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Research paper

Unraveling the antifungal activity of a South American rattlesnake toxin crotamine

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ABSTRACT

Crotamine is a highly basic peptide from the venom of *Crotalus durissus terrificus* rattlesnake. Its common gene ancestry and structural similarity with the β-defensins, mainly due to an identical disulfide bond pattern, stimulated us to assess the antimicrobial properties of native, recombinant, and chemically synthesized crotamine. Antimicrobial activities against standard strains and clinical isolates were analyzed by the colorimetric microdilution method showing a weak antibacterial activity against both Gram-positive and Gram-negative bacteria [MIC (Minimum Inhibitory Concentration) of 50–>200 μg/mL], with the exception of *Micrococcus luteus* [MIC ranging from 1 to 2 μg/mL]. No detectable activity was observed for the filamentous fungus *Aspergillus fumigatus* and *Trichophyton rubrum* at concentrations up to 125 μg/mL. However, a pronounced antifungal activity against *Candida* spp., *Trichosporon* spp., and *Cryptococcus neoformans* [12.5–50.0 μg/mL] was observed. Chemically produced synthetic crotamine in general displayed MIC values similar to those observed for native crotamine, whereas recombinant crotamine was overridingly more potent in most assays. On the other hand, derived short linear peptides were not very effective apart from a few exceptions. Pronounced ultrastructure alteration in *Candida albicans* elicited by crotamine was observed by electron microscopy analyses. The peculiar specificity for highly proliferating cells was confirmed here showing potential low cytotoxic effect of crotamine against nontumoral mammal cell lines (HEK293, PC12, and primary culture astrocyte cells) compared to tumoral B16F10 cells, and no hemolytic activity was observed. Taken together these results suggest that, at low concentration, crotamine is a potentially valuable anti-yeast or candicidal agent, with low harmful effects on normal mammal cells, justifying further studies on its mechanisms of action aiming medical and industrial applications.

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Abbreviations: MIC, minimal inhibitory concentration; AMP, antimicrobial peptide; CPP, cell penetrating peptide; ATCC, American Type Culture Collection; IOC, Oswaldo Cruz Institute Collection; CD, circular dichroism; CC50, 50% cytotoxic concentration; TEM, transmission electron microscopy.

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1. Introduction

All animals, from arthropods to humans, express peptides able to kill microbial cells. Antimicrobial peptides (AMPs) are essential components of innate immunity for essentially all organisms, including invertebrates, vertebrate, and plants, acting as the first line of defense against invading microbes by disrupting cytoplasmic membrane functions [1]. AMPs are potential candidates for a new antibiotic generation against multiresistant bacterial strains [2,3].

Interestingly AMPs and cell penetrating peptides (CPPs) are similar in several aspects: they share physical and chemical characteristics (short length, cationic, amphipathic, and structured arranged peptides) and, consequently, the fundamental property of strong interaction with lipid membranes. However, AMPs predominantly translocate into the cell by transient pore formation in the membrane, while entry of CPPs has been shown to include both endocytic and non-endocytic routes [4–6], sometimes involving more than one single mode [7,8]. Whatever the pathway CPPs use to translocate membrane (trafficking) systems, they can serve to deliver molecules into cells and their internal compartments, including endosomes, nuclei, and mitochondria [9–11].

Crotamine is the major toxic component toward rodents found in the venom of the South American rattlesnake *Crotalus durissus terrificus*. It was initially described as being responsible for the hind limb paralysis observed in mice after venom injection [12]. The primary structure of crotamine is very similar to those of other small, non-enzymatic, myonecrotic toxins identified in different rattlesnake venoms [13,14]. The 3D structure of crotamine in solution, determined by proton NMR spectroscopy, showed that both its cysteine-pairing pattern and global fold are similar to those of the human β-defensins AMPs, regardless of their low primary structure identity [15,16]. Our group was the first to demonstrate that crotamine is also a CPP with a high specificity for actively proliferating cells [17,18]. We demonstrated that internalization of this polycationic peptide involves endocytosis and is dependent on cell membrane heparan sulfate proteoglycans [19]. Subsequently, we studied the cytotoxic effect of this toxin on highly proliferative cells and suggested the lysosomes as the primary target for crotamine toxic activity at the cellular level [20]. This led us to study and propose the potential application of native crotamine as a theranostic agent [18,21].

Crotamine is a highly basic and amphipathic toxin, particularly rich in Lys, whose 42-residues long polypeptide [YKQCH KKGGHCFPKEKICLPPSSDFGKMDCRWRWKCCKKGSG] folds into a compact structure containing an antiparallel β-sheet and an α-helix stabilized by three disulfide bridges [15,16]. This type of fold and amphipathic structure is also found in the aforementioned β-defensins [1,22], which compose the major family of AMPs [23]. Defensins possess direct antimicrobial activities against a broad range of Gram-negative and Gram-positive bacteria [15,23,24], and the interaction with glycosaminoglycans was also recently demonstrated for human β-defensin 2 (HBD2) [25]. Moreover, it has been suggested that crotamine and β-defensin share a common ancestry [16,26,27], and a potential antimicrobial activity was recently attributed to crotamine [28,29].

In the present work we aimed to characterize the antimicrobial activity of crotamine against Gram-negative and Gram-positive bacteria, and several fungi species, including clinical resistant strains. For comparison, recombinant and chemically synthesized forms of crotamine were included in these studies. In addition, denatured full-length crotamine and two synthetic linear crotamine fragments, comprising half of the positively charged amino acid residues in each, were also evaluated here. The results indicate that crotamine has strong potential as an anti-yeast or candicidal agent, with no hemolytic activity and low harmful effects on normal mammal cells. The effective antifungal activity against a number of clinical yeast strains point out the potential of crotamine as a structural model compound for the development of a new generation of antimicrobial drugs against clinical strains, potentially able to overcome the microbial resistance challenge.

2. Materials and methods

2.1. Materials

C.d. terrificus venom was extracted from snakes maintained at the Faculdade de Medicina de Ribeirão Preto (FMRP) serpentarium, São Paulo University, and dried under vacuum. All other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA). Gomesin was the same as previously described [30]. The cDNA encoding the full-length crotamine [GenBank Acc. No. AF044674] subcloned in frame into the expression plasmid vector pQE-1 (Qiagen, Hilden, Germany), named 127pQE, was kindly provided by Dr. Gandhi Rádis-Baptista (Instituto de Ciências do Mar, Universidade Federal do Ceará, Fortaleza, Brazil).

2.2. Preparation and biochemical characterization of the native, synthetic, and recombinant crotamine

2.2.1. Purification of native crotamine from snake venom

Six hundred milligrams of crude venom were dissolved in 5 mL of 0.25 M ammonium formate buffer pH 3.5, and the bulk of crotoxin, the major venom component, was eliminated by slow speed centrifugation as a heavy precipitate that formed upon slow addition of 20 mL of cold water to the solution. Tris-base [1 M] was then added dropwise to the supernatant to raise the pH to 8.8 and the solution was applied to a CM-Sepharose FF (1.5 × 4.5 cm; former Amersham-Pharmacia, GE Healthcare, Buckinghamshire, UK) column, equilibrated with 0.04 M Tris–HC1 buffer pH 8.8, containing 0.064 M NaCl. After washing the column with 100 mL of equilibrating solution, crotamine was recovered as a narrow protein peak by raising the NaCl concentration of the diluting solution to 0.64 M. The material was thoroughly dialyzed against water (benzoylated membrane, cut off MW = 3000) and was lyophilized. Amino acid analysis after acid hydrolysis of a sample (4 N $MeSO_3H$ + 0.1% tryptamine; 24 h at 115 °C) indicated a yield of 72 mg (14.7 μmol) of crotamine and trace amounts of Thr, Ala and Val (purity > 98%).

2.2.2. Expression of recombinant crotamine in bacteria

The expression and purification of the recombinant protein were performed essentially as previously described [31], except for the use of Ni–NTA agarose (Qiagen Inc., Valencia, CA) instead of glutathione-sepharose beads for crotamine. Briefly, bacteria transformed with 127pQE were grown under antibiotic selection to an absorbance reading of about 0.6 at 600 nm, when the expression of recombinant crotamine, carrying a 6xHis-tag at its C-terminus, was induced by addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG), followed by incubation for 3 h at 30 °C. The bacteria were then collected by centrifugation, re-suspended in 20 mM Tris–HCl pH 8.5, 100 mM KCl, 20 mM imidazole, 10% (v/v) glycerol buffer, and then lysed by two cycles of freezing and thawing, followed by sonication. After removal of bacterial debris, the supernatant was purified by affinity chromatography using Ni–NTA magnetic agarose beads, which had been exhaustively washed with 20 mM Tris–HCl pH 8.5, 1 M KCl, 10% (v/v) glycerol buffer, in order to remove undesired bacterial proteins. The recombinant crotamine was eluted using 20 mM Tris–HCl pH 8.5, 100 mM KCl, and 100 mM

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