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Betulinic acid induces cell death by necrosis in *Trypanosoma cruzi*

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ABSTRACT

Chagas' disease is a neglected disease caused by the protozoan parasite *Trypanosoma cruzi* and constitutes a serious health problem worldwide. The treatment is limited, with variable efficacy of benznidazole and nifurtimox. Betulinic Acid (BA), a triterpene, can be found in medicinal herbs and has a wide variety of biological and pharmacological activities. The objective was to evaluate betulinic acid effects on the cell death mechanism in *Trypanosoma cruzi* strain Y. BA inhibited the growth of epimastigotes in periods of 24 h (IC_{50} = 73.43 μM), 48 h (IC_{50} = 119.8 μM) and 72 h (IC_{50} = 212.2 μM) of incubation; of trypomastigotes (IC_{50} = 51.88 μM) in periods of 24 h and intracellular amastigotes (IC_{50} = 25.94 μM) in periods of 24 and 48 h of incubation, no toxicity on LLC-MK_2 cells at the concentrations used. Analysis of the possible mechanism of parasite cell death showed alterations in mitochondrial membrane potential, alterations in cell membrane integrity, an increase in the formation of reactive oxygen species and increase swelling of the reservosomes. In conclusion, betulinic acid was be able to inhibition all developmental forms of *Trypanosoma cruzi* Y strain with necrotic mechanism and involvement of mitochondrial membrane potential alteration and increase in reactive oxygen species.

1. Introduction

Chagas' disease is one of the seventeen Neglected Tropical Diseases (NTDs), caused by the parasite *Trypanosoma cruzi*. It is considered one of the greatest public health problems in Latin America and an estimated 7 million individuals are infected with *T. cruzi* worldwide, causing more than 7000 deaths a year (WHO, 2015). Benznidazole and nifurtimox, drugs used for the treatment, have important side effects and limited efficacy (Bermudeza et al., 2016). Thus, the search for new therapeutic agents it is necessary.

Betulinic acid (BA), a pentacyclic lupane-type triterpene, is widely found in the medicinal herbs and plants. BA and/or its derivatives exhibits a wide variety of biological and pharmacological activities, including anti-human immunodeficiency virus (HIV), antibacterial, antimalarial, anti-inflammatory, anthelmintic, antinociceptive, anti-herpes simplex viruses-1 (HSV-1), immune-modulatory, antiangiogenic and anticancer activity (Lee et al., 2015). Furthermore, it has been demonstrated that betulinic acid derivatives inhibit growth of epimastigote forms *T. cruzi* strain Tulahuen (Domínguez-Carmona et al., 2010) and reduces the viability of trypomastigote forms of *T. cruzi* strain Y (Meira et al., 2016). In this short communication, it is reported the effect of betulinic acid on cell death mechanism in *Trypanosoma cruzi* strain Y.

2. Materials and methods

2.1. Trypanocidal activity of betulinic acid

Epimastigote forms of *T. cruzi* Y strain were plated in LIT medium (HiMedia Laboratories, Mumbai, IND) supplemented with 10% FBS and antibiotics (100 IU/mL Penicillin and 100 μg/mL streptomycin). Trypomastigote forms were obtained from infected LLC-MK2 cells in T-25/75 cm^2 sterile flasks using DMEM medium (Vitrocell, São Paulo, Brazil) supplemented with 1% antibiotics and 2% of FBS, cultured at 37 °C in an atmosphere with 5% CO_2 (Aparicio et al., 2004) for 24 h. Both epimastigote and trypomastigote forms were incubated in the presence of BA (Sigma–Aldrich™, St. Louis, USA) (1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200 μM) or benznidazole (BZ; ROCHE, São Paulo, BR) (1.56, 3.12, 6.25, 12.5, 25, 50 μg/mL) for 24, 48 and 72 h (epimastigote) and 24 h (trypomastigote) in culture conditions. Parasite growth inhibition was quantified in a Neubauer chamber (Rodrigues et al., 2014; Adade et al., 2014).

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To evaluate the action of BA on the amastigote form, the LLC-MK2 cells were seeded in well plates containing glass coverslips cultivated in DMEM medium, supplemented with 10% FBS and antibiotics for 24 h at 37 °C in a 5% CO_2. Furthermore, the cells were infected with trypomastigote forms at 5×10^6 parasite/mL in DMEM medium supplemented with 2% FBS. After 48 h of infection, the parasites were washed with PBS to remove the non-interiorized parasites. After that, the infected LLC-MK2 cells were treated with BA at concentrations of 25.94 μM (½ IC_{50}) and incubated for 24 and 48 h. The coverslips were washed with PBS, fixed in Bouin's solution, stained with Giemsa and permanently mounted. The number of intracellular amastigotes per 100 cells was determined by counting 300 cells in triplicate (Zingales et al., 1998; Zingales et al., 1997; Andrews and Colli, 1982).

2.2. Cytotoxicity assay of betulinic acid against LLC-MK2 cells

LLC-MK2 cell were plated in DMEM medium supplemented with 10% FBS and antibiotics, and then incubated in a 5% CO_2 at 37 °C for 24 h. The cells were then incubated with BA (200, 400, 800 and 1600 μM) and BZ (1.56, 3.12, 6.25, 12.5, 25, 50 μg/mL) for 24 h. Cell viability was measured using a standard MTT assay (AMRESCO, Ohio, USA) (Mosmann, 1983). The absorbance at 570 nm was analyzed in a microplate reader (Biochrom® Asys Expert Plus). Cell viability was calculated in comparison with the control group.

2.3. Mechanism of action evaluation

a) *Cytometry flow analysis:* in order to evaluate BA action mechanism, treated cells were analyzed by flow cytometry after staining with specific markers. It was used a FACSCalibur flow cytometer and at least 10,000 live events were counted and analyzed using Cell Quest software (Becton-Dickinson, San Jose, CA). Epimastigote forms treated with BA (73.43 μM) for 24 h were stained with annexin V-PE/7-AAD according to the manufacturer's instructions (BD Biosciences, California, USA) to evaluate cell death by apoptosis or necrosis. For mitochondrial transmembrane potential evaluation, treated parasites were analyzed after Rhodamine 123 labeling (10 μg/mL). Reactive Oxygen Species (ROS) measurement was also assessed, using DCFH-DA (20 mML). For investigation of reservosome swelling, acridine orange (5 μg/mL) was used as marker of acidic vesicles.
b) *Confocal microscopy:* Epimastigote forms were incubated with IC_{50} (73.43 μM) of BA for 24 h at 28 °C. After that, the parasites were washed and labeled with Rhodamine 123 (10 μg/mL) to assess the mitochondrial transmembrane potential. The cells were analyzed under a LSM 710 confocal laser scanning microscope (Zeiss, Germany).

2.4. Statistical analysis

The data were expressed as SEM of at least three independent experiments and were analyzed using two-way or one-way analysis of variance (ANOVA) followed by Bonferroni post-tests or Student's *t*-test. Values of $p \leq 0.05$ were considered statistically significant. Growth capacity 50% of the cellular growth (IC50) estimated by non-linear regression and the percentage of viable parasites. The statistical analysis was performed using GraphPad Prism 5.0 program (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

In the present study, the strain Y of T. cruzi was selected, since presents partial resistance to benznidazole (Cherkesova et al., 2014). BA inhibited the growth of epimastigote forms after 24, 48 and 72 h incubation (1.96–200 μM) and reduced the viability of trypomastigote forms after 24 h of treatment (Table 1). These data corroborate previous studies assessing the effect of betulinic acid and its derivatives on epimastigote forms of *T. cruzi* strain Tulahuen (Domínguez-Carmona et al., 2010) and trypomastigote forms of *T. cruzi* strain Y (Meira et al., 2016).

To evaluate the cytotoxic effects of BA, LLC-MK2 cells were treated with different concentrations of BA using the MTT assay. It wasn't observed toxicity on cells at the concentrations used (Fig. 1A). The IC_{50} was 391.8 μM after 24 h treatment (Table 1). The IC_{50} was compared with the activity against trypomastigotes (IC_{50}), yielding the selectivity index (SI; ratio of the IC_{50} in $LLCMK_2$ cells to the IC_{50} in protozoa). BA was shown to be more toxic to the protozoa than to mammalian cells (SI = 7.5 for trypomastigotes) (Table 1). The BA showed a superior selectivity index than benznidazole, the standard drug, and exhibited a potential selectivity when tested on $LLC\text{-}MK_2$.

The present study shows for the first time the effects of BA on intracellular forms of *T. cruzi*, with decrease on the number of amastigote per 100 cells (Fig. 1B) and the percentage of infected cells (Fig. 1C) after 24 and 48 h of incubation (25.94 μM), when compared to untreated infected cells with BA. BZ, the reference drug, also caused antiamastigote activity, with 64.4 ± 3.6% of infected cells at 257 μM and 48.8 ± 5.5% at 514 μM.

BA trypanocidal effect is supported by other studies, where it displays antiparasitic effect on other protozoa, as such as *Leishmania major* (Begum et al., 2014), *L. tarentolae* and *Plasmodium falciparum* (Graziose et al., 2012). Furthermore, AB and its derivates also have shown leishmanicidal effect on promastigote forms of *L. amazonensis*, *L. braziliensis* and *L. donovani* (Domínguez-Carmona et al., 2010).

After this, flow cytometry assays were performed to evaluate changes on morphological and biochemical characteristics on *T. cruzi* cells treated with BA. These changes are useful to identify specific cell death profiles and identify BA biological effect. Some events, as loss of plasma membrane integrity, characterize necrotic mechanisms, while externalization of phosphatidylserine (PS) and loss of mitochondrial membrane potential (ΔYm) occurs in apoptotic cells (Menna-Barreto and Castro, 2015).

Experiments using stained-Rho 123 epimastigotes were performed to evaluate ΔΨm. It was observed a decrease on Rho 123 accumulation after treatment with BA (73.43 μM), as shown by flow cytometry (Fig. 1D) and by confocal microscopy (Fig. 1E). This data suggests alteration on mitochondrial transmembrane potential in BA-treated parasites. In addition, it was observed an increase on ROS production (Fig. 1F), verified by enhance on DCF-labeled cells.

Additionally, *T. cruzi* cells were stained with acridine orange (AO) after treatment with BA. This dye accumulates in acidic compartments, and it is used as a marker of reservosome on hemoflagellate parasites (Klionsky et al., 2016). In this study, it was observed an increase on the level of AO labeling, indicating reservosome swelling. This event is characteristic of autophagic mechanisms, through permeabilization of acidic vesicles and enzymatic digestion of intracellular components (Krysko et al., 2008).

Finally, 7-AAD and annexin V were used as markers of necrosis and apoptosis, respectively. As shown in Fig. 1H, epimastigote forms treated with BA exhibited a significant increase in double-marked (DM) fluorescence and, especially, those marked with 7AAD fluorescence intensity after 24 h of treatment compared with untreated parasites.

Altogether, the results demonstrates that BA effect on *T. cruzi* epimastigotes is related to necrosis induction, with increase on ROS production, loss of mitochondrial integrity and reservosome swelling. These data corroborate with previous studies, that demonstrated that betulinic acid causes death in *T. cruzi* trypomastigotes mainly by necrosis (Meira et al., 2016). In contrast, BA causes phosphatidylserine externalization and DNA fragmentation assays in *Leishmania infantum* promastigote forms (Sousa et al., 2014), suggesting that different cell death mechanisms may be involved on its action mechanisms on parasites.

In conclusion, betulinic acid was able to inhibit all developmental

Table 1
Anti-parasitic activity of Betulinic Acid on *T. cruzi* and host cell cytotoxicity.

	Epimastigote *(T. cruzi)*		Trypomastigote (*T. cruzi)*		LLC-MK_2[a]		SI	
	BA IC_{50} ± SEM (μM)	BZ IC_{50} ± SEM (μM)	BA IC_{50} ± SEM (μM)	BZ IC_{50} ± SEM (μM)	BA IC_{50} ± SEM (μM)	BZ IC_{50} ± SEM (μM)	BA	BZ
24 h	73.43(± 0.09)	218(± 55)	51.88(± 0.14)	25(± 20)	391.8(± 0.05)	616(± 61)	7.5	2.3
48 h	119.8(± 0.06)	61(± 10)	–	–	–	–	–	–
72 h	212.2(± 0.13)	16,5 (± 3)	–	–	–	–	–	–

BA = betulinic acid. BZ = benznidazole. IC_{50} = inhibitory concentration at 50%. SI = Selective index. SEM = Standard error mean. Values are expressed as mean ± SEM of three independent experiments performed in triplicate.

[a] Cell viability of LLC-MK_2 determined 24 h after treatment.

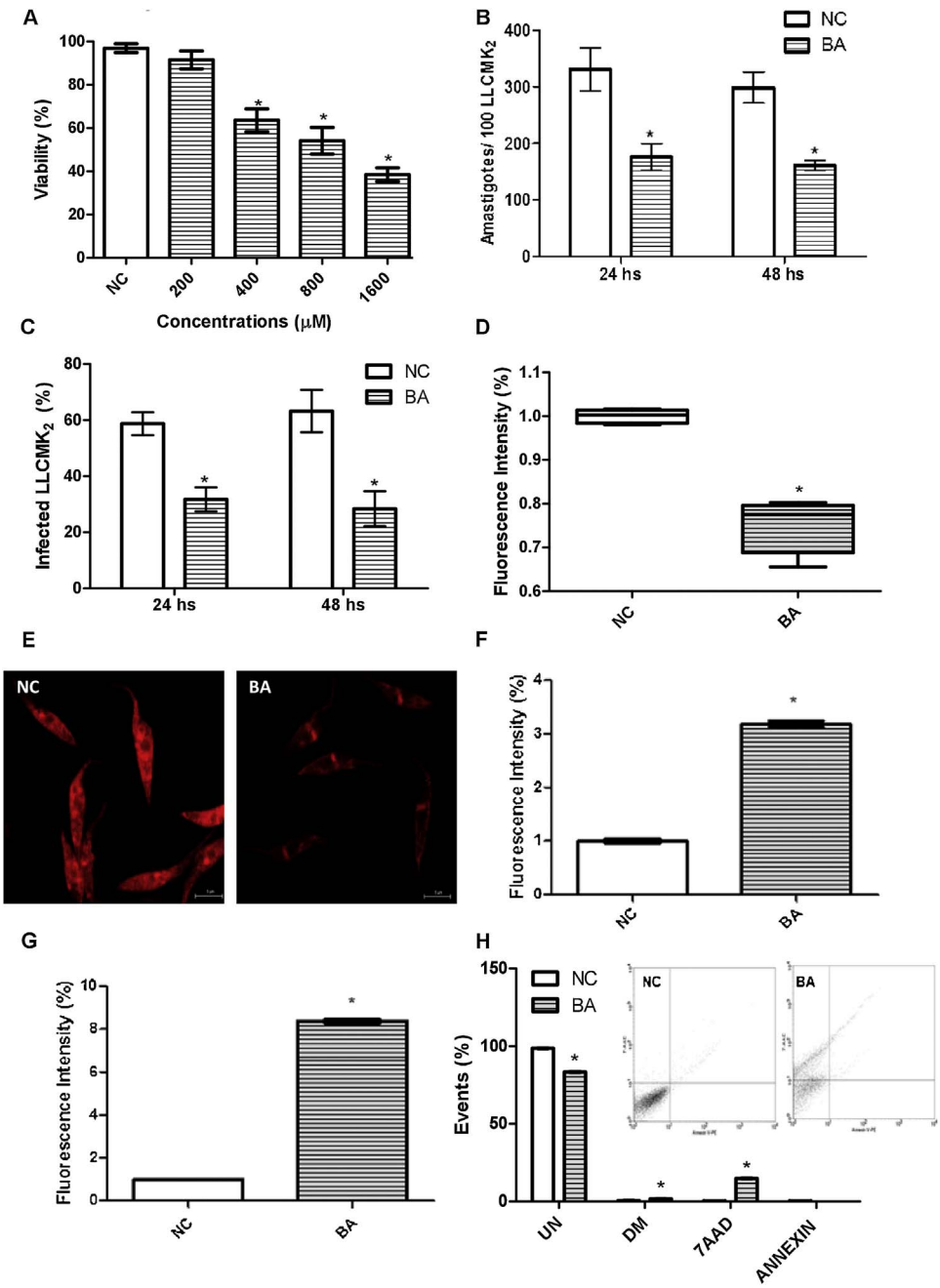

Fig. 1. (A) Cytotoxic effect of Betulinic Acid (BA) for 24 h on LLC-MK_2 cells in MTT assay. (B) Effect of BA on amastigote/100 cells counting after 24 and 48 h of incubation. (C) Effect of BA on percentage of infected cells after 24 and 48 h of incubation. (D) Evaluation of mitochondrial transmembrane potential (ΔΨm) of epimastigote forms treated with BA for 24 h measured by Rhodamine 123 by flow cytometry analysis. (E) Evaluation of mitochondrial transmembrane potential (ΔΨm) of epimastigote forms treated with BA (IC_{50}) for 24 h measured by Rhodamine 123 staining with confocal laser scanning microscopy. (F) Total ROS production of epimastigote forms treated with BA for 24 h and stained with the cell-permeable probe H_2DCFDA staining, by flow cytometry. (G) Epimastigote forms treated with BA for 24 h and stained with the cell-permeable probe Orange Acridine (OA) by flow cytometry analysis. (H) Evaluation of cell death pathway of epimastigote forms treated with BA for 24 h measured by annexin V (AX) and 7AAD staining and detected by flow cytometry. Values are expressed as mean ± SEM of three independent experiments performed in triplicate. *Significantly different from the control group (two-way and one-way ANOVA and Bonferroni post-test; Student's *t*-test *$p < 0.05$).

forms of *Trypanosoma cruzi* Y strain. Additionally, its mechanism is related to necrotic induction, mitochondrial membrane potential disruption, increase on ROS production and reservosome swelling.

Conflicts of interest

No conflicts of interest are declared.

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