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Rhodamine B-conjugated encrypted vipericidin nonapeptide is a potent toxin to zebrafish and associated with *in vitro* cytotoxicity

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ABSTRACT

Background: Animal venoms contain a diverse array of proteins and enzymes that are toxic toward various physiological systems. However, there are also some practical medicinal uses for these toxins including use as anti-bacterial and anti-tumor agents.
Methods: In this study, we identified a nine-residue cryptic oligopeptide, KRFKKFFKK (EVP50) that is repeatedly encoded in tandem within vipericidin sequences.
Results: EVP50 displayed *in vivo* potent lethal toxicity to zebrafish larvae (LD_{50} = 6 μM) when the peptide's N-terminus was chemically conjugated to rhodamine B (RhoB). *In vitro*, RhoB-conjugated EVP50 (RhoB-EVP50) exhibited a concentration-dependent cytotoxic effect toward MCF-7 and MDA-MB-231 breast cancer cells. In MCF-7 cells, the RhoB-EVP50 nonapeptide accumulated inside the cells within minutes. In the cytoplasm, the RhoB-EVP50 induced extracellular calcium influx and intracellular calcium release. Membrane budding was also observed after incubation with micromolar concentrations of the fluorescent EVP50 conjugate.
Conclusions: The conjugate's interference with calcium homeostasis, its intracellular accumulation and its induced membrane dysfunction (budding and vacuolization) seem to act in concert to disrupt the cell circuitry. Contrastively, unconjugated EVP50 peptide did not display neither toxic nor cytotoxic activities in our *in vivo* and *in vitro* models.
General significance: The synergic mechanism of toxicity was restricted to the structurally modified encrypted vipericidin nonapeptide.

1. Introduction

Vipericidins (crotalicidin, batroxicidin, lutzicidin, and lachesicidin) together with Bf-CRAMP, Na-CRAMP and Oh-CRAMP comprise the cathelicidin-related antimicrobial peptides (CRAMPs) from the venom gland of the pit vipers and elapid snakes, respectively [1–3]. The cathelicidin family of peptides encompasses hundreds of sequences expressed in the tissues and cells of the immune systems of several species of vertebrates. Vipericidins and elapid CRAMPs are structurally arranged as prepropeptides with a signal peptide of ~20 residues, a conserved cathelin domain (cathepsin L inhibitor protein) and a hypervariable carboxyl-terminal stretch where a range of natural antimicrobial activities are found. Mature vipericidins and elapid CRAMPs share a high level of amino acid similarity, with most of their 34 residues being identical or strictly conserved substitutions. Due to the high proportion of lysine residues alternating with hydrophobic amino acid residues, these peptides have a net positive charge and have an amphipathic character, as observed in other membrane-active venom peptides, like anoplin [4] and mastoparans [5]. One distinctive structural feature in snake venom CRAMPs when compared to other familial members of cathelicidins is the presence of an extra acidic patch (or acidic signature) at the C-terminal end of the prosequence, which is rich in aspartic acid and uncharged residues (Fig. 1). Snake venom CRAMPs (vipericidins and elapid cathelicidins) possess effective broad-spectrum activity against clinical isolates and standard strains of bacteria and yeast [1–3,6,7]. They are particularly active against Gram-negative bacteria, even rivaling organic antimicrobial compounds. Interestingly, the cathelicidin of *Bungarus fasciatus* (BF-30) was shown to annihilate *in vitro* melanoma B16F10 cells in a dose- and time-dependent

Abbreviations: EVP, encrypted vipericidin peptide; RhoB, rhodamine B; CRAMP, cathelicidin-related antimicrobial peptide; dpf, days post-fertilization; ACP, acidic connecting peptide

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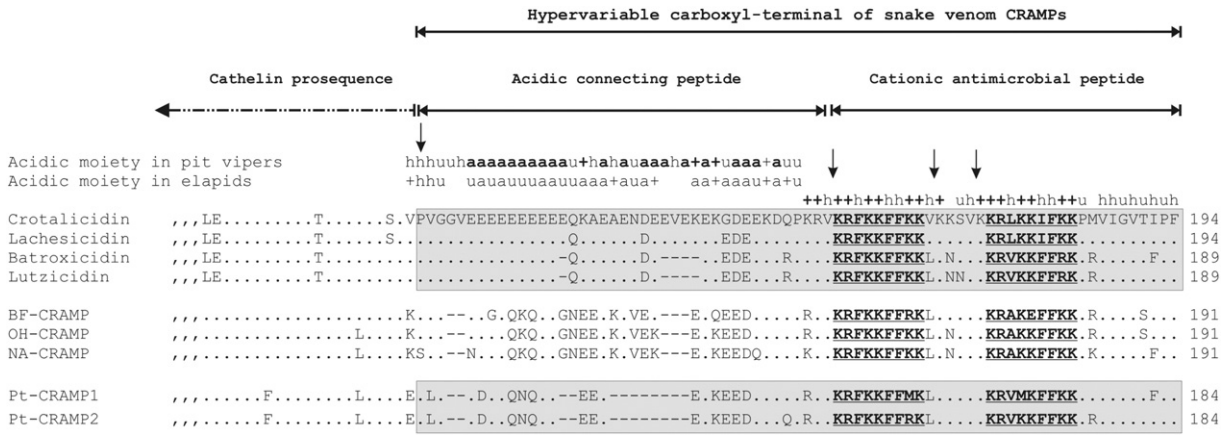

Fig. 1. Hypervariable C-terminal region of the snake venom CRAMPs and putative proteolytic cleavage sites. The acidic and cationic/amphiphatic domains of vipericidins and elapid CRAMPs were aligned. Identical amino acid residues are represented by dots, and gaps are represented by hyphens. The hypervariable anionic linkage sequences followed by the cationic antimicrobial peptides are preceded by the highly conserved cathelin prosequence (indicated by a slash). The *in tandem* repetitive cryptic nonapeptides embedded in the mature antimicrobial sequences are in bold and underlined. Arrows indicate putative proteolytic cleavage sites. The "a", "h" and "u" lowercase letters and the "+" symbol represent acidic, hydrophobic, uncharged and basic amino acid residues, respectively. Numbers on the right side of each polypeptide sequence refer to the number of residues that a given cathelicidin-related pre-propeptide contains. The vipericidins (crotalicidin, lachesicidin, batroxicidin and lutzicidin) and *P. textilis* CRAMPs (PT-CRAMP1 and 2) C-termini are shown in gray.

fashion and inhibit melanoma growth and metastasis in grafted mice [8]. On the same spectrum of activity, a Lys-16 mutant of BF-30 (BF30-K16) displayed selective *in vitro* cytotoxicity against lung cancer cell propagation in a concentration range that was not toxic to non-tumor cell lines [9].

Pre-propeptides, like the cathelicidin precursors, are processed by specific proteases that release the active portion of the polypeptide responsible for a given biological effect. Animal toxin peptides are also processed into their mature active form by precise proteolysis. For instance, crotamine and crotamine-like peptides from snake venom and lizards [10,11], cytolysins from sea anemones [12,13], apamin, melittin and mastoparan-B from hymenoptera venom [14,15], conotoxins from the marine gastropods *Conus* [16,17] and antimicrobial and cytolytic peptides from spider venom [18] are all toxins that mature by proteolytic cleavage. A less obvious maturation process (post-translational modification) is the proteolytic release of a number of bioactive peptides that are encrypted within longer polypeptides. Classic examples of cryptic peptides that contain natural physiologically active human cryptomes include the proteolytically activated prohormone precursor pro-opiomelanocortin (POMC) and the extracellular-matrix-derived crypteins [19]. In the first example, dozens of diverse active hidden opioid peptides with distinct pharmacological functions are released from POMC by tissue-specific convertases (proteases). In the second example, encrypted peptides with anti-angiogenic, anti-tumor, immunomodulation, antimicrobial and chemotaxis activities are proteolytically cleaved from proteins in the extracellular matrix, such as collagens, laminin and perlecan [19]. Thus, biological systems utilize proteolytic cleavage to generate encrypted peptides and to increase the structural, molecular and pharmacological diversity of a given peptidome to elicit complex physiological and pathophysiological responses in mammals.

Snake venom cathelicidins contain several proteolytic cleavage sites that can potentially produce a diverse array of smaller peptides [1,2]. It was proposed that elastase-like proteases are involved in the processing of bovine and porcine cathelicidins and potentially fowlicidins — cathelicidins from chicken [20]. Thus, it was anticipated that vipericidins and elapid CRAMPs might give rise to several hidden bioactive peptides from the lysine-rich, α-helical, carboxyl-terminal antimicrobial sequences. We focused our attention on the C-terminal hypervariable region of vipericidins and prepared synthetic peptides conjugated to rhodamine-B. The toxic and cytotoxic activities of encrypted vipericidin peptides were evaluated *in vivo* with zebrafish model of vertebrate organism and *in vitro* with two lines of human breast cancer cells, MCF-7 and MDA-MB231. The cell uptake and intracellular accumulation, the membrane disturbance and the interference with calcium homeostasis were observed for a criptic vipericidn nonapeptide (KRFKKFFKK) that was covalently linked to rhodamine B.

2. Materials and methods

2.1. Peptide sequences and synthesis

The hypervariable C-terminus of the vipericidins, which served as template sequences for synthesis of the encrypted peptides, was from lachesicidin (GenBank accession number AGS36142.1), which shares a high degree of sequence identity with crotalicidin (99%, AGS36138.1), batroxicidin (85%, AGS36140.1), lutzicidin (84%, AGS36141.1), Bf-CRAMP (65%, B6D434), Oh-CRAMP (65%, B6S2X2.1), and Na-CRAMP (64%, B6S2X0.1). Batroxicidin was also used for the design and synthesis of an acidic cryptic peptide (Fig. 1). All peptides were synthesized by solid phase chemistry and obtained at a purity grade over 95% and confirmed by the presence of a single peak in analytical reverse-phase HPLC and mass spectrometry analysis (Cellmano Biotech Limited, Hefei, China). The fluorescent dye Rhodamine B was covalently linked to the N-terminal end of the peptide series, directly to N-deblocked chain of peptide attached to the resin. Complete deprotection and cleavage was carried out essentially with trifluoroacetic acid in water. The crude peptides were precipitated out by the addition of chilled ether. Then, the crude peptide was purified by HPLC, freeze-drying and re-tested by HPLC to make sure that it is qualified. Table 1 summarizes the synthetic encrypted vipericidin peptides used in this study.

2.2. Zebrafish maintenance

The enhanced green fluorescent protein (EGFP) was specifically expressed in the endothelial cells of *Tg*(*fli-1:EGFP*) zebrafish larvae. The zebrafish used in our study were maintained as previously described [21]. Natural pair-wise mating (3–12 months old) was used to generate zebrafish embryos. The embryos were raised and maintained in "embryo medium" at 28.5 °C. Ethic approval for the animal experiments was granted by the Animal Research Ethics Committee in University of Macau, University of Macau, China.

2.3. Acute toxicity of encrypted vipericidin peptides toward zebrafish

Zebrafish larvae at three days post-fertilization (3dpf) were separated into a 24-well plate and exposed to 2-logs (from 1 to 100 μM)