# Antichagasic effect of crotalicidin, a cathelicidin-like vipericidin, found in *Crotalus durissus terrificus* rattlesnake's venom gland

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## Abstract

Cathelicidins are antimicrobial peptides produced by humans and animals in response to various pathogenic microbes. Crotalicidin (Ctn), a cathelicidin-related vipericidin from the South American *Crotalus durissus terrificus* rattlesnake's venom gland, and its fragments have demonstrated antimicrobial and antifungal activity, similarly to human cathelicidin LL-37. In order to provide templates for the development of modern trypanocidal agents, the present study evaluated the antichagasic effect of these four peptides (Ctn, Ctn[1-14], Ctn[15-34] and LL-37). Herein, Ctn and short derived peptides were tested against the epimastigote, trypomastigote and amastigote forms of *Trypanosoma cruzi* Y strain (benznidazole-resistant strain) and cytotoxicity in mammalian cells was evaluated against LLC-MK2 lineage cells. Ctn inhibited all *T. cruzi* developmental forms, including amastigotes, which is implicated in the burden of infection in the chronic phase of Chagas disease. Moreover, Ctn showed a high selective index against trypomastigote forms (>200). Ctn induced cell death in *T. cruzi* through necrosis, as determined by flow cytometry analyses with specific molecular probes and morphological alterations, such as loss of membrane integrity and cell shrinkage, as observed through scanning electron microscopy. Overall, Ctn seems to be a promising template for the development of antichagasic agents.

## Introduction

Chagas disease, also known as American trypanosomiasis, is an important public health problem in Latin America and it is increasingly spreading in other areas. It is estimated that around six million people are affected worldwide and approximately 7000 deaths occur annually (WHO, 2016). The current treatment for Chagas disease includes two nitro-heterocyclic drugs – benznidazole (Bz) and nifurtimox – which are only effective against the parasite in the acute phase of infection. The use of these drugs is limited due to their poor bioavailability and side-effects, such as allergic dermatitis, pruritus and gastrointestinal manifestations among others (WHO, 2016; Chatelain, 2017). These facts highlight an urgent need for the development of more effective and safer drugs to be used alone or in combination with other chemotherapeutic agents for the treatment of Chagas disease.

In the last decades, several antimicrobial peptides (AMPs) have been discovered. AMPs are naturally designed to target the cell membranes of pathogens and are widely seen as a promising alternative to conventional antimicrobials considering the rise in multi-antibiotic resistance (Sani and Separovic, 2016). Cathelicidins comprise one of these AMP families and are characterized by a conserved cathelin domain and a variable C-terminal cationic antimicrobial domain (Wódz and Brzezińiska-Błaszczyk, 2015). Recently, our research group have found and characterized cathelicidin precursors from the venom gland cDNA libraries of several species of South American pit viper snakes, called vipericidins (Rádis-Baptista, 2015). Crotalicidin (Ctn), a vipericidin from the *Crotalus durissus terrificus*, has shown antibacterial (Falcão *et al.* 2014) and antiproliferative activities against several cancer cell lines (Falcão *et al.* 2015). *In silico* dissection of Ctn yielded the fragments Ctn[1–14] and Ctn[15–34], which were tested to establish to what extent they reproduced the structure and activity of the parent peptide. Indeed, Ctn[15–34] displayed antibacterial, anti-tumour (Falcão *et al.* 2015) and antifungal activity (Cavalcante *et al.* 2016) with very low haemolytic effect (Falcão *et al.* 2015). Recently, Ctn antiviral activity [15–34] was also demonstrated in an *in vitro* model of shrimp haemocytes (Vieira-Girão *et al.* 2017).

The trypanosomicidal activity of animal venom components has been described by other authors (Adade *et al.* 2013; Souza *et al.* 2016). In general, these venom antimicrobial components are amphipathic molecules mainly constituted of positively charged (hydrophilic) and hydrophobic residues, which can interact with and disrupt parasite cell membranes (Mcgwire and Kulkarni, 2010; Teixeira *et al.* 2012). Thus, the aim of this work was to evaluate

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the antichagasic effect of cathelicidin Ctn and its short fragments (Ctn[1–14] and Ctn[15–34]) in comparison with the paradigmatic human cathelicidin LL-37 (hCAP18). Additionally, the main mechanisms of Ctn and Ctn peptides that trigger *T. cruzi* cell death were also investigated.

## Materials and methods

### Peptides and Bz

Ctn, Ctn[1–14] and Ctn[15–34] were obtained as described by Falcão *et al.* (2014, 2015). The human cathelicidin LL-37/hCAP18 was synthesized according to Falcão *et al.* (2014). The 1 mM peptide stock solutions were prepared by separately weighting and dissolving the right amount of each peptide in deionized water and kept at 4 °C for up to 6 weeks. Their amino acid sequences were analysed using the 'Peptide property calculator' (http://www.pepcalc.com) and 'Heliquest' (http://heliquest.ipmc.cnrs.fr) software programmes to obtain some physicochemical properties. Bz ($C_{12}H_{12}N_4O_3$; MW = 260.249 g mol$^{-1}$) was obtained from Roche® (Basileia, Switzerland) and stock solutions were prepared with 1000 mM (1 M) of PBS and kept at 4 °C until its use within 6 weeks.

### Trypanosoma cruzi and LLC-MK2 cell cultures

The Rhesus monkey kidney cells LLC-MK2 (ATCC CCL-7) were cultured in DMEM medium (Vitrocell, São Paulo, Brazil) supplemented with 10% fetal calf serum (FBS) and 1% antibiotics (100 U mL$^{-1}$ penicillin and 100 $\mu$g mL$^{-1}$ streptomycin) solution in T-25/75 cm$^2$ flasks and were maintained at 37 °C in a humidified atmosphere with 5% CO$_2$. These cells were subcultured each time they reached 80–90% of confluence after being harvested with a solution containing 0.25% of trypsin and 2.21 mM EDTA. *Trypanosoma cruzi* Y strain were isolated at the Chemistry Institute, Department of Biochemistry of the University of São Paulo.

### Anti-T. cruzi activity assay against epimastigote forms

Epimastigote forms of *T. cruzi* Y strain were added to 96-well plates (10$^6$ parasites mL$^{-1}$) containing 2-fold serial dilutions of Ctn, or Ctn[1–14], or Ctn[15–34], or LL-37 or Bz in liver infusion tryptose medium supplemented with 1% antibiotics (100 U mL$^{-1}$ penicillin and 100 $\mu$g mL$^{-1}$ streptomycin) and 10% of FBS. Final concentration ranges were: peptides at 0.19–100 $\mu$M and Bz at 15.6–1000 $\mu$M. Parasite 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 only with sterile PBS medium as negative controls (100% viability) and all experiments were carried out in triplicate.

### Anti-T. cruzi activity assay against trypomastigote forms

*T. cruzi* trypomastigote forms were obtained by infecting LLC-MK2 cells with trypomastigote forms in T-25/75 cm$^2$ flasks at 37 °C in an atmosphere with 5% CO$_2$ in DMEM medium (Vitrocell, São Paulo, Brazil) supplemented with 1% antibiotics (100 U mL$^{-1}$ penicillin and 100 $\mu$g mL$^{-1}$ streptomycin) and 2% FBS for 24 h. Thereafter, trypomastigote forms were plated (96-well plate) and 2-fold serial dilutions of either peptides or Bz in DMEM were added at the same final concentration ranges used against the epimastigote forms. After 24 h of 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 only with sterile PBS medium as negative controls and all experiments were carried out in triplicate.

### Anti-T. cruzi activity assay against amastigote forms

LLC-MK2 cells were seeded in 24-well plates (5 × 10$^5$ cells/well) containing glass coverslips (13-mm diameter) in DMEM medium supplemented with 10% FBS and 1% antibiotics (100 U mL$^{-1}$ penicillin and 100 $\mu$g mL streptomycin), and maintained for 24 h at 37 °C in a 5% CO$_2$ atmosphere. Next, the medium 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 Ctn (0.22 or 0.44 $\mu$M) or Bz (282 or 564 $\mu$M) 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). Non-treated infected LLC-MK2 cells were used as controls. 300 cells were counted in triplicate and intracellular amastigotes per 100 cells and percentage of infected cells were calculated.

### Trypanosoma cruzi epimastigote death pathway assays

Flow cytometry experiments were performed to evaluate *T. cruzi* death pathways when treated with Ctn. The epimastigote forms (10$^6$ cells mL$^{-1}$) were treated with Ctn and Bz at their half maximal effective concentration (EC$_{50}$) concentrations (4.47 and 218 $\mu$M, respectively) in liver infusion tryptose medium. After incubation for 24 h at 28 °C, the parasite cells were washed and stained with fluorescein isothiocyanate-annexin V (AX) and/or 7-aminoactinomycin D (7-AAD) according to the manufacturer's instructions (BD Pharmingen, Franklin Lakes, NJ, USA) to detect cell death by apoptosis or necrosis, respectively. In separate experiments, the loss of mitochondrial transmembrane potential (MTP), the outburst of reactive oxygen species (ROS) and the swelling of reservosomes were also investigated. After the treatment of *T. cruzi* epimastigote forms with Ctn and Bz at their EC$_{50}$ (4.47 and 218 $\mu$M, respectively) for 24 h at 28 °C, the parasites were stained with 10 $\mu$g mL$^{-1}$ rhodamine 123 or 20 mM L$^{-1}$ dichloro-dihydro-fluorescein diacetate (DCFH-DA) or 5 $\mu$g mL$^{-1}$ acridine orange (AO) (Sigma – Aldrich™, St. Louis, MO, USA), according to the manufacturer's instructions, respectively. Next, these cells were run in a FACSCalibur flow cytometer and 10 000 live events were analysed using the Cell Quest software (Becton-Dickinson, San Jose, CA, USA). The results were established by determining the fold change (treated/non-treated cell ratio) of the geometric mean of fluorescence. Epimastigotes without Ctn treatment were used as controls and all experiments were carried out in triplicate.

### Cytotoxicity test in mammalian cells (MTT assay)

Cell viability was also measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Vanden Berghe *et al.* 2013). LLC-MK2 cells were plated in DMEM medium, treated with different concentrations of Ctn, Ctn[1–14] and Ctn [15–34] (1.56–100 $\mu$M) and incubated at 37 °C for 24 h. MTT (Amresco, Ohio, USA; 5 mg mL$^{-1}$) was added and the cells were incubated for 4 h, when 10% sodium dodecyl sulphate (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 using cells treated only with sterile PBS medium as negative controls and all experiments were carried out in triplicate. The Selectivity Index (SI) was calculated through the ratio of EC$_{50}$ of LLC-MK2 cells/EC$_{50}$ of trypomastigote forms (Nwaka and Hudson, 2006).

**Table 1.** Amino acid sequence and some physicochemical properties of the peptides

| Peptide | One-letter amino acid sequence[a] | Charge[b] | Hydrophobicity, H[c] | Molecular weight[d] |
|---|---|---|---|---|
| Ctn | KRFKKFFKKVKKSVKKRLKKIFKKPMVIGVTIPF | +16 | 0.263 | 4151.41 |
| Ctn[1–14] | KRFKKFFKKVKKSV | +9 | −0.012 | 1797.30 |
| Ctn[15–34] | KKRLKKIFKKPMVIGVTIPF | +8 | 0.455 | 2371.10 |
| LL-37 | LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES | +6 | 0.201 | 4493.26 |

[a]Ctn and fragments had C-terminal amides.
[b]At neutral pH, from the 'Peptide property calculator' software (http://www.pepcalc.com).
[c]Obtained from the 'Heliquest' software (http://heliquest.ipmc.cnrs.fr).
[d]Previously determined (Falcão *et al.* 2015).

### Scanning electron microscopy

Epimastigote forms were treated with Ctn peptide at its $EC_{50}$ (4.47 $\mu$M) concentration in liver infusion tryptose medium. After incubation for 12 h at 28 °C, the parasites were washed with PBS and fixed with a 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) solution. After fixation for 2 h at room temperature, parasites were washed with PBS, and then dehydrated with ethanol. Next, samples were dried with $CO_2$, coated in gold and, finally, observed in a FEI Quanta 450 FEG scanning electron microscope (FEI, Oregon, USA). Digital images were acquired and stored (Lima *et al.* 2016).

### Statistical analysis

Three independent experiments were performed in triplicate for all assays. The statistical analysis was performed using the GraphPad Prism 5 programme (GraphPad Software, San Diego, CA, USA). The $EC_{50}$ values were determined by non-linear regression. Data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's post-test. Significance was defined as $*P < 0.05$.

### Results

The obtained peptides with their respective amino acid sequences and physicochemical properties such as net charge, hydrophobicity and molecular weight are shown in Table 1. They were screened against all *T. cruzi* morphological forms to assess and compare their antiprotozoa activities with the standard drug Bz.

Against the epimastigote forms, the only peptide that showed activity against *T. cruzi* within the tested concentration range was Ctn, with an $IC_{50}$ of 4.47 (±0.9) $\mu$M reached after 24 h of incubation, which did not change up to 72 h after treatment. Bz required very high molar concentrations, as expected, against the Y strain ($IC_{50}$/24 h of 218 ± 15 $\mu$M) (Table 2) and could not reach an $IC_{50}$ value close to Ctn concentrations even after 72 h of treatment ($IC_{50}$/72 h of 16.5 ± 1 $\mu$M).

Against the trypomastigote forms, Ctn and its fragments showed different activities against *T. cruzi*. While Ctn[1–14] and Ctn[15–34] was able to inhibit 50% of the trypomastigotes with concentrations of 33.1 and 9.5 $\mu$M, respectively, Ctn only required 0.22 $\mu$M to inhibit 50% of that *T. cruzi* morphological form. Next, the cytotoxicity of the peptides to LLC-MK2 cells without the parasites was verified.

*T. cruzi* amastigote forms inside LLC-MK2 were also treated with Ctn or Bz at their respective $EC_{50}$s against the trypomastigote forms. According to Fig. 1, Ctn significantly reduced the number of amastigotes for each 100 LLC-MK2 cells when compared to untreated infected cells (control) after 24 h of incubation (Fig. 1A). In addition, Ctn at 0.22 $\mu$M significantly reduced the percentage of infected LLC-MK2 cells when compared to the control after 48 h of treatment (Fig. 1B). Similar results were also observed with Bz at 282 $\mu$M.

In addition, flow cytometry analyses and scanning electron microscopy (SEM) were performed to evaluate the death pathway(s) of *T. cruzi* epimastigote forms. After 24 h of treatment with Ctn at 4.4 $\mu$M ($EC_{50}$), the remaining live epimastigotes were mainly labelled with either 7-AAD only (41.6%) or 7-AAD and AX (39.6%) markers, which indicate cell death by necrosis or necrosis/late apoptosis, respectively (Fig. 2). Bz was able to increase the labelling with 7-AAD and AX (8.0%) and more significantly the labelling with 7-AAD only (65.6%) on epimastigotes treated with the $EC_{50}$ after 24 h (Supplementary Fig. S1A).

Additional flow cytometry experiments (Fig. 3) were then carried out to clarify the epimastigote death pathway, such as the generation of ROS and the loss of MTP. In Fig. 3A, Ctn at 4.47 $\mu$M for 24 h induced a burst of ROS, increased DCFH-DA signal (shift to the right), by as much as 3-fold when compared to untreated parasites (control). In a separate assay, a reduction in MTP was observed, decreasing rhodamine 123 signal by 50% (shift to the left), when the epimastigotes were treated with Ctn at the $EC_{50}$ compared to non-treated *T. cruzi* (Fig. 3B). Bz showed a similar profile, decreasing MTP by approximately 30% and increasing by 70% ROS generation in epimastigotes treated with the $EC_{50}$ after 24 h (Supplementary Fig. S1B–C).

**Table 2.** $EC_{50}$ values (in $\mu$M) and Selectivity Index (SI) to trypomastigote forms of the peptides and Bz against epimastigote and trypomastigote forms of *Trypanosoma cruzi* Y strain and LLC-MK2 mammalian cells

| | Ctn | Ctn[1–14] | Ctn[15–34] | LL-37 | Bz |
|---|---|---|---|---|---|
| Epimastigote 24 h | 4.47 ± 0.9 | >100 | >100 | >100 | 218.0 ± 15 |
| 48 h | 4.37 ± 0.5 | >100 | >100 | >100 | 61.1 ± 3 |
| 72 h | 6.21 ± 0.4 | >100 | >100 | >100 | 16.5 ± 1 |
| Trypomastigote | 0.22 ± 0.02 | 33.1 ± 15 | 9.5 ± 2 | >100 | 282 ± 20 |
| LLC-MK2 | 44.7 | >100 | >100 | >100 | 614.8 + 30 |
| SI | 203.2 | >3 | >10.5 | nd | 2.18 |

Data are expressed as means ± s.e.m. of triplicate of three independent experiments (nd: not determined).
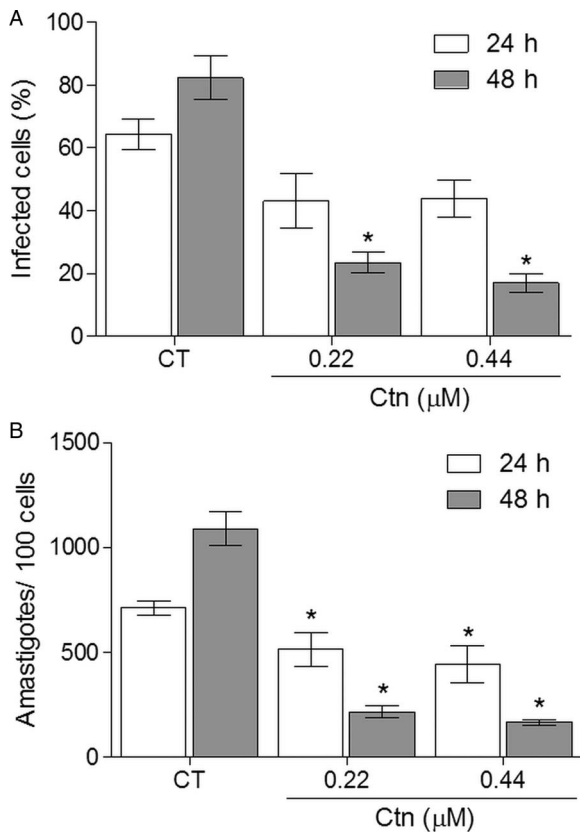
**Fig. 1.** Effect of crotalicidin on intracellular amastigotes in infected cells (A) amastigote/100 cells (B) at 24 and 48 h of incubation (CT – control treated with PBS; *$P < 0.05$).

We also investigated an alternative cell death pathway, the autophagy, utilizing AO staining to evaluate the increase of acidic vesicles, the reservosomes. However, after 24 h, epimastigote forms treated with Ctn at the $EC_{50}$ did not show any increase in the AO fluorescent signal compared to the control group (Supplementary Fig. S2), neither did Bz (Supplementary Fig. S1D).

Based on the abovementioned results, which provided an insight that Ctn kills *T. cruzi* by inducing necrosis and late apoptosis, SEM images (Fig. 4) were acquired to visualize the morphological alterations and, thus corroborate the flow cytometry findings. Compared to a non-treated *T. cruzi* epimastigote (Fig. 4A), parasites treated for 12 h with Ctn at the $EC_{50}$ showed

cell shrinkage (Fig. 4B) and loss of membrane integrity with the presence of pores (Fig. 4C–D).

## Discussion

In this present study, we assessed the potential anti-*T. cruzi* activity of full-size Ctn, Ctn[1–14] and Ctn[15–34] short Ctn derivatives and LL-37 against all parasite morphological forms and compared them with Bz, a commercially available antichagasic drug of choice, as well as studied the death pathway(s) induced by the most promising biologically active peptide. Against the epimastigotes, Ctn was the only peptide that showed activity at the tested concentration range. By observing the physicochemical properties of the analysed peptides, it seems that both bulky and highly positively charged peptides are required for anti-*T. cruzi* activity against the epimastigote forms. Similar results against *T. cruzi* were previously reported with the use of batroxicidin (BatxC), a vipericidin from *Bothrops atrox* that has a molecular weight of 4258.63 and net charge of +16 (Falcao *et al.* 2014; Mello *et al.* 2017). In addition, after 24 h of treatment, Ctn $EC_{50}$ (4.47 $\mu$M) was more than one order of magnitude lower than Bz $IC_{50}$ (218 $\mu$M), which did not reach values close to the peptide even after 72 h of incubation.

The peptides were also evaluated against trypomastigotes and amastigotes. These *T. cruzi* morphological forms were obtained after infecting LLC-MK2 kidney cells, which have been used for more than 30 years as mammalian cell models of *T. cruzi* infection (Andrews and Colli, 1982; Zingales *et al.* 1982; Zingales *et al.* 1997). Interestingly, while the *in vitro* Bz concentration killed 50% of the trypomastigotes (282 $\mu$M), it essentially did not change when compared to epimastigotes after 24 h of incubation. In contrast, the Ctn $EC_{50}$ against the trypomastigote forms (0.22 $\mu$M) showed a 20-fold lower effect against epimastigotes (4.47 $\mu$M). Moreover, Ctn at 0.22 $\mu$M also significantly reduced the number of amastigotes and the percentage of infected LLC-MK2 cells when compared to non-treated infected cells. Therefore, *T. cruzi* trypomastigote and amastigote forms seem to be more susceptible to Ctn than epimastigotes. This increased susceptibility can be related to the prevalence of negatively charged molecules on the parasite cell membrane, especially in trypomastigote forms, such as mucin glycoproteins and sialic acid, which are implicated in host–parasite interactions (de Souza *et al.* 2010). These anionic molecules made *T. cruzi* trypomastigote forms susceptible even to Ctn[1–14] and Ctn[15–34] fragments, which have only half of the net charge of Ctn, particularly the mid-sized and highly hydrophobic Ctn[15–34].
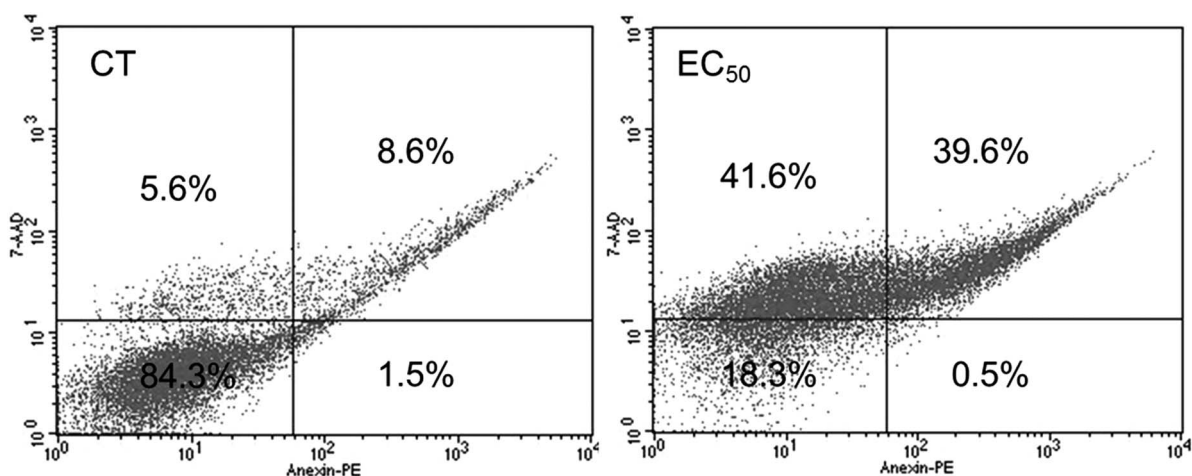


**Fig. 2.** Representative flow cytometry scattering graphs of *Trypanosoma cruzi* epimastigotes treated with crotalicidin at 4.47 $\mu$M for 24 h. Percentage values are averages of 10 000 live events from three independent experiments (CT – control treated with PBS).
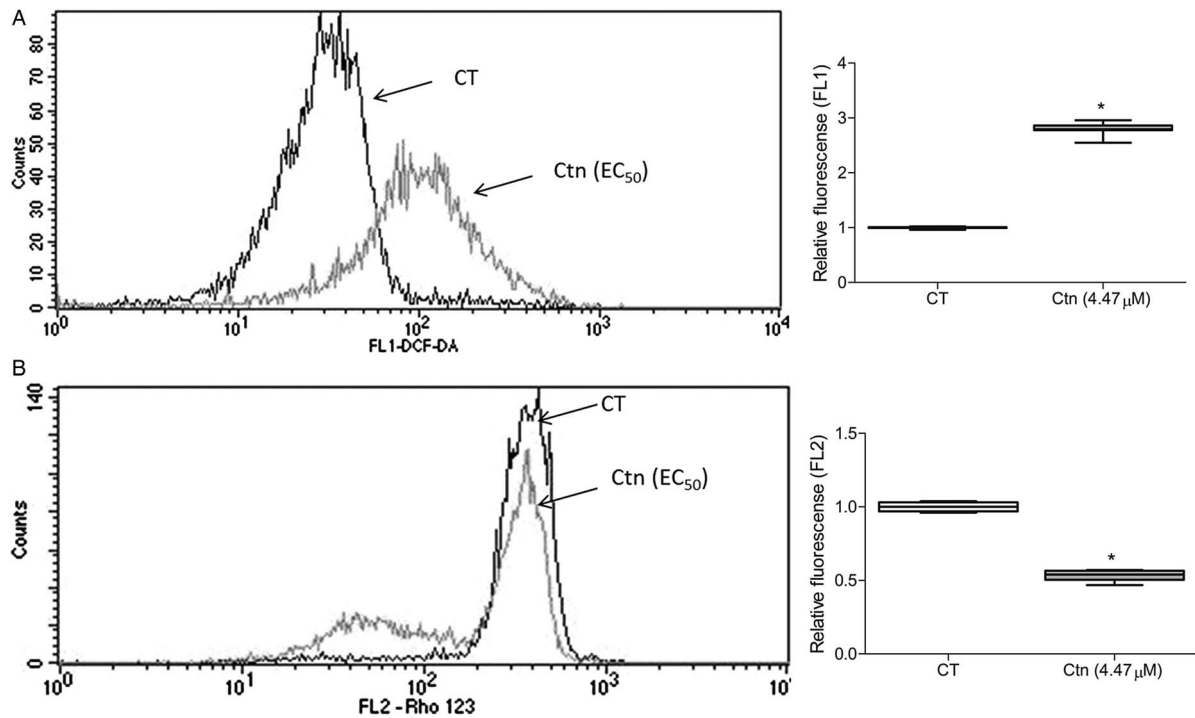
**Fig. 3.** A representative histogram of DCFH-DA fluorescent signal and relative fluorescence values (from three independent experiments) from *Trypanosoma cruzi* epimastigotes that were initially treated with crotalicidin at 4.47 $\mu$M for 24 h (A) and a representative histogram of rhodamine 123 (Rho 123) fluorescent signal and relative fluorescence values (from three independent experiments) from *T. cruzi* epimastigotes initially treated with crotalicidin at 4.47 $\mu$M for 24 h (B) (CT – control treated with PBS).
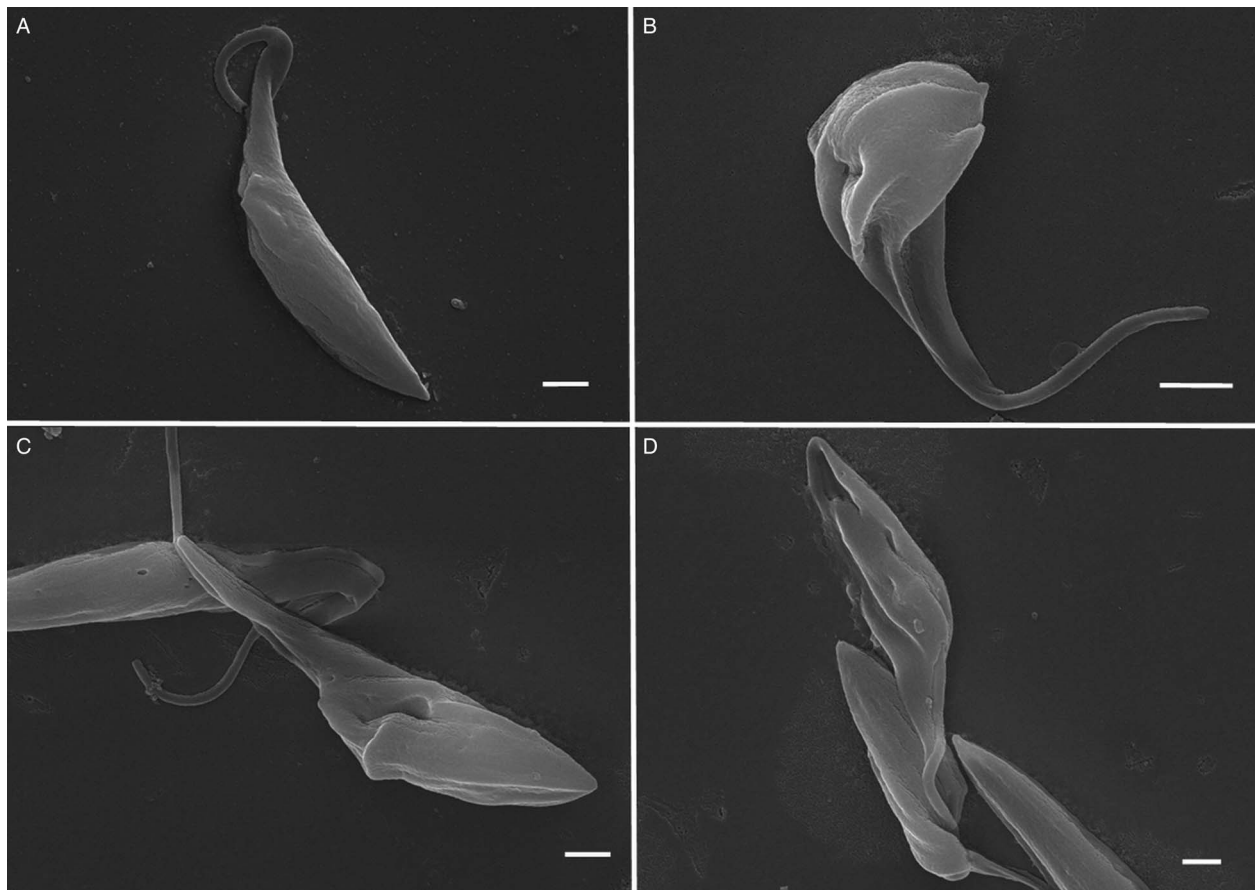


**Fig. 4.** Scanning electron microscopy of control epimastigotes (A) and treated epimastigotes with EC$_{50}$ of crotalicidin (B–D). Morphological alterations were observed, such as cell shrinkage (B) and membrane integrity loss (C–D). Scale bars: 1 $\mu$m.

The cytotoxicity of the drugs (peptides and Bz) against uninfected LLC-MK2 cells was also verified. Considering the $IC_{50}$ concentrations against trypomastigotes, Ctn showed the greatest selectivity (SI = 203.2) among the other peptides and Bz. Furthermore, Ctn met two important criteria for a potential anti-*T. cruzi* drug candidate to be considered as a success and, thus, the most promising evaluated peptide: trypomastigote $IC_{50}$ below 1 $\mu$g mL$^{-1}$ (0.22 $\mu$M or 0.913 $\mu$g mL$^{-1}$) and SI above 50 (Nwaka and Hudson, 2006). Furthermore, the Ctn selectivity against parasite-infected cells seems to be another advantage for the clinical development of target therapy for *T. cruzi* infection.

The necrotic pathway observed in Ctn was also observed when the epimastigotes were treated with the vipericidin BatxC at the $EC_{50}$ (Mello *et al.* 2017). *Trypanosoma cruzi* cell death by apoptosis and autophagy was also observed when other AMPs were used, such as melittin (Adade *et al.* 2013).

Cathelicidins are typically potent trypanolytic AMPs. Similar peptides such as cathelicidins SMAP-29 and protegrin-1 have been tested in the intraperitoneal route and showed interesting results, such as decrease in parasitaemia and prolonged survival of mice infected with *Trypanosoma brucei* (McGwire *et al.* 2003). The evaluation of Bz-induced cell death in epimastigote forms also showed a necrotic profile, meaning Ctn is promising as a template for the designing of a new antichagasic agent, because it has a similar cell death profile to the reference drug and an almost 10-fold higher selective index.

In conclusion, Ctn, a cathelicidin-related peptide obtained from the venom gland of *C. durissus terrificus* rattlesnake, not only has antibacterial and anti-tumour properties but also exhibited anti-*T. cruzi* activity against all morphological forms of a Bz-resistant strain. In addition, Ctn killed the epimastigote parasite form by inducing necrosis. Remarkably, when toxicity to healthy mammalian cells was compared, the peptide's greater selectivity for *T. cruzi* when compared to Bz makes Ctn a promising drug candidate or a lead for the development of new peptide-based drugs to treat Chagas disease.

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