Toxicon 130 (2017) 56–62

Contents lists available at ScienceDirect

Toxicon

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

Evaluation of the antichagasic activity of batroxicidin, a cathelicidin-related antimicrobial peptide found in *Bothrops atrox* venom gland

Clarissa Perdigão Mello [a], Danya Bandeira Lima [a], Ramon Róseo Paula Pessoa Bezerra de Menezes [b], Izabel Cristina Justino Bandeira [a], Louise Donadello Tessarolo [a], Tiago Lima Sampaio [b], Claudio Borges Falcão [a], Gandhi Rádis-Baptista [c], Alice Maria Costa Martins [a, *]

[a] Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Ceará, Fortaleza, CE, Brazil
[b] Departamento de Fisiologia e Farmacologia, Faculdade de Medicina, Universidade Federal do Ceará, Fortaleza, CE, Brazil
[c] Instituto de Ciências do Mar, Universidade Federal do Ceara, Fortaleza, CE, Brazil

ARTICLE INFO

Article history:
Received 22 January 2017
Received in revised form
23 February 2017
Accepted 24 February 2017
Available online 27 February 2017

Keywords:
Batroxicidin
Trypanosoma cruzi
Chagas' disease
Necrosis
Cathelicidin
Antimicrobial peptide

ABSTRACT

Antimicrobial peptides (AMPs) are potential alternatives to conventional antibiotics, as they have a fast mode of action, a low likelihood of resistance development and can act in conjunction with existing drug regimens. We report in this study the effects of batroxicidin (BatxC), a cathelicidin-related AMP from *Bothrops atrox* venom gland, over *Trypanosoma cruzi*, a protozoan that causes Chagas' disease. BatxC inhibited all *T. cruzi* (Y strain: benznidazole-resistant) developmental forms, with selectivity index of 315. Later, separate flow cytometry assays showed *T. cruzi* cell labeling by 7-aminoactinomycin D, the increase in reactive oxygen species and the loss of mitochondrial membrane potential when the parasite was treated with BatxC, which are indication of necrosis. *T. cruzi* cell death pathway by a necrotic mechanism was finally confirmed by scanning electron microscopy which observed loss of cell membrane integrity. In conclusion, BatxC was able to inhibit *T. cruzi*, with high selectivity index, by inducing necrosis.

1. Introduction

Chagas' disease also known as American trypanosomiasis is one of the world's 13 neglected diseases. According to the World Health Organization an estimated 10 million people are infected worldwide and more than 28 million are at risk of the disease. The causative agent is the flagellated protozoan *Trypanosoma cruzi*, which is transmitted by a triatomine insect (Volpato et al., 2015; WHO, 2016).

The current available drugs to treat Chagas' disease, nifurtimox and benznidazole, are only effective in the acute phase, but fails to eradicate the intracellular form of parasites in chronic phase (Morilla and Romero, 2015). Futhermore, these medicines causes undesirable adverse effects in up to 40% of patients, which include nausea, vomiting, abdominal pain and several neurological effects (Marin-Neto et al., 2009).

The search for new molecules with potential anti-T. cruzi activity has been intensified in the field of natural products, with a special emphasis on selective polypeptides from animal venoms. These venom polypeptides and their fractions have stimulated many studies for drug discovery, with some notable successes (Harvey, 2014). A specific group of molecules derived from such venoms with potential anti-protozoa activities comprises the so-called antimicrobial peptides (AMPs), which have shown activity not only against *T. cruzi* but also against African trypanosomes and Leishmania spp. Also named host defense peptides, they are amphipathic molecules mainly composed of positively charged (hydrophilic) and hydrophobic residues, which can interact with and disrupt parasite cell membranes (Mcgwire and Kulkarni, 2010; Teixeira et al., 2012). An important class of these AMPs regards to the cathelicidins, which are found as multi-effector molecules of the innate immunity in numerous vertebrate animals. All cathelicidins share in common a conserved N-terminal preproregion composed of the signal peptide and the cathelin, cathepsin L inhibitor, domain and a variable C-terminal region which contains the mature sequence responsible for the antimicrobial activity (Gao

* Corresponding author.
E-mail address: martinsalice@gmail.com (A.M.C. Martins).

http://dx.doi.org/10.1016/j.toxicon.2017.02.031
0041-0101/

et al., 2015).

Recently, our research groups have found and characterized cathelicidin precursors from the venom gland cDNA libraries of several species of South American pit viper snakes, including *Crotalus durissus terrificus*, *C.d. cascavella*, *Lachesis muta muta*, *Bothrops atrox* and *B. lutzi*; the deduced mature sequences of these snake venom cathelicidins were collectively named vipericidins (Rádis-Baptista, 2015). Batroxicidin, vipericidin from *B. atrox*, was obtained and it shows good antibacterial activity, especially against Gram-negative species (Falcão et al., 2014).

In this study, we have evaluated the anti-parasitic effect of batroxicidin, focusing on its potential activity against *T. cruzi*. This aim was pursued by verifying the cathelicidin-related peptide trypanocidal activity against all protozoan morphological forms. Additionally, the main mechanism that trigger *T. cruzi* cell death was also investigated followed of the treatment with batroxicidin.

2. Materials and methods

2.1. Chemicals and cells

Batroxicidin (BatxC) (KRFKKFFKKLKNSVKKRVKKFFRKPRVIGVTFPF-amide; MW = 4258.63 g/mol) was obtained as described by Falcão et al. (2014). The peptide stock solutions were prepared at 1000 μM with deionized water as required and kept at 4 °C for up to six weeks. Beznidazole (Bz) ($C_{12}H_{12}N_4O_3$; MW = 260.249 g/mol) was obtained from Roche® (Basileia, Switzerland) and stock solutions were prepared at 1000 mM with PBS and kept at 4 °C for up six weeks. The Rhesus monkey kidney cells LLC-MK2 (ATCC CCL-7) were cultured in DMEM medium (Vitrocell, São Paulo, Brazil) supplemented with 10% FBS and 1% antibiotics solution in T-25/75 cm^2 flasks maintained at 37 °C in a humidified atmosphere with 5% CO_2. These cells were split each time they reached 80–90% confluence after being harvested with a solution containing 0.25% trypsin and 2.21 mM EDTA. *Trypanosoma cruzi* Y (benznidazole-resistant) strain were isolated in Laboratory of parasites Biochemistry, University of São Paulo (São Paulo – Brasil) and donated in epimastigote and trypomastigote forms.

2.2. Batroxicidin activity assay against T. cruzi epimastigote forms

Epimastigote forms of *T. cruzi* Y stain were added to 96-well plates (10^6 parasites/mL) containing 2-fold serial dilutions of BatxC or Bz in Liver Infusion Tryptose medium supplemented with 1% antibiotics and 10% of FBS. Final concentration ranges were: 0.19–100 μM of BatxC and 15,6–1000 μM of Bz. Parasites growth inhibition was quantified in a Neubauer chamber after incubation at 28 °C for 24, 48 and 72 h (Rodrigues et al., 2014). Relative viability was determined with parasites treated with only sterile PBS in medium as negative controls (100% viability) and experiments carried out in triplicate.

2.3. Batroxicidin activity assay against T. cruzi trypomastigote forms

The trypomastigote forms of *T. cruzi* were obtained by infecting of LLCMK2 cells with the protozoa (trypomastigote) in T-25/75 cm^2 flasks at 37 °C and 5% CO_2 in DMEM medium (Vitrocell, São Paulo, Brazil) supplemented with 1% antibiotics and 2% FBS for 24 h. After that time trypomastigote forms were seeded in 96 well plates and 2-fold serial dilutions of either BatxC or Bz in DMEM were added at the same final concentrations ranges of BatxC and Bz used against the epimastigote forms. After 24 h incubation at 37 °C, parasite growth inhibition was quantified in a Neubauer chamber (Adade et al., 2014). Relative cell viability was calculated with cells treated with only sterile PBS in medium as negative controls and experiments were carried out in triplicate.

2.4. BatxC cytoxicity evaluation toward LLC-MK2 cells

Cell viability was also measured using a standard MTT assay (Vanden Berghe et al., 2013). LLC-MK2 cells were plated in the DMEM medium, treated with different concentrations of BatxC and incubated at 37 °C for 24 h. MTT (Amresco, Ohio, USA; 5 mg/mL) was added and the cells were incubated for 4 h, when 10% Sodium dodecyl sulphate (SDS; Vetec, São Paulo, Brazil) was added to solubilize the formazan product. Cell viability measurements were performed at 570 nm on a microplate reader (Biochrom® Asys Expert Plus). Relative cell viability was calculated with cells treated with only sterile PBS in medium as negative controls and experiments were carried out in triplicate. The Selectivity index (SI) was calculated by the ratio of cytotoxic/trypanocidal activity (Nwaka and Hudson, 2006).

2.5. Batroxicidin activity assay against T. cruzi amastigote forms

LLC-MK2 cells were seeded in 24-well plates (5×10^5/well) containing at the bottom glass coverslips (13-mm diameter) and cultured in DMEM medium supplemented with 10% FBS and 1% antibiotics, at 37 °C, in a 5% CO2 atmosphere, for 24 h. Next, the media was removed from the wells and LLC-MK2 cells were infected with trypomastigote forms (parasite: host cell ratio of 20:1) in DMEM medium containing 2% FBS. After 48 h of incubation, the cells were washed to remove the non-internalized parasites and treated with either BatxC (0.44 or 0.88 μM) or Bz (282 or 564 μM) in DMEM medium with 2% FBS. After incubation for 24 or 48 h at 37 °C, the coverslips were collected, washed with PBS, fixed in Bouin's solution and stained with Giemsa (Lima et al., 2016). Infected LLC-MK2 not treated with either Bz and BatxC peptide cells were used as controls and the number of intracellular amastigotes per 100 cells was counted in triplicates.

2.6. T. cruzi epimastigote death pathway assays

Experiments of flow cytometry were performed to investigate and evaluate the pathways by which BaxtC induces *T. cruzi* death. The epimastigote forms were treated with BatxC at their respective IC_{50} and 2x IC_{50} concentrations (11.3 and 22.6 μM) in Liver Infusion Tryptose medium. After incubation for 6 and 24 h at 28 °C, the parasite cells were washed and stained with FITC-annexin V (Ax) and/or 7-AAD according to the manufacturer's instructions (BD Pharmingen, California, USA) to evaluate cell death by apoptosis or necrosis. In separate experiments, the mitochondrial transmembrane potential, the reactive oxygen species (ROS) and the swelling of reservosomes were also assessed. After treatment of *T. cruzi* epimastigote forms with BatxC at its IC50 (11.3 μM) for 6 and 24 h at 28 °C, the parasites were stained with 10 μg/mL Rhodamine 123 or 20 mMol/L DCFH-DA or 5 μg/mL Acridine Orange (Sigma – Aldrich™, St. Louis, MO), according to the manufacturer's instructions, respectively. Next, these parasite samples were run in a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA), in which 10,000 live events were collected and analyzed by means of the Cell Quest software (Becton-Dickinson, San Jose, CA). Epimastigotes without BatxC treatment were used as controls and experiments were carried out in triplicate.

2.7. Scanning electron microscopy (SEM)

Epimastigote forms were treated with BatxC peptide at its IC_{50}