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Antiparasitic evaluation of betulinic acid derivatives reveals effective and selective anti-*Trypanosoma cruzi* inhibitors

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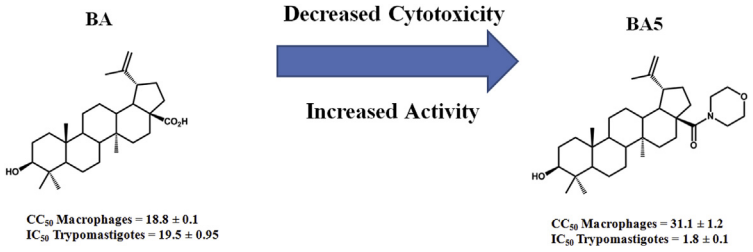
HIGHLIGHTS

- Terpenoids are potent and selective trypanocidal agents.
- BA5 destroys parasite cells by necrotic death.
- Betulinic acid derivatives inhibit the growth of intracellular amastigotes of *T. cruzi*.
- Combination of BA5 and benznidazole showed synergistic effects.

GRAPHICAL ABSTRACT



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ABSTRACT

Betulinic acid is a pentacyclic triterpenoid with several biological properties already described, including antiparasitic activity. Here, the anti-*Trypanosoma cruzi* activity of betulinic acid and its semi-synthetic amide derivatives (BA1-BA8) was investigated. The anti-*Trypanosoma cruzi* activity and selectivity were enhanced in semi-synthetic derivatives, specially on derivatives BA5, BA6 and BA8. To understand the mechanism of action underlying betulinic acid anti-*T. cruzi* activity, we investigated ultrastructural changes by electron microscopy. Ultrastructural studies showed that trypomastigotes incubated with BA5 had membrane blebling, flagella retraction, atypical cytoplasmic vacuoles and Golgi cisternae dilatation. Flow cytometry analysis showed that parasite death is mainly caused by necrosis. Treatment with derivatives BA5, BA6 or BA8 reduced the invasion process, as well as intracellular parasite development in host cells, with a potency and selectivity similar to that observed in benznidazole-treated cells. More importantly, the combination of BA5 and benznidazole revealed synergistic effects on trypomastigote and amastigote forms of *T. cruzi*. In conclusion, we demonstrated that BA5 compound is an effective and selective anti-*T. cruzi* agent.

1. Introduction

Chagas disease is a neglected disease caused by the protozoan parasite *Trypanosoma cruzi* and constitutes a serious public health problem worldwide (Pinto-Dias, 2006). It affects about 8–10

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million people, mainly in Latin American countries, where this disease is endemic (Rassi et al., 2010). Pharmacotherapy is based on nifurtimox and benznidazole, which are recommended to treat all infected people (Urbina and Docampo, 2003). Treatment with benznidazole is associated with side effects, prolonged treatment time and low and variable efficacy in chronic phase of infection, which is the most prevalent form of the disease (Urbina and Docampo, 2003; Viotti et al., 2009; Morillo et al., 2015). This scenario emphasizes a need to develop safer and more effective drugs.

Natural products play an important role in drug discovery and development (Newman and Cragg, 2012). Naturally occurring terpenoids represent an important class of bioactive compounds that exhibit several medicinal properties (Yin, 2015). This is exemplified by betulinic acid, a lupane-type pentacyclic triterpenoid abundant in the plant kingdom, which can be isolated from several plant species or obtained from its metabolic precursor, betulin (Yogeeswari and Sriram, 2005). Betulinic acid and its derivatives possess anti-HIV activity, anti-bacterial, antihelmitic, anti-inflammatory and a potent cytotoxic activity against a large panel of tumor cell lines (Baglin et al., 2003; Chandramu et al., 2003; Yogeeswari and Sriram, 2005; Drag et al., 2009; Costa et al., 2014; Chakraborty et al., 2015).

Betulinic acid and other triterpenoids, both naturally-occurring and semi-synthetic, have also been investigated as antiparasitic agents (Hoet et al., 2007; Innocente et al., 2012). More specifically, the activity of betulinic acid and its derivatives against the erythrocitic stage of a chloroquine-resistant *Plasmodium falciparum* strain, as well as antileishmanial activity on different *Leishmania* species, were reported (Alakurtti et al., 2010; Chen et al., 2010; Innocente et al., 2012; Sousa et al., 2014). Regarding the anti-*Trypanosoma cruzi* activity, it was previously shown that betulinic acid and ester derivatives inhibit epimastigote proliferation (Domínguez-Carmona et al., 2010). In view of these findings, betulinic acid is considered to be a prototype for the design and synthesis of antiprotozoal agents. Chemical modifications of the carboxyl group have suggested that this part of the molecule can produce derivatives with enhanced antiprotozoal activity when compared to betulinic acid (Gros et al., 2006; Domínguez-Carmona et al., 2010; Da Silva et al., 2011; Sousa et al., 2014; Spivak et al., 2014). Based on these facts, the purpose of our work was to evaluate the trypanocidal potential of new semi-synthetic amide derivatives of betulinic acid.

2. Material and methods

2.1. Chemistry

Betulinic acid (BA) was extracted from the bark of *Ziziphus joazeiro* Mart. (Rhamnaceae) by using a previously described method (Barbosa-Filho et al., 1985). Semi-synthetic compounds (BA1 to BA8) were prepared from betulinic acid (Fig. 1). Betulinic acid was initially converted to mixed anhydride by using isobutyl chloroformate (Sigma-Aldrich), followed by addition of the respective secondary amines. This methodology allowed the synthesis of compounds BA1 to BA8 (Fig. 1), with yields varying from 30 to 41% after HPLC purification.

2.2. General procedure for the synthesis

Two mmol (0.1 g) of betulinic acid were added to a 100 mL round bottom flask under magnetic stirring and dissolved in dichloromethane (10 mL). The mixture was cooled to −10 °C and then 4-dimethylaminopiridine isobutyl chloroformate (3.0 mmol, 0.4 g) in dichloromethane (1 mL) was slowly added during 30 min. The mixture was maintained under stirring for 3 h. The reaction was transferred to a separating funnel, to which 50 mL of ethyl ether were added and the organic phase was quickly washed with a saturated solution of sodium bisulfite (1 × 50 mL), washed with water and with saturated NaCl solution (2 × 50 mL). The organic phase was separated, dried over $MgSO_4$, and evaporated under reduced pressure to give a white thick oily residue, which was crystallized in cyclohexane. Purity was analyzed by HPLC and structures examined by HRESIMS. Mass spectrometry was performed in a Q-TOF spectrometer (nanoUPLC-Xevo G2 Tof, Waters). ESI was carried out in the positive ion mode. HPLC analysis was carried out in Beckmann Coulter using UV detector in a C_{18} column (100 Å, 2.14 × 25 cm) with a linear gradient of 5–95% MeCN/H_2O in 0.1% TFA. Compound **BA1**. 95% (HPLC). HRESIMS Anal. Calc. (Found)/Error for $C_{34}H_{55}NO_2$: 509.4232 (510.4713, $[M+H^+]$)/6.5 ppm. Compound **BA2**. 94% (HPLC). HRESIMS Anal. Calc. (Found)/Error for $C_{35}H_{58}N_2O_2$: 538.4498 (539.4491, $[M+H^+]$)/5.7 ppm. Compound **BA3**. 98% (HPLC). HRESIMS Anal. Calc. (Found)/Error for $C_{35}H_{57}NO_2$: 523.4389 (524.5031, $[M+H^+]$)/5.0 ppm. Compound **BA4**. 98% (HPLC). HRESIMS Anal. Calc. (Found)/Error for $C_{34}H_{56}N_2O_2$: 524.4341 (524.4249, $[M+H^+]$)/4.0 ppm. Compound **BA5**. 95% (HPLC). HRESIMS Anal. Calc. (Found)/Error for $C_{33}H_{53}NO_3$: 511.4025 (512.3177, $[M+H^+]$)/10 ppm. Compound **BA6**. 94% (HPLC). HRESIMS Anal. Calc. (Found)/Error for $C_{33}H_{53}NSO_2$: 527.3797 (529.1641, $[M+H^+]$)/20 ppm. Compound **BA7**. 95% (HPLC). HRESIMS Anal. Calc. (Found)/Error for $C_{40}H_{58}NFO_2$: 603.4451 (604.4085, $[M+H^+]$)/4.0 ppm. Compound **BA8**. 98% (HPLC). HRESIMS Anal. Calc. (Found)/Error for $C_{40}H_{59}NO_2$: 585.4545 (589.7109, $[M+H^+]$)/10 ppm.

2.3. Cytotoxicity to mammalian cells

Peritoneal exudate macrophages were obtained by washing, with cold RPMI medium, the peritoneal cavity of BALB/c mice 4–5 days after injection of 3% thioglycolate in saline (1.5 mL per mice). Then, cells were placed into 96-well plates at a density 1×10^5 cells/well in RPMI-1640 medium without phenol red (Sigma-Aldrich, St. Louis, MO) supplemented with 10% of fetal bovine serum (FBS; Cultilab, Campinas, Brazil), and 50 μg/mL of gentamycin (Novafarma, Anápolis, Brazil) and incubated for 24 h at 37 °C and 5% CO_2. After that time, each compound was added in triplicate at eight concentrations ranging from 0.04 to 100 μM and incubated for 6 or 72 h. Twenty μL/well of AlamarBlue (Invitrogen, Carlsbad, CA) was added to the plates during 10 h. Colorimetric readings were performed at 570 and 600 nm. CC_{50} values were calculated using data-points gathered from three independent experiments. Gentian violet (Synth, São Paulo, Brazil) was used as a cytotoxicity control, at concentrations ranging from 0.04 to 10 μM.

2.4. Cytotoxicity for trypomastigotes

Bloodstream trypomastigotes forms of *T.* cruzi (Y strain) were obtained from supernatants of LLC-MK2 cells previously infected and maintained in RPMI-1640 medium supplemented with 10% FBS, and 50 μg/mL gentamycin at 37 °C and 5% CO_2. Parasites (4×10^5 cells/well) were dispensed into 96-well plates and the test inhibitors were added at eight concentrations ranging from 0.04 to 100 μM in triplicate, and the plate was incubated for 24 h at 37 °C and 5% of CO_2. Aliquots of each well were collected and the number of viable parasites was assessed in a Neubauer chamber and compared to untreated cultures. Benznidazole (LAFEPE, Recife, Brazil) was used as positive control in the anti-*Trypanosoma cruzi* studies. Three independent experiments were performed.

2.5. In vitro T. cruzi infection assay

Peritoneal exudate macrophages were plated at a cell density of