advocated during last 25 years to improve treatment option (Sykes et al., 2012). Despite this, there is still a need to pursue research to have additional trypanocidal compounds that are safe, cheap, easy to administer, active against all African trypanosome species and effective against both the early- and late-stages of the disease.

In the field of drug discovery, efforts are now being shifted profoundly towards natural products due to their unlimited variety of

Abbreviations: MTT, 3 (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; DAPI, 4, 6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle's Medium; PI, propidium iodide; ROS, Reactive Oxygen Species; NAC, N-acetyl cysteine; MMP, Mitochondrial Membrane Potential; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; CPT, Camptothecin; GSH, reduced glutathione

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novel structures with the possibility of its modification, selective toxicity and cost effectiveness. According to various reports, ~75% of the drugs developed for anti-infectious diseases have their natural origin (Tagboto and Townson 2001).

Andrographolide (andro) is a labdane diterpenoid derived from the herb *Andrographis paniculata* which is a well known traditional remedy in Asia to treat a variety of ailments (Chao and Lin 2010; Wong et al., 2016). Andro has also been reported to exhibit anticancer (Sheeja and Kuttan 2007; Banerjee et al., 2016), anti-inflammatory, antioxidant (Wasman et al., 2011), cardioprotective and hepatoprotective (Yoopan et al., 2007) properties. However, no study was undertaken to evaluate the role of andro as a potential antitrypanosomal agent. Liposomal andrographolide was found to be effective in dropping the parasitic load in the spleen and in relieving *L. donovani* infection related toxicity as well (Sinha et al., 2000). Previous study also revealed filaricidal activities of andro (Niranjan et al., 2010). Andro was also reported to have antiplasmodial activity when tested against the erythrocytic stages of *Plasmodium falciparum* with an IC_{50} of 9.1 μM (Mishra et al., 2011). Being nontoxic to the in vivo system, andro has been extensively used for antimalarial drug development, thus objective of this study was to assess its potency as anti-trypanosomal agent with the comparable inhibitory dose. In the present study, structural and physiological alterations in *T. brucei* following the treatment of andro were investigated and emphasis was given to elucidate the possible mechanism of action on cell death and other apoptotic features.

2. Materials and methods

2.1. Reagents

Andrographolide was purchased from Santa Cruz Biotechnology (CA, USA; naturally synthesized and purity ≥98%), dissolved in 100% DMSO and kept at 4 °C at a concentration of 40 mM. N-Acetyl-L-cysteine (NAC), glutathione, Carbonyl cyanide m-chloro-phenylhydrazone (CCCP), Propidium iodide (PI), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and 4, 6-diamidino-2-phenylindole (DAPI), purchased from Sigma-Aldrich (Saint Louis, MO) were dissolved and stored according to the manufacturer's instructions.

2.2. Parasite culture and maintenance

Procyclic forms (PCF) of *Trypanosoma brucei* (ATCC PRA-380) were maintained at 27 °C under 5% CO_2 in SDM-79 medium supplemented with haemin (7.5 μg/ml) and 10% heat-inactivated fetal bovine serum as described previously (Cross and Manning, 1973). For experimental purposes, exponentially growing PCF trypanosomes were harvested, washed once in phosphate-buffered saline (PBS) and incubated with andro at the given concentrations. Each experiment was performed at least in triplicate.

2.3. Cell viability assay

In vitro anti-trypanosomal activity of andro was determined according to Faria et al. (2015) with slight modifications. Exponentially growing parasites were allowed to grow into 96-well black plates (2×10^5 cells/well) in the absence and presence of andro (0–200 μM) for 72 h. Pentamidine (Sigma-Aldrich, St Louis, MO) (0–25 μM) was considered as a positive control. Cells were then lysed by adding 5 × SYBR Green I (Invitrogen, CA) in lysis buffer containing 0.012% saponin and 0.12% Triton X-100. After incubation for 1 h in the dark, fluorescence signal was measured using SpectraMax M5 microplate reader (Molecular Devices, USA), at an excitation of 485 nm and emission of 530 nm. The results were expressed as percentage inhibition in parasite growth compared to the control (no effect) comprised of 0.5% DMSO. The 50% inhibitory concentration (IC_{50}) of andro was determined by logarithmic regression analysis of the data. Andro was further tested for its cytotoxic effect on RAW 264.7 mammalian cells with increasing concentrations of compound (0–400 μM) and IC_{50} was determined in a similar way. A selectivity index (SI) was calculated and is defined as the RAW IC_{50} value divided by the *T. b brucei* IC_{50} value. Doxorubicin (Sigma-Aldrich) was kept as a positive control drug for the cytotoxicity assay on RAW 264.7.

2.4. Analysis of cytopathological changes

2.4.1. Giemsa staining

T. brucei procyclic cells were treated with 8.3 μM and 17.4 μM (IC_{50} and IC_{90} respectively) concentrations of andro. The treated parasites were then harvested, smeared on slide, stained with Giemsa and examined under light microscope (magnification, x100).

2.4.2. Scanning electron microscopy (SEM)

To observe the morphological alterations, the parasites were treated for 72 h with concentrations that corresponded to the IC_{50} and IC_{90} for andro, harvested with a cell density of 2×10^6 cells/ml and fixed in 2.5% glutaraldehyde (Sigma-Aldrich) for 1 h. Parasites were then adhered on poly-L-lysine-coated coverslips, dehydrated in an ascending series of EtOH, critical-point-dried in CO_2, coated with a thin gold layer (Gluenz et al., 2015) and observed under a scanning electron microscope (Zeiss EVO® LS 10, Germany).

2.5. Flow cytometric analysis of cell cycle progression

FACS analysis was performed as described by Hammarton et al. (2003) with the following modifications: exponentially grown *T. brucei* cells (1×10^7 cells/ml) were treated with an IC_{50} dose of andro (8.3 μM) for 24 and 48 h at 27 °C. Cells were then harvested, washed in 1 × PBS, fixed in ice-cold 70% methanol and kept at 4 °C for 24 h. The cells were then pelleted (1000 *g*, 10 min, 4 °C), rehydrated in PBS, incubated with propidium iodide (PI) staining solution (200 μg/ml RNAse A, 50 μg/ml PI in PBS) for 30 min in the dark at room temperature and acquired in a flow cytometer (FACSVerse™, BD Biosciences, USA). Data were analysed using FACSuite™ software. A total of 10,000 events were acquired.

2.6. Externalization of phosphatidylserine

Phosphatidyl serine externalization from inner to the outer cell membrane, a hallmark of apoptosis, was studied using FITC-conjugated Annexin V (BD Biosciences, CA), a Ca^{2+} −dependent phospholipid-binding protein, following manufacturer's protocol and acquired in a FACSVerse™ (BD Biosciences, USA) flow cytometer equipped with FACSuite™ software. A total of 10,000 events were acquired. Cells were considered necrotic when they were positive only for PI and apoptotic that were stained with Annexin-V (PI-positive or −negative) (Jimenez et al., 2008).

2.7. Detection of chromatin condensation and lipid droplet accumulation

Chromatin condensations in andro treated parasites were detected under confocal microscope after staining with DAPI (4′, 6′-diamidino-2-phenylindole) with a slight modification of previous report (Fernandes Rodrigues et al., 2008). The parasites were treated for 72 h with the concentrations corresponding to the IC_{50} and IC_{90} of andro and were then harvested, washed in PBS, allowed to adhere on 0.01% poly-L-lysine coated slides, fixed with 4% paraformaldehyde and directly stained with DAPI (4 μg/ml) for 20 min at room temperature. Images were captured under a confocal laser scanning microscope (Leica TCS SP8, Leica Microsystems, Germany) using a 63X oil-immersion lens with 360 nm excitation and 454 nm emission and analysed by Leica Application Suite X (LASX) software.

The cytoplasmic lipid bodies in the parasites were similarly detected