Full length article

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

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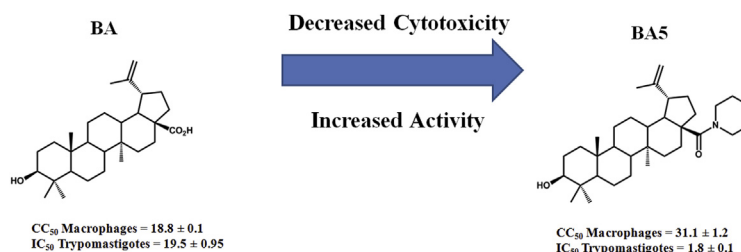[c] Universidade do Estado da Bahia, Departamento de Ciências da Vida, Salvador, BA, Brazil
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HIGHLIGHTS

- 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

BA → Decreased Cytotoxicity / Increased Activity → BA5

CC$_{50}$ Macrophages = 18.8 ± 0.1
IC$_{50}$ Trypomastigotes = 19.5 ± 0.95

CC$_{50}$ Macrophages = 31.1 ± 1.2
IC$_{50}$ Trypomastigotes = 1.8 ± 0.1

ARTICLE INFO

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 blebbing, 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.

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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

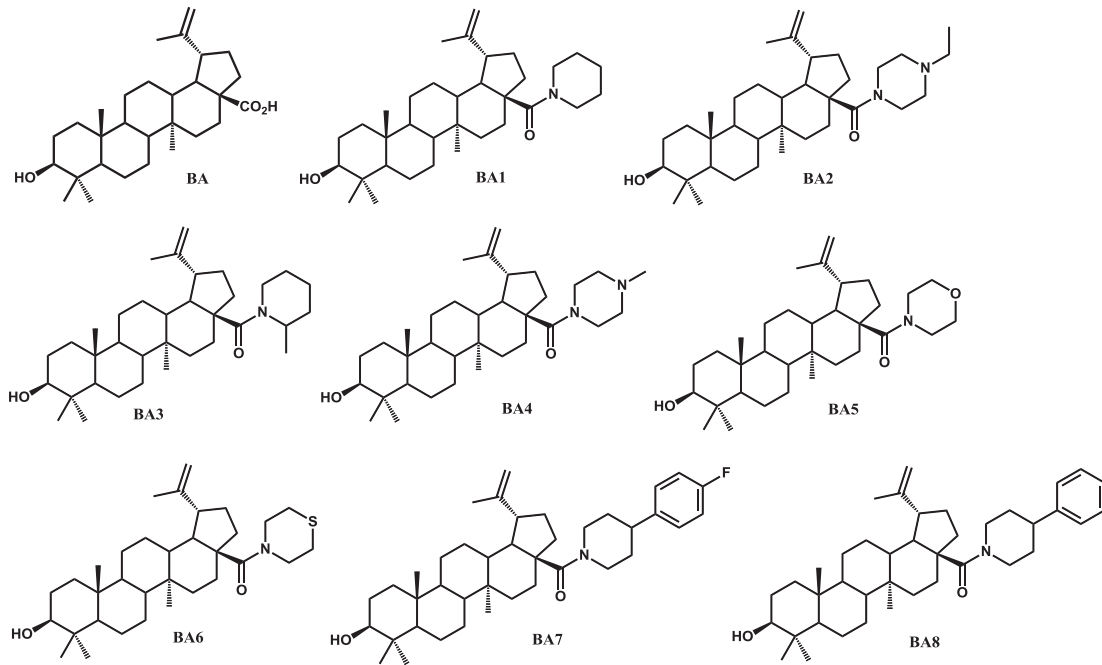Peritoneal exudate macrophages were plated at a cell density of

Fig. 1. Structure of betulinic acid and its derivatives.

2×10^5 cells/well in 24-well plates with sterile coverslips on the bottom in RPMI supplemented with 10% FBS and incubated for 24 h at 37 °C and 5% CO$_2$. Cells were then infected with trypomastigotes at a ratio of 10 parasites per macrophage for 2 h. Free trypomastigotes were removed by successive washes using saline solution. Cultures were incubated in complete medium alone or with the compounds under investigation in different concentrations for 6 h. The medium was replaced with fresh medium and the plate was incubated for 3 days (Soares et al., 2012). Cells were fixed in absolute alcohol and the percentage of infected macrophages and the mean number of amastigotes/100 macrophages was determined by manual counting after hematoxylin and eosin staining in an optical microscope (Olympus, Tokyo, Japan). The percentage of infected macrophages and the relative number of amastigotes per macrophage was determined by counting 100 cells per slide. Experiments were performed three times.

2.6. Trypanosoma cruzi invasion assay

Peritoneal exudate macrophages (10^5 cells) were plated onto sterile coverslips in 24-well plates and kept for 24 h. Plates were washed with saline solution and trypomastigotes were then added at a cell density of 1×10^7 parasites/well along with the addition of BA5, or BA6 or BA8 (50 μM). The plate was incubated for 2 h at 37 °C and 5% CO$_2$, followed by successive washes with saline solution to remove extracellular trypomastigotes. Plates were maintained in RPMI medium supplemented with 10% FBS at 37 °C for 2 h. Infected macrophages were examined for the presence of amastigotes by optical microscopy using a standard hematoxylin and eosin staining. Amphotericin B (Gibco Laboratories, Gaithersburg, MD) was used as a positive control in this assay. Three independent experiments were performed.

2.7. Ultrastructural studies

Trypomastigotes at a cell density of 1×10^7 cells/mL in 24 well-plates were treated with test inhibitor BA5 (2 or 4 μM) or not for 24 h. Parasites were then fixed with 2% formaldehyde and 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in sodium cacodylate buffer (0.1 M, pH 7.2) for 1 h at room temperature. After fixation, parasites were washed 3 times with sodium cacodylate buffer (0.1 M, pH 7.2), and post-fixed with a 1.0% solution of osmium tetroxide containing 0.8% potassium ferrocyanide (Sigma) for 1 h. Cells were subsequently dehydrated in increasing concentrations of acetone (30, 50, 70, 90 and 100%) for 10 min at each step and embedded in polybed resin (PolyScience family, Warrington, PA). Ultrathin sections on copper grids were contrasted with uranyl acetate and lead citrate and observed under a ZEISS 109 transmission electron microscope. For scanning electron microscopy, trypomastigotes treated with or without BA 505 (2 or 4 μM) and fixed in the same conditions were washed in 0.1 M cacodylate buffer, and allowed to adhere in coverslips pre-coated with poly-L-lysine (Sigma). Cells were then post-fixed with a solution of osmium tetroxide containing 0.8% of potassium ferrocyanide for 30 min and dehydrated in crescent concentrations of ethanol (30, 50, 70, 90 and 100%). The samples were dried until the critical point, metallized with gold and analyzed in a JEOL JSM-6390LV scanning electron microscope. Two independent experiments were performed.

2.8. Propidium iodide and annexin V staining

Trypomastigotes 1×10^7/mL in 24 well-plates were treated with 5 or 10 μM of BA5 in RPMI supplemented with FBS at 37 °C for 24 or 72 h and labeled for propidium iodide (PI) and annexin V using the annexin V-FITC apoptosis detection kit (Sigma), according to the manufacturer's instructions. Acquisition and analyses was performed using a FACS Calibur flow cytometer (Becton Dickinson, San Diego, CA), with FlowJo software (Tree Star, Ashland, OR). A total of 10,000 events were acquired in the region previously established as that corresponding to trypomastigotes forms of T. cruzi. Two independent experiments were performed.

Table 1
Cytotoxicity against macrophages and anti-*Trypanosoma cruzi* activity against trypomastigotes forms of betulinic acid and its derivatives.

Compound	CC$_{50}$ (μM)[a]	IC$_{50}$ Try. (μM)	SI
BA	18.8 (±0.1)	19.5 (±0.9)	0
BA1	>100	>100	–
BA2	>100	>100	–
BA3	>100	13.7 (±2.3)	7.3
BA4	39.7 (±0.5)	10.2 (±1.0)	3.9
BA5	31.1 (±1.2)	1.8 (±0.1)	17.3
BA6	28.7 (±1.1)	5.4 (±1.3)	5.3
BA7	>100	55 (±0.6)	1.8
BA8	53.5 (±0.4)	5 (±0.5)	10.7
Gentian violet	0.5 (±0.1)	–	–
Benznidazole	>100	11.4 (±1.4)	8.8

CC$_{50}$ = cytotoxicity concentration 50%. IC$_{50}$ = inhibitory concentration. SI = Selective index. 50%. Values are means ± SD of three independent experiments performed in triplicate.

[a] Cell viability of BALB/c mouse macrophages determined 72 h after treatment.

2.9. Drug combination

For *in vitro* drug combinations, doubling dilutions of each drug (BA5 and benznidazole), used alone or in fixed combinations were incubated with trypomastigotes or intracellular parasites followed the protocols described above. The analysis of the combined effects was performed by determining the combination index (CI), used as cutoff to determine synergism, by using Chou-Talalay CI method (Chou and Talalay, 2005) and through the construction of isobologram using the fixed ratio method, as described previously (Fivelman et al., 2004).

2.10. Statistical analyses

To determine the cytotoxicity concentration 50% of BALB/c mice macrophages (CC$_{50}$) and the inhibitory concentration 50% (IC$_{50}$) of the trypomastigotes and amastigotes forms of *T. cruzi*, we used nonlinear regression. The selectivity index (SI) was defined as the ratio of CC$_{50}$ by IC$_{50}$ (trypomastigotes or amastigotes). The one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine the statistical significance of the group comparisons in the *in vitro* infection studies and cell invasion study. Results were considered statistically significant when $P < 0.05$. All analyses were performed using Graph Pad Prism version 5.01 (Graph Pad Software, San Diego, CA).

3. Results

3.1. Trypanocidal and cytotoxicity activity

First the compounds had their anti-*T. cruzi* activity determined against trypomastigote forms (Table 1). The structural design of the
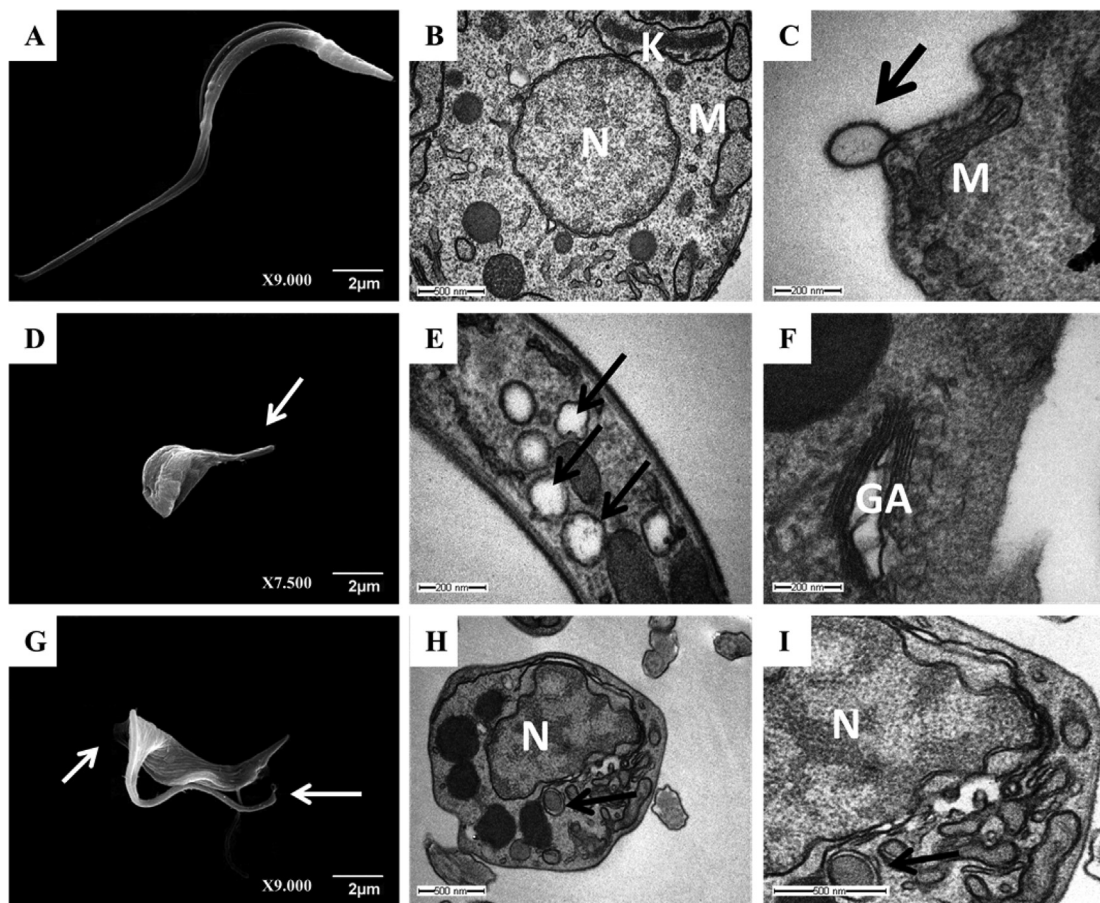

Fig. 2. Electron microscopy of trypomastigotes of *T. cruzi* treated with BA5 for 24 h. Using SEM, we observed alterations in cell shape and flagella retraction (2 μM; D) and loss of plasma membrane integrity and body deformation (4 μM; G), untreated cells (A). White arrows indicate the alterations reported. Scale bars = 2 μm. Using TEM, we observed appearance of membrane blebbling (2 μM; C), the formation of numerous and atypical vacuoles within the cytoplasm (2 μM; E), dilatation of some Golgi cisternae (2 μM; F) and (4 μM; H and I) appearance of profiles of endoplasmatic reticulum involving organelles accompanied by the formation of autophagosomes, (B) untreated cells. Black arrows indicate changes in the organelles.
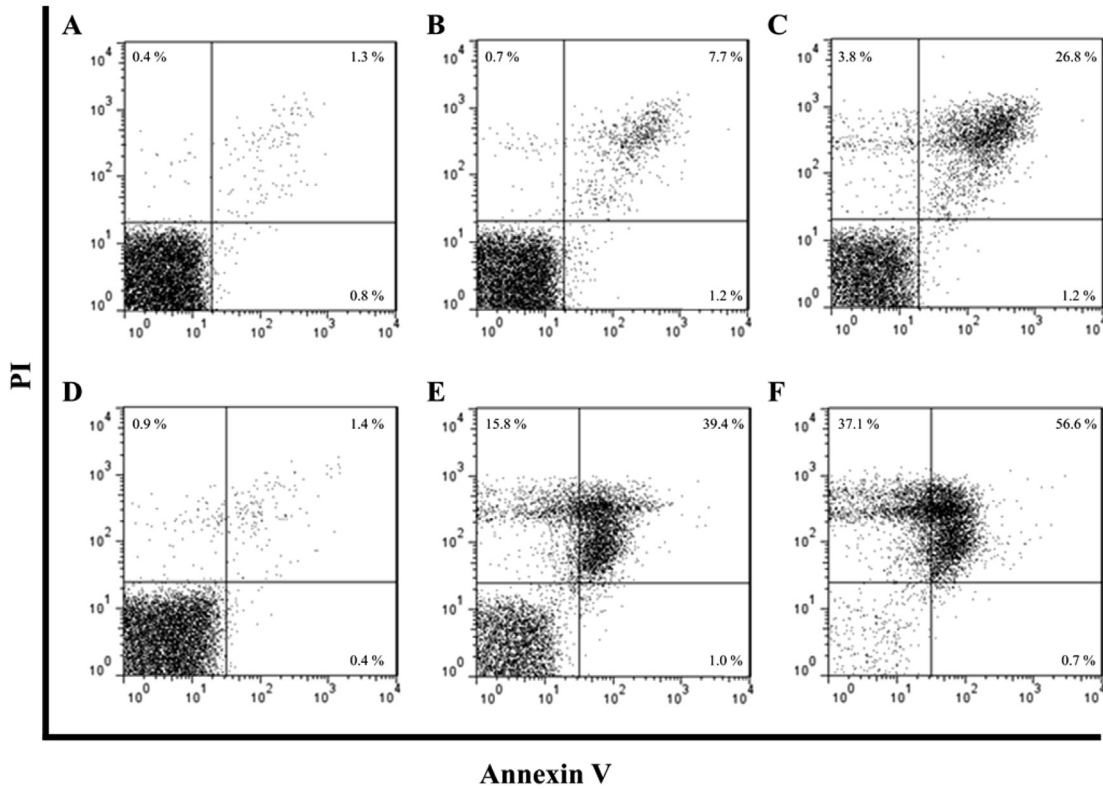
Fig. 3. Flow cytometry analysis of trypomastigotes treated with BA5 and incubated with propidium iodide (PI) and annexin V. (A) Untreated trypomastigotes after 24 h of incubation; (B and C) trypomastigotes treated with 5 and 10 μM of BA5, respectively by 24 h. (D) Untreated trypomastigotes after 72 h of incubation. (E and F) Trypomastigotes treated with 5 or 10 μM of BA5, respectively, for 72 h.
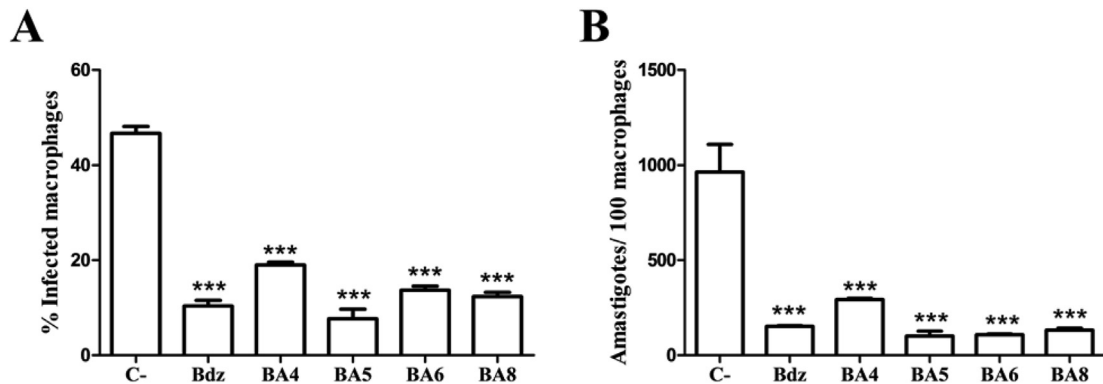

Fig. 4. The betulinic acid derivatives inhibit the growth of intracellular amastigotes of T. cruzi. Mouse peritoneal macrophages were infected with Y strain trypomastigotes for 2 h and treated with betulinic acid derivatives (50 μM) or benznidazole (50 μM) for 6 h. Infected cells were stained with hematoxylin and eosin and analyzed by optical microscopy. Bdz = benznidazole. Values represent the mean ± SEM of triplicate. ***, $P < 0.001$ compared with untreated cultures.

Table 2
Antiparasitic activity in intracellular parasite, host cells cytotoxicity and selectivity index of derivatives.

Comp.	Amastigotes $IC_{50} \pm$ S.D.(μM)[a]	Macrophages $CC_{50} \pm$ S.D.(μM)[b]	Selectivity index[c]
BA4	18.9 (±1.2)	>100	>5.3
BA5	10.6 (±0.8)	>100	>9.4
BA6	12.4 (±1.7)	>100	>8
BA8	13.2 (±1.6)	>100	>7.6
Bdz	13.5 (±1.3)	>100	>7.4

IC_{50} and CC_{50} values were calculated using concentrations in triplicate, in three independent experiments. IC_{50} = inhibitory concentration at 50%. CC_{50} = cytotoxic concentration at 50%. S.D. = standard deviation. Bdz = benznidazole.
[a] Cells were exposed to derivatives for 6 h and activity was determined 3 days after incubation with derivatives.
[b] Cell viability of BALB/c mouse macrophages determined 6 h after treatment.
[c] SI is selectivity index, calculated by the ratio of CC_{50} (macrophages) and IC_{50} (amastigotes).
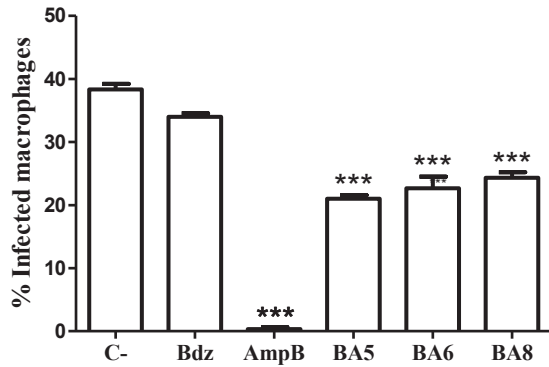
Fig. 5. The betulinic acid derivatives impair *T. cruzi* trypomastigote invasion in macrophages. Mouse macrophages were simultaneously infected with 10^7 Y strain trypomastigotes and treated with betulinic acid derivatives (50 μM) or benznidazole (50 μM) for 2 h. Bdz = benznidazole; AmpB = amphotericin B. ***, $P < 0.001$ compared with untreated cultures.

betulinic acid derivatives was performed to investigate the piperidinyl moiety attached to the C28 position of carboxylic acid function, varying thus the hydrogen-bond sites. In fact, most of semi-synthetic derivatives had IC_{50} values lower than betulinic acid and, in some cases (BA4, BA5, BA6 and BA8), the values of IC_{50} was also lower than benznidazole, the reference drug. More specifically, the attachment of morpholyl, thiomorpholyl or a 4-methyl-phenylpiperidine group produced the most potent compounds.

Next, the cytotoxicity of the compounds to mouse macrophages was analyzed. As shown in Table 1, all semi-synthetic derivatives presented values of CC_{50} higher than betulinic acid. The derivatives BA5 and BA8 showed the best profiles of selectivity with values of SI of 17.3 and 8.8 respectively.

3.2. Investigating the mechanism of action

We used scanning electron microscopy (SEM) to study the morphology of trypomastigotes treated or not with the most potent derivative (BA5). Untreated trypomastigotes had the typical elongated shape of the parasite without visible alterations in the plasma membrane or in cell volume (Fig. 2A). On the other hand, trypomastigotes treated for 24 h with BA5 (2 or 4 μM) had flagella retraction (Fig. 2D), loss of plasma membrane integrity and body deformation (Fig. 2G).

Transmission electron microscopy (TEM) was also used to examine ultrastructural alterations. Thin sections of untreated trypomastigotes observed by TEM revealed normal appearance of organelles, intact plasma membrane and cytoplasm without alterations (Fig. 2B). However, treatment with BA5 (2 or 4 μM) caused plasma membrane alterations (Fig. 2C), the formation of numerous atypical vacuoles within the cytoplasm (Fig. 2E), dilatation of Golgi cisternae (Fig. 2F) and profiles of endoplasmatic reticulum involving organelles as nucleus accompanied by the formation of autophagosomes (Fig. 2H and I).

To understand the mechanism by which compound BA5 causes parasite death, a double staining with annexin V and propidium iodide (PI) was performed for flow cytometry analysis. In untreated cultures, most cells were negative for annexin V and PI staining, demonstrating cell viability. In contrast, a significant increase in the number of PI-positive parasites was observed in cultures treated with BA5 at 5 and 10 μM. Treatment with 5 μM of BA5 for 72 h resulted in 15.8% and 39.4% of cells positively stained for PI and PI$^+$ annexin V$^+$, respectively (Fig. 3). These results suggest that the BA5 derivative induces a necrotic process in *Trypanosoma cruzi* trypomastigotes.

3.3. In vitro infection studies

We next investigated the ability of BA4, BA5, BA6 and BA8

Table 3
Concentration reductions and combination indexes for anti-*T. cruzi* activity by BA5 and benznidazole on trypomastigotes and amastigotes forms of *T. cruzi*.

Compounds	$IC_{50} \pm S.D$ (μM)[a] Trypomastigotes		$IC_{50} \pm S.D$ (μM)[a] Amastigotes		CI[b]	
	Drug alone	Combination	Drug alone	Combination	Trypo	Ama.
BA5	2.6 ± 0.2	0.9 ± 0.1	11.2 ± 0.9	3.7 ± 0.8	0.45 ± 0.1	0.84 ± 0.1
Bdz	10.6 ± 0.9	1.3 ± 0.4	13.6 ± 0.1	5.8 ± 0.6		

[a] IC_{50} values were calculated using concentrations in triplicates and two independent experiments were performed.
[b] Combination index (CI). Cutoff: CI value of 0.3–0.7, synergism; 0.7–0.85, moderate synergism; 0.85–0.9, slight synergism; 0.9–1.1, additively; > 1.1, antagonism.
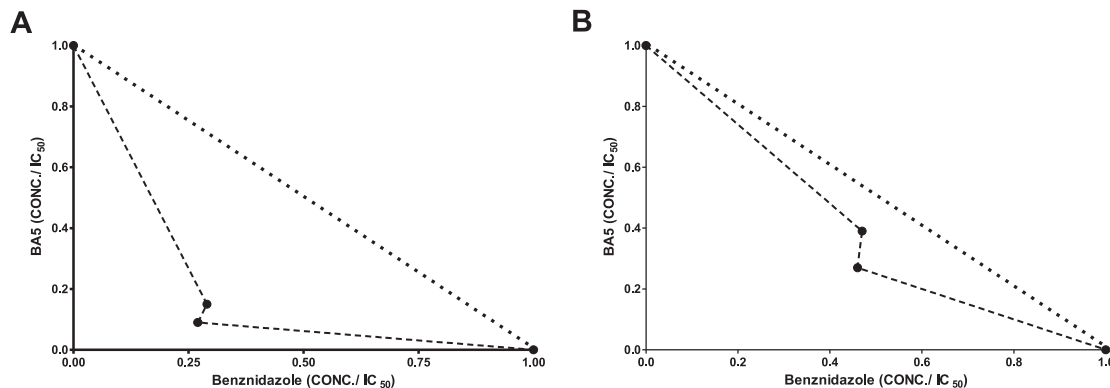S.D = standard deviation.

Fig. 6. Isobologram describing the synergistic effects of BA5 and benznidazole on *T. cruzi* trypomastigotes (**A**) and amastigotes (**B**). Dotted lines correspond to the predicted positions of the experimental points for additive effects.

derivatives to inhibit the development of parasites in host cells. Macrophages infected with *Trypanosoma cruzi* trypomastigotes treated with 50 μM of the compounds were stained with hematoxylin and eosin for analysis by optical microscopy. As shown in Fig. 4, treatment with the semi-synthetic compounds significantly decreased the percentage of infected macrophages ($P < 0.001$) and the relative number of amastigotes per 100 macrophages ($P < 0.001$) when compared with untreated cultures. When tested at different concentrations, it was possible to calculate the IC_{50} value of the derivatives against intracellular parasites. As shown in Table 2, the derivatives had an equipotent activity when compared to benznidazole. Cytotoxicity measured at 6 h of drug exposure demonstrated that benznidazole, as well as all derivatives testes are not cytotoxic at the tested concentrations ($CC_{50} > 100$ μM). These data demonstrate that the derivatives are selective compounds.

We also evaluated the effect of the most potent derivatives on the invasion process. In this assay, peritoneal macrophages were exposed to trypomastigotes and at the same time treated with the semi-synthetic BA5, BA6 and BA8 derivatives (50 μM) for 2 h. After this time, the cells were washed with saline solution to remove extracellular parasites and incubated for 2 additional hours. Cells were stained with hematoxylin and eosin and analyzed by optical microscopy. Amphotericin B was used as a positive control for this experiment. As shown in Fig. 5, the derivatives significantly inhibited the parasite invasion in comparison to untreated cells ($P < 0.001$), but not as efficiently as the positive control amphotericin B, although they were more effective than benznidazole, that show no significant activity on this assay.

Finally, the antiparasitic effect of BA5 and benznidazole in combination was investigated against trypomastigote and amastigote the forms of *Trypanosoma cruzi*. In comparison to the drugs alone, the combination of BA5 and benznidazole reduced IC_{50} values in both forms of the parasite. In fact, the IC_{50} of benznidazole decreased in average by 88% and 43% when combined with EEPA BA5 against trypomastigotes and amastigotes forms of *T. cruzi* respectively (Table 3).

An isobologram analysis and CI calculation, which can distinguish between the synergistic, additive and antagonistic effects of two compounds, confirmed that the combination of BA5 and benznidazole resulted in a remarkable synergistic effect on trypomastigotes and a moderate synergistic effect on amastigotes (Table 3; Fig. 6).

4. Discussion

Terpenoids, such as betulinic acid, are some of the most investigated source of antiparasitic compounds in terms of potency and selectivity (Newman and Cragg, 2012). In the present study, we investigated the anti-*Trypanosoma cruzi* activity of amide semi-synthetic betulinic acid derivatives containing substituents attached in the lupane backbone. We observed that an incorporation of an amide on C-28 enhanced the anti-*T. cruzi* activity. This led to the identification of compound BA5, which exhibited a potency superior to benznidazole, the current standard drug. Several reports describe the chemical modifications of betulinic acid at C-28 position to produce semi-synthetic derivatives with enhanced antimalarial, anti-tumor and anti-viral activities (Jeong et al., 1999; Baltina et al., 2003; Domínguez-Carmona et al., 2010). This is the first report, however, regarding the contribution of the incorporation of amides on C-28 as drug design strategy to enhance the anti-*T. cruzi* activity.

An examination of parasite morphology revealed that compound BA5 is parasiticidal, by altering parasite ultrastructure. It induced flagella retraction, loss of plasma membrane integrity and notable cell body deformation. Interestingly, the treatment with

BA5 also led to the formation of numerous and atypical vacuoles within the cytoplasm, as well as the dilatation of Golgi cisternae. Lack of membrane integrity and cytoplasmic vacuolization are often associated to necrotic parasitic death (Rodriguez et al., 2006; Zong and Thompson, 2006). Through flow cytometry assays, we confirmed the parasitic death by necrosis. In addition, we observed that the treatment produced endoplasmatic reticulum profiles involving organelles as nucleus accompanied by the formation of autophagosomes, typical features of autophagy (Tsujimoto and Shimizu, 2005; Fernandes et al., 2012).

Most importantly, the semi-synthetic derivatives were able to prevent the parasite development and invasion into host cells, crucial events for *T. cruzi* infection establishment, with potency similar to benznidazole. As a limitation, these compounds didn't eliminate intracellular amastigotes even in the highest concentration tested. However, the derivative BA5 exhibited synergistic activity when used in combination to benznidazole. These results encourage further investigations, since the combination of drugs is becoming increasingly attractive to combat parasitic diseases (Alirol et al., 2013; Diniz et al., 2013; Sousa et al., 2014).

Altogether, these findings reinforce that terpenoids are potent and selective trypanocidal agents. Therefore, the screening for structurally-related BA5 derivative for Chagas disease treatment is an attractive line of drug development.

5. Conclusion

Here, we have reported the synthesis and anti-*T. cruzi* evaluation of betulinic acid and its new amide semi-synthetic derivatives. By varying substituents attached to the amide group, we could find substituents that retain, enhance or greatly increase the trypanocidal activity, in comparison to betulinic acid. Specifically, we identified the betulinic acid derivative BA5 as a selective anti-*T. cruzi* agent, which destroys parasite cells by necrotic death and acts synergistically in combination to benznidazole.

Acknowledgements

This work was supported by grants from CNPq (grant number 562655/2010-7), PRONEX (grant number 0002/2014), FAPESB (grant number 0042/2013) and FINEP (grant number 01.04.0320-00).

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