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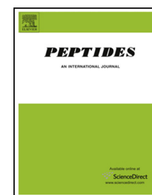

Effects of cyclotides against cutaneous infections caused by *Staphylococcus aureus*

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ABSTRACT

The main bacterium associated with skin infection is *Staphylococcus aureus*, occurring especially in infections acquired *via* surgical wounds, commonly leading to lethal hospital-acquired infections, emphasizing the importance of identifying new antimicrobial compounds. Among them, cyclotides have gained interest due to their high stability and multifunctional properties. Here, cycloviolacin 2 (CyO2) and kalata B2 (KB2) were evaluated to determinate their anti-staphylococcal activities using a subcutaneous infection model. Anti-staphylococcal activities of 50 mM for KB2 and 25 mM for CyO2 were detected with no cytotoxic activities against RAW 264.7 monocytes. In the *in vivo* assays, both cyclotides reduced bacterial load and CyO2 demonstrated an increase in the phagocytosis index, suggesting that the CyO2 *in vivo* anti-staphylococcal activity may be associated with phagocytic activity, additionally to direct anti-pathogenic activity.

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Introduction

The skin is the largest and the most exposed organ of the human body [26]. Although it is extremely effective in protecting host-organisms against external environmental threats, skin infections are common and may be caused by bacteria, fungi or viruses [26,28]. The main bacterium associated with skin infections is *Staphylococcus aureus* [2,27]. The majority of infections can be simply treated, but some are less tractable, especially those associated with surgical wounds, which are the most common hospital-acquired infections and a major cause of morbidity and mortality [9,16]. These infections are increasingly caused by pathogens that are resistant to conventional treatments, which highlights the importance of identifying new and more effective antimicrobial therapies [1,32].

Antimicrobial peptides (AMPs) have emerged as a promising new therapy with potential for the treatment of infectious diseases [1,32]. Among AMPs, cyclotides have shown great promise in a range of drug design applications. These head-to-tail cyclized peptides, originally isolated from plants, incorporate six conserved cysteine residues in their structure, which form three interconnected disulfide bonds organized in a cyclic cystine knot (CCK) motif. This motif makes cyclotides highly stable and has generated interest in them as peptide-based scaffolds for designing medicines [6,10,19]. The cyclotides can be divided into mainly two subfamilies known as Möbius (Fig. 1A) and bracelet cyclotides (Fig. 1B); the main characteristic that distinguishes one from the other is the presence of a conserved cis-proline residue in loop 5 of the Möbius subfamily, creating a twist in the circular backbone [4,8,10,29,40].

Here, the cyclotides cycloviolacin O2 (CyO2) and kalata B2 (KB2), respectively members of the bracelet and Möbius subfamilies (Fig. 1), were evaluated to characterize their cytotoxic and anti-staphylococcal activities against *S. aureus* in an *in vitro* microdilution assay and also *in vivo* using in a subcutaneous wound

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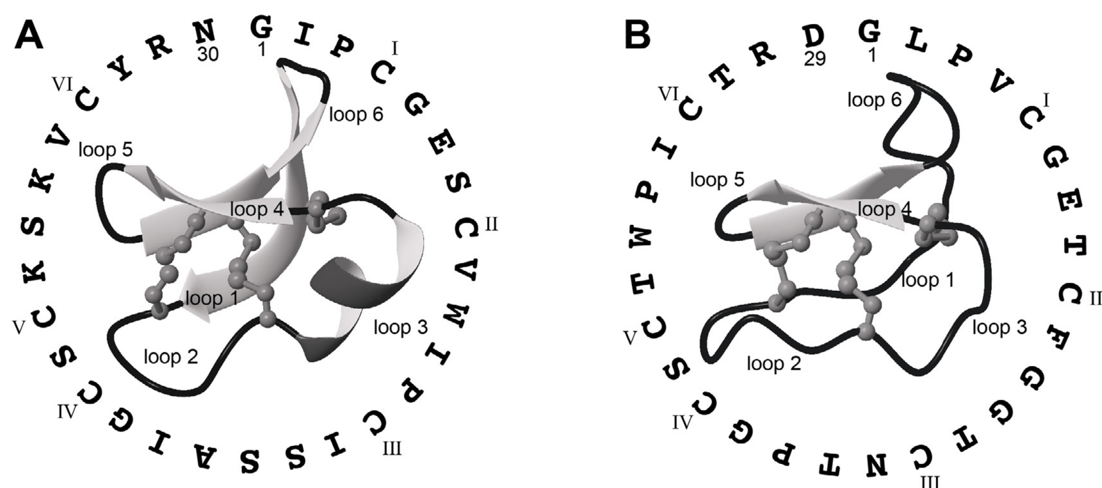

Fig. 1. Sequences and structures of cyclotides – (A) cycloviolacin O2 (CyO2) and (B) kalata B2 (KB2). All cyclotides contain six cysteines (I-VI) connected by disulfide bonds to form six loops (1–6). CyO2 and KB2 belong to the bracelet and Möbius subfamily, respectively. The two subfamilies differ by the presence of conserved cis-Pro in loop 5 in Möbius cyclotides that gives a conceptual backbone twist resembling a Möbius strip.

infection model. This study is the first report defining the efficacy of cyclotides in an *in vivo* infection model.

Materials and methods

Peptides

The peptides used in this study, CyO2 and KB2, were extracted from the aerial parts of the plants *Viola odorata* (CyO2) and *Oldenlandia affinis* (KB2) with a 1:1 solution of dichloromethane/methanol for 12 h. The extract was partitioned with water and the methanol/water fraction was rotary evaporate to remove the methanol and lyophilized. The dried product was resuspended in water and purified by RT-preparative HPLC using a C18 column. Peptide masses were analysed using an ES-TOF micromass LCT mass spectrometer [7].

Bactericidal microdilution assays

Minimum inhibitory concentrations (MICs) of the cyclotides against *S. aureus* ATCC 25923 were determined using a standardized dilution method according to NCSLA guidelines [39]. A single bacterial colony was inoculated in Mueller-Hinton broth (MH) (Himedia, India) and incubated for 12 h at 37 °C. Overnight-cultured *S. aureus* was transferred to MH medium and cultured to exponential phase (OD_{600} of ∼0.6). The culture was centrifuged and resuspended in sterile PBS and adjusted to a final amount of 1×10^5 colony-forming units (CFU) mL^{-1} by use of the equation CFU mL^{-1} = OD_{600} nm $\times 2.5 \times 10^8$ [35]. For MIC determination, peptides were applied at various concentrations (1–200 μM) from a stock solution. Ten microliters of each concentration of peptide solution was added to each corresponding well of a 96-well plate (Becton & Dickinson, USA) and 90 μL of bacteria (1×10^5) in MH medium was added. The polypropylene plates (TPP, Switzerland) were incubated at 37 °C for 24 h. MICs were determined as the lowest tested concentration that leads to complete inhibition (100%) compared to the negative control group [33].

Animals

C57BL/6 mice weighting 18–22 g were used in this study, provided by the animal facility of the Catholic University of Brasilia. All animals were housed in separate cages under a constant temperature (22 °C) and humidity, with a 12-h light/dark cycle and access to food and water *ad libitum* throughout the study. The mice were euthanized by CO_2 or ether inhalation at the end of the experiments. All procedures including care and handling of the animals were approved by the Ethics Committee of the Catholic University of Brasilia number 005/13.

Cell cytotoxicity assays

Murine monocytes RAW 264.7 cells (Rio de Janeiro Cell Bank) were seeded in 96-well microtiter plates in a concentration of 1.0×10^5 cells per well, in DMEM medium, supplemented with various concentrations of the tested cyclotides (0.3–15 μM). After 24 and 48 h incubation, a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell viability assay was performed. Briefly, 60% of the medium was removed, and 10 μL of MTT (5 mg mL^{-1}) (Sigma, USA) solution was added to each well and the plate was incubated for 4 h in 5% CO_2 at 37 °C. The blue formazan product produced was dissolved by the addition of 100 μL of 100% DMSO (Mallinckrodt, Germany) per well. Plates were then gently swirled for 5 min at room temperature to dissolve the precipitate. The absorbance was monitored at 575 nm using a microplate spectrophotometer (Bio-Tek, USA). Cytotoxicity was determined as a percentage of the maximum value after subtracting the background. The results were stated as the percentage of each sample matched to the negative control (PBS buffer, pH 7.4) and cell culture was incubated in a lysis buffer (10 mM Tris, pH 7.4, 1 mM ethylenediamine tetraacetic acid (EDTA), and 0.1% Triton X-100) [39].

Induction of peritonitis and peritoneal cell collection

Peritonitis was induced as described by Leendertse et al. [23]. Mice were injected intraperitoneally with 200 μL of 3% thioglycollate solution. Peritonitis was induced 6 h after injection [23]. After this period, mice were anesthetized by 10% ketamine (80 mg kg^{-1}) (Agener Union – Brazil) and xylazine (10 mg kg^{-1}) (Agener Union – Brazil), euthanasiated, and a peritoneal lavage was performed with 5 mL of RPMI 1670 medium using an 18-gauge needle. After collection of peritoneal lavage fluid (PLF), cellular content was immediately placed on ice. The numbers of total cells and neutrophils were counted using a microscope.

Phagocytosis assays

Phagocytosis assays were conducted using neutrophils (10^5 cells per well). Controls (untreated and treated with DMSO 2.5% and gentamicin 15 μM) and treatments with the previously determined cyclotide MIC concentration were conducted in RPMI 1640 medium. The cells were infected with *S. aureus* at a multiplicity of infection (MOI) of 1:1 and incubated for 90 min. Following incubation, the cells were centrifuged, placed on slides and stained with fast panoptic dye [34]. For neutrophil phagocytosis assay assessments, at least 100 cells on each slide with or without phagocytosis were counted. Additionally, the number of phagocytosed particles and phagocytic index was calculated. In all experiments the phagocytosis confirmatory counts were conducted by an independent observer. The phagocytic index (PI) was calculated using the formula: PI equals the percentage of cell phagocytosis multiplied by the average number of particles phagocytosed by each cell.

Murine surgical wound infection model

The murine surgical wound infection model was performed as described by McRipley and Whitney [24] with minor modifications. Mice ($n = 5$/group) were anesthetized, their dorsal surface shaved and the surgical area disinfected with 70% ethanol. A puncture was performed on the dorsal surface using 6-mm punch biopsy needles (Stiefel Laboratories, UK), and then 10 μL of *S. aureus* ATCC 25923 suspension (2×10^9 mL^{-1}; previously cultured as described in the section on bactericidal microdilution assays) was introduced into the puncture wound, and the skin was closed with one silk suture [41]. Wounds were treated every 24 h with 20 μL of a solution containing 0.75–3 mg kg^{-1} of cyclotide. Vetaglos® (gentamicin 0.5%, sulfanilamide 5% and sulfadiazine 5%) ointment (Vetnil, Brazil) was used as a positive control and PBS as negative control. Mice were euthanized 7 days post-surgery; the wounded muscle tissue was excised, weighed, and homogenized in 1 mL of PBS. Serial homogenate dilutions were plated in triplicate on mannitol salt agar (Himedia, India) and the results were stated as CFU g^{-1} of tissue [18,35].

Statistical analysis

Data are presented as mean ± SD of all samples. Values of $p < 0.05$ were considered statistically significant. GraphPad Prism software v5.0 (GraphPad Software, USA) was used for all statistical analyses [33].

Results

In vitro antibacterial activity

The activities of the cyclotides against *S. aureus* were evaluated *in vitro* and MICs determined by microdilution tests (data not shown). The MIC for KB2 was 50 μM and that for CyO2 was 25 μM.

Effects of cyclotides in cell viability/metabolism

Cytotoxicity evaluation against RAW 264.7 cells for CyO2 (Fig. 2A) and KB2 (Fig. 2B) did not show significant deleterious activities at the concentrations tested when compared with the control, demonstrating that these peptides show no cytotoxicity to RAW

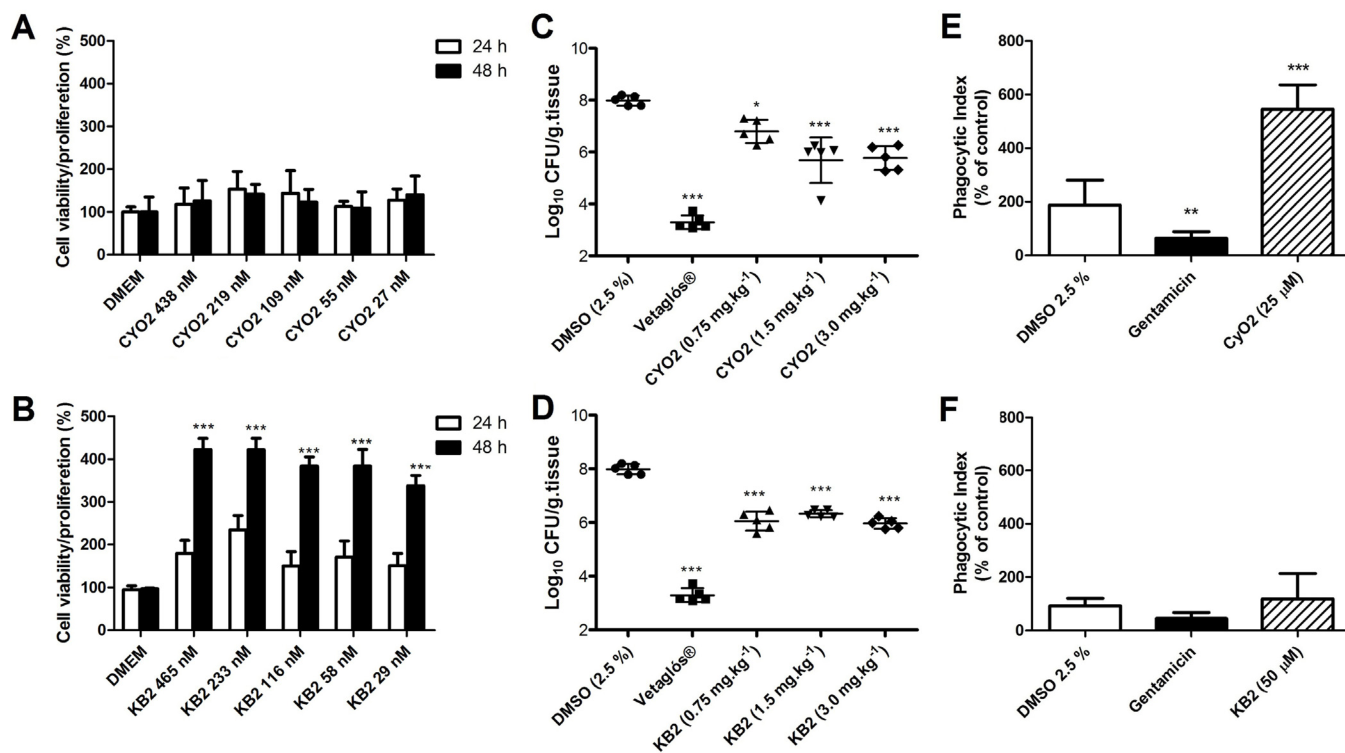

Fig. 2. Effect of cyclotides CyO2 (A) and KB2 (B) on viability of murine cell line RAW 264.7. Cells were incubated for 24 and 48 h with CyO2 (27–438 nM), and KB2 (29–465 nM). Values represent the mean ± SD and data were analyzed by one-way ANOVA and Bonferroni test: * $p < 0.5$; ** $p < 0.01$; *** $p < 0.001$ compared to untreated group. Recovery of bacterial load of the wound from the C57BL/6 mice infected with *S. aureus* ATCC 25923 after 6 days of treatment. The wounds were inoculated with 2×10^9 cells mL^{-1} of bacteria and treated with: DMSO 2.5%, Vetagls® (gentamicin 5 mg kg^{-1}, sulphanilamide 50 mg kg^{-1}, sulfadiazine 50 mg kg^{-1}); (C) CyO2 (0.75 mg kg^{-1}, 1.5 mg kg^{-1}, 3.0 mg kg^{-1}); (D) KB2 (0.75 mg kg^{-1}, 1.5 mg kg^{-1}, 3.0 mg kg^{-1}). Values represent the mean ± SD and data were analysed by two-way ANOVA and Tukey's test, with $p < 0.05$ as significant: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to the negative control. For the phagocytosis assay, neutrophils extracted from healthy animals were incubated for 1.5 h with controls: DMSO (2.5%), gentamicin (15 μM) or DMEM; and treated with: (E) CyO2 (25 μM) or (F) KB2 (50 μM). Values represent the mean ± SD and data were analysed by one-way ANOVA and Bonferroni test: * $p < 0.5$; ** $p < 0.01$; *** $p < 0.001$ compared to untreated group.

264.7 cells, at the evaluated concentrations (CyO2 ranging from 437.8 to 27.4 nM and KB2 ranging from 465.2 to 29.1 nM).

Verification of bacterial burdens recovered from wounds

Mice treated with 0.75 mg kg^{-1} of CyO2 showed a slight reduction in bacterial load of 10^8 to $\sim 10^7$. When treated with 1.5 and 3.0 mg kg^{-1}, the bacterial load was reduced to $\sim 10^6$ (Fig. 2C). In animals treated with 6.0 mg kg^{-1} of CyO2, a clear decrease in bacterial load of 10^8–10^5 was observed (data not shown). The infected mice treated with KB2 showed a decrease in bacterial load of 10^8 to $\sim 10^6$ in all tested concentrations (0.75, 1.5 and 3.0 mg kg^{-1}) (Fig. 2D).

Evaluation of phagocytosis

To evaluate possible indirect antibacterial activity through stimulation of the host, we measured the activity of cyclotides on the phagocytic activity of neutrophils following *S. aureus* infection. Neutrophils treated with KB2 (Fig. 2E) showed no variation in the phagocytic index compared with controls. However, CyO2 increased the phagocytic index by ~545% (Fig. 2F), suggesting that it has the capability of stimulating phagocytosis of *S. aureus* by neutrophils.

Discussion

With the rapidly increasing prevalence of multidrug-resistant bacteria and the scarcity of new antibiotic research in the pharmaceutical industry, the use of AMPs could be a promising alternative to conventional antibiotic treatments. AMPs are multifunctional mediators of the innate immune response with direct antimicrobial and immunomodulatory activities. Among AMPs, cyclotides are notable for being small cyclic peptides with six conserved cysteine residues that form three interconnected disulfide bonds, thereby giving them greater stability than linear AMPs [13,17,30]. Thus, cyclotides could potentially overcome some of the limitations of peptides as drugs, such as sensitivity to proteolytic degradation [12,13,30].

In the present study the antibacterial activities of KB2 and CyO2 against *S. aureus* ATCC 25923 were evaluated. CyO2 had the best anti-staphylococcal activity *in vitro* whereas KB2 had only slightly lower potency. In the literature there are two studies in which cyclotides were tested against *S. aureus*. Tam et al. [36], reported that KB1, circulin A and B, and cyclopsychotride showed MICs against *S. aureus* of 0.26 μM, 0.19 μM, 13.5 μM and 39 μM, respectively under low salt conditions but no activity under physiological salt conditions [36]. Pranting et al. [31] evaluated CyO2, KB1, KB2, Vaby A and D for activity against *S. aureus*, but no deleterious activities against *S. aureus* were obtained using the radial diffusion assay, performed with 4×10^6 CFU in a culture medium based on sodium phosphate buffer containing 0.03% (w/v) trypticase soy broth and 1% agarose, with wells made using a 3 mm gel punch added 5 μL of sample, then incubated for 18–24 h, 37 °C. However, in microdilution assays, these cyclotides inhibited *S. aureus* growth: CyO2 (>50 μM), KB1 (>100 μM), KB2 (35 μM), Vaby A and D (>90 μM) [31].

Given the confirmation in our study of the anti-staphylococcal activity of cyclotides KB2 and CyO2, we tested them for effects on cell viability of murine RAW 264.7 monocytes and found that low doses did not cause any harmful effect. Otherwise any evaluation was performed at higher concentrations. Only KB2 showed slight cytotoxicity at higher doses (>15 μM) (data not shown). Similar data were obtained by Grundemann et al. [11], where *O. affinis* extract and KB1 (1.8–14.0 μM) reduced peripheral blood mononucleated cell (PBMC) proliferation. That study showed that at concentrations of 1.8–14 μM a dose-dependent correlation decrease in cell proliferation was observed.

Since *in vitro* antibacterial activity, but no mammalian cytotoxicity, was observed in the two cyclotides tested here, the next step was to evaluate their *in vivo* anti-staphylococcal activity using a surgical *S. aureus* wound infection model. To our knowledge, there has been no previous report of cyclotide antimicrobial activity in an animal infection model. The cyclotides showed similar responses, and both decreased bacterial load. Interestingly, a progressive dose response was not observed, probably due to the low concentrations tested. A similar phenomenon was observed with the antimicrobial peptide PXL150, which all tested concentrations showed activity toward *S. aureus* [25].

A number of other studies have reported *in vivo* antibacterial activity of AMPs using the surgical wound model used here. For example, coprisin (5 mg kg^{-1}), in addition to reducing the bacterial load, accelerated the wound healing process [21]. In another study, PEP35 exhibited poor antimicrobial activity *in vitro*, with MIC50 values for various clinical strains of *S. aureus* isolates of approximately 500 μg mL^{-1}. Nevertheless, this peptide showed immunomodulatory activity, and when tested *in vivo* in the surgical wound model, reduced the bacterial load to approximately 10^2 CFU g^{-1} tissue, probably recruiting neutrophils to the infection site [22]. In another study, epinecidin peptide-1 (EPI-1) was evaluated against MRSA (10^6 CFU), with treatments placed on the wound 5 min after infection, which was covered after 30 min to prevent interference by the animal [15]. Groups treated with EPI-1 showed an improved survival rate with a decrease in bacterial load of 10^6 to ~20 to CFU g^{-1} tissue [15]. In a study using the same methodology, the pardaxin peptide (GE33) led to complete bacterial clearance in a surgical wound model infected with 10^6 CFU of *S. aureus*. The activities of the cyclotides tested here are comparable to those reported in these previous studies.

To observe whether the *in vivo* effects of the cyclotides could be related simply to direct antibacterial activity or also to additional indirect host activity, a phagocytosis assay was performed to assess the stimulation of neutrophils to increase *S. aureus* phagocytosis. KB2 showed no change compared with the controls, suggesting that the mechanism of action is based predominantly on bactericidal activity. By contrast, CyO2 showed an increase in phagocytic index of approximately 550%, suggesting that its *in vivo* activity against *S. aureus* might be associated with both bactericidal and phagocytic induction in the host animal. It is interesting that this dual strategy is not seen with KB2. Other antimicrobial peptides have been reported to induce phagocytic activity, such as LL-37, which was able to enhance phagocytosis of opsonized Gram-negative and Gram-positive bacteria, besides improving the phagocytosis of non-opsonized *Escherichia coli* in human macrophages [38]. Another peptide that leads to improved phagocytic activity in macrophages is hLF1-11, which was shown to improve the phagocytosis of not only *S. aureus*, but also *Candida albicans* [37].

In conclusion, CyO2 and KB2 might represent a starting point for a novel therapeutic approach for the local treatment of infections. Cyclotides can be produced recombinantly [3] or chemically [5], which opens new frontiers for the production of cyclotide-based medicines. The exceptional stability and cell penetrating properties of cyclotides make them possible scaffolds for the grafting of known active peptides for the engineering of peptide-based drugs [20]. Such an approach might be used to enhance the natural antimicrobial activity of the cyclotide framework, which appears to be driven mainly by membrane binding and disruption [14]. However, more studies are needed to evaluate the applicability of these cyclic AMPs as well as to shed additional light on their *in vivo* antibactericidal mechanism of action.

Conflicts of interest

The authors declare no conflicts of interest, financial or otherwise, in relation to this manuscript.

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