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Review

Crotamine and crotalicidin, membrane active peptides from *Crotalus durissus terrificus* rattlesnake venom, and their structurally-minimized fragments for applications in medicine and biotechnology

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ABSTRACT

A global public health crisis has emerged with the extensive dissemination of multidrug-resistant microorganisms. Antimicrobial peptides (AMPs) from plants and animals have represented promising tools to counteract those resistant pathogens due to their multiple pharmacological properties such as antimicrobial, anticancer, immunomodulatory and cell-penetrating activities. In this review, we will focus on crotamine and crotalicidin, which are two interesting examples of membrane active peptides derived from the South America rattlesnake *Crotalus durrisus terrificus* venom. Their full-sequences and structurally-minimized fragments have potential applications, as anti-infective and anti-proliferative agents and diagnostics in medicine and in pharmaceutical biotechnology.

1. Introduction

1.1. Antimicrobial drug resistance

The surge and dissemination of drug resistant microorganisms have become major public health concerns worldwide [1,2]. One of the main causes to antibiotic resistance is the inappropriate use of the available antimicrobial agents. The delays in the initiation of the appropriate therapy, the wrongly chosen antibiotics and regimens have all contributed to the acceleration of antimicrobial resistance over the last decades. Such drug and multidrug resistant (MDR) microorganisms can be found in community-acquired infections, but most of the emergence of these pathogens has happened in hospital settings [3,4].

Patients with infections due to MDR bacteria have increased morbidity of their diseases and increased length of stay in the hospital. This longer length of stay is generally accompanied by more surgical procedures and drug treatments which will increase overall costs to the patients, their families and health insurance companies and/or governmental agencies compared to patients with susceptible strains. Actually, many of those patients have died because there can be no antibiotics available to treat their MDR infections [5,6].

1.2. Cancer chemotherapy resistance

Drug resistance issues can also be found in malignant tumors. Even though the mechanisms of chemotherapy resistance in cancer cells [7,8] and in microorganisms [9,10] are obviously different, a common feature to these resistance pathways is that they all have developed after selective pressure exerted by the cytotoxic and antimicrobial agents, respectively. In this way, patients affected with resistant cancers have similar burdens (e.g., increased morbidity, prolonged hospital stays, etc.) compared to individuals with MDR infections. Interestingly, some studies have shown that a cancer chemotherapy treatment can itself promote a selection of resistant microorganisms in the gut that can become harmful to the host/patient [11,12]. Thus, new antimicrobial and anticancer agents, as well as related chemotherapeutic adjuvants, are urgently needed to overcome those resistant microorganisms and tumors.

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1.3. Antimicrobial peptides (AMPs)

Among potential antibiotic drug candidates, the antimicrobial peptides (AMPs) have represented a significant group that can become new therapeutic strategies to fight infections caused by MDR bacteria. Also called host defense peptides, these AMPs are found in plants and animals from diverse origins and used by them to prevent infections by invading pathogens. Different from conventional antibiotics which were discovered to be products of enzymatic pathways, all AMPs are derived from gene precursors. The host innate immune cells express and store these AMPs as prepropeptides (precursors). Upon stimulation, such as the lipopolysaccharides (LPS) from Gram-negative bacteria, these precursors are released and further cleaved by proteases in the extracellular environment to generate smaller peptide fragments (usually not more than 60 residues) that have the antimicrobial activity (mature peptides) [13,14].

In terms of structure, the mature AMPs are mainly composed of hydrophilic, basic (positively charged at neutral pH), and hydrophobic residues. These peptides can adopt diverse amphipathic structures, such as α-helices, anti-parallel β-sheets and random coils, depending on their amino acid compositions and sequences, when they are in solution or when they interact directly with bacteria. That initial AMP-bacteria contact is facilitated by electrostatic interactions between the positive charges of most AMPs and the anionic phospholipids of the bacterial cell membranes. Later, the hydrophobic residues of the peptides interact with the hydrophobic tails of the phospholipids by van der Waals forces, and the bacterial plasma membrane is then destabilized, which leads to bacterial cell death. The proposed biophysical mechanisms of such destabilizations can be essentially classified in transmembrane pore (toroidal and barrel-stave) and non-pore (carpet) models [15,16].

Due to that general mechanism of action, direct cell membrane destabilization, AMPs have shown anti-proliferative activities against not only bacteria but also fungi [17], protozoa [18] and viruses [19]. AMPs can also interfere with cell walls because they are able to bind teichoic acids from Gram-positive bacteria and to inhibit chitin synthesis and the β-glucan synthase enzyme in fungi [20,21].

Some AMPs have also demonstrated antimicrobial properties through an indirect mechanism, which involves immunomodulation. Examples of such peptides are the defensins and the cathelicidins, two important classes of AMPs, which are present in vertebrates including humans. These peptides are able to stimulate immune cells (e.g., macrophages and neutrophils) to secrete chemotactic cytokines that attract other immune cells to an infection site. At the same time, these defensins and cathelicidins can bind endotoxins (e.g., LPS) and prevent the release of toxic cytokines, such as tumor necrosis factor alpha (TNF-α), by these same immune cells (i.e., an anti-inflammatory effect). Therefore, these AMPs such as the human cathelicidin LL-37 are promising pharmacological tools for a possible new antimicrobial therapy because of their dual beneficial role in immunomodulation: to help attract more immune cells to a site of infection and to prevent the deleterious effects of bacterial toxins [22–24].

1.4. Anticancer properties of AMPs

Further anti-proliferative properties of some AMPs have been found against cancer cells. These peptides have preferential activity towards tumor-derived cells compared to healthy eukaryotic cells because there is the prevalence of negatively charged molecules on cancer cell membranes, such as phosphatidylserine phospholipids, O-glycosylated mucins and sialic acid-derived gangliosides. Meanwhile, the plasma membrane of healthy eukaryotic cells is mainly composed of zwitterionic phospholipids which are electrically neutral [25–27]. Some of these AMPs can still interact with intracellular targets, such as nucleic acids and proteins [28], as well as on organelles like mitochondria, which were evolutionary ancient bacteria, where apoptotic cell death can be triggered [29,30].

1.5. AMPs as cell-penetrating peptides

Few AMPs have shown additional extraordinary biological activities, such as cell-penetrating properties. Those properties consist in the AMPs and certain cationic peptides' ability to bind cell membranes and translocate to the cytosol without killing the host cells, when these peptides are utilized in lower concentrations than the required for their anti-proliferative and/or cytotoxic actions. The proposed mechanisms involved in such properties are direct translocation and/or endocytosis. Those mechanisms are dependent on essentially the same physicochemical characteristics required for the peptides to have antimicrobial activities: positively charged and hydrophobic residues (i.e., amphipathicity). Remarkably, cell-penetrating peptides (CPPs) can be conjugated to different cargos, either through electrostatic or covalent interactions, and these complexes can be delivered intracellularly. Such properties have made CPPs powerful research tools to investigate the delivery of nucleic acids, peptides, proteins, drugs and drug carriers *in vitro* and *in vivo* [31,32].

1.6. Challenges and strategies for the development of AMPs as potential drug candidates

Although AMPs have all those described potential therapeutic and biotechnological applications, very few of them have reached the market and their clinical uses have been restricted to topical applications [33,34]. Some of the problems involved that have hindered AMP development as pharmaceuticals are their susceptibilities to salt concentrations and their low levels of selectivity, because AMPs can damage cell membranes of not only microorganisms and cancer cells but eventually also normal healthy cells. Additional issues that have also hampered AMPs development, common to other therapeutic peptides, are their high costs of manufacturing, especially for bigger and more structurally complex molecules, and their low bioavailabilities. Due to their relatively higher molecular weights than small drugs, net charges and hydrophilicities, AMPs have reduced permeabilities through biological membranes. Even when AMPs are injected intravenously, they can be quickly captured by circulating proteins in the plasma or metabolized and excreted before they are able to reach their site of action [35,36].

To overcome AMPs poor bioavailabilities and increase their stabilities to degrading enzymes, the current approaches under studies are chemical modifications and formulation strategies. Chemical modifications can involve the inclusion, at a peptide N- and/or C-termini, of less reactive groups (e.g., acetylation at the N-terminal and/or amidation at the C-terminal) [37], substitution of L-amino acids for D-amino acids [38], peptidomimetics [39–41] and peptide cyclization [42,43]. In the formulation strategies, peptides can be conjugated to or encapsulated in polymeric and/or lipid micro/nanocarriers [44,45]. Moreover, the searches for minimal structures from these AMPs that can maintain and/or enhance their biological activities (i.e., the pharmacophores) have contributed to reduce the production costs and to facilitate further peptide developments [46,47].

1.7. Animal venoms as sources of AMPs: crotamine and crotalicidin

Venoms have evolved in a number of animal species of different taxa, like arthropods (e.g., insects, arachnids such as scorpions and spiders) and reptiles (e.g., lizards and snakes), for a variety of purposes such as self-defense from predators, and prey capture and digestion. Since these venoms are complex mixture of polypeptides which have greatly differentiated from one to another in terms of structure, molecular and cellular targets and pharmacological activities, they have been explored for possible biomedical uses. The advancement of some scientific areas such as molecular biology, "omics" sciences and computational biology has allowed the identifications of the sequences of the polypeptides and, later, the cloning and preparation of selected

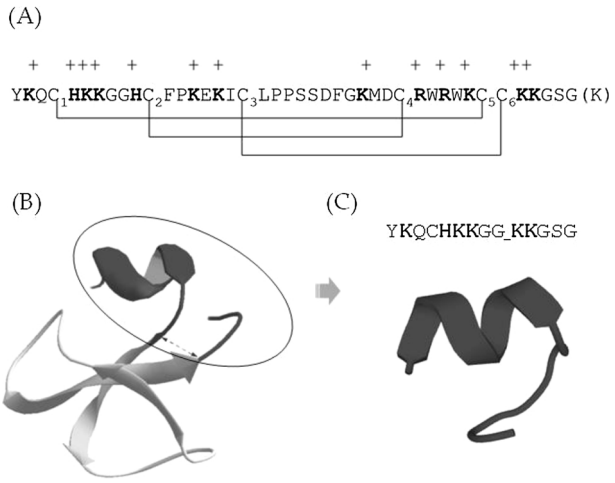

Fig. 1. Crotamine primary and tridimensional structures, highlighting the design of the first nucleolar-targeting peptide (NrTP1). (A) Primary structure of crotamine (42 amino acids), showing the distribution of positively charge residues and six cysteine residues involved in three disulfide bonds (Cys^4-Cys^{36}, Cys^{11}-Cys^{30} and Cys^{18}-Cys^{37}) that stabilize the peptide tertiary structure (B). (C) The N-terminal ^{1}YKQCHKKGG^{10} and C-terminal ^{38}KKGSG^{42} residues of crotamine are in dark gray to show the amino acid domains of the nucleolar-targeting peptide 1 (NrTP1; YKQCHKKGGKKGSG) and their spatially close proximity in the parent peptide.

polypeptide sequences for biological studies [48,49].

In this review, we will describe two antimicrobial-related peptides derived from the rattlesnake *Crotalus durissus terrificus* venom, crotamine and crotalicidin, and will address their discovered biological activities, up until now, as antimicrobial, antitumoral, immunomodulatory and cell penetrating agents. Furthermore, rationally designed structural minimizations of these AMPs will also be presented, with or without additional chemical modifications, which have maintained and/or enhanced those pharmacological activities. Special emphases will be given to membrane-peptide interactions and to examples of potential applications of these peptides as tools in medicine, biotechnology and future biopharmaceuticals.

2. Crotamine and the nucleolar-targeting peptides (NrTPs)

2.1. Crotamine

Crotamine (YKQCHKKGGHCFPKEKICLPPSSDFGKMDCRWRWKCCKKGSG – Fig. 1) is a 42-residue peptide initially isolated from the South American rattlesnake *Crotalus durrisus terrificus* crude venom. As part of such venom, crotamine has myotoxic properties that impair several sodium and potassium ion channels and promote hind limb paralyses *in vivo* [50]. Structurally, crotamine has eleven basic amino acids (nine residues of lysine and two arginine), which make the peptide highly cationic at neutral pH, and six cysteine residues that pair (Cys^4-Cys^{36}, Cys^{11}-Cys^{30} and Cys^{18}-Cys^{37}) to result in three internal disulfide bonds. These bridges contribute to stabilize the 3-D conformation, determined by proton NMR spectroscopy and later by X-ray crystallography, which consists of one α-helix and three stranded antiparallel β-sheets arranged in a $\alpha\beta_1\beta_2\beta_3$ topology (Fig. 1). That folding is highly similar to β-defensins, which suggests a common gene ancestry, despite their primary amino acid sequences are quite different. This so-called defensin-like arrangement provides for crotamine a compact molecule with a large surface area capable of hydrophobic and electrostatic interactions that are needed for the peptide biological activities [51–53].

2.2. Crotamine as an AMP

The antimicrobial properties of crotamine were mainly found against eukaryotic microorganisms. The peptide was cytotoxic to standard and clinical yeast strains belonging to the *Candida* genus. Some of these *Candida* spp. are part of the human normal microbiota, but they are capable of causing local and systemic infections, especially in patients with deficiencies in their immune systems [54,55]. When these yeast infections were evaluated *in vivo* utilizing the nematode *Caenorhabditis elegans* [56], Dal Mas and collaborators found that crotamine alone at 20 μM was lethal to the worm. The toxicity mechanisms that caused *C. elegans* death were then investigated, and it was discovered that crotamine disrupted the nematode lysosomes, which allowed the leakage of acidic components from these organelles to the cytosol [57]. Later, that lysomotropic property of the peptide was also examined against *Plasmodium falciparum*, one of the causative agents of malaria. Crotamine accumulation inside such parasites, either isolated in culture or in infected Red Blood Cells (RBCs) but not in healthy RBCs, destabilized their lysosomal membranes and reduced blood parasitemia in a dose-dependent manner [58]. In addition, crotamine presented activity against *Leishmania brasiliensis* in both promastigote [59] and, when encapsulated in polylactic-glycolic acid (PLGA) microparticles, amastigote [60] forms.

There are still uncertainties about the antibacterial effects of crotamine. A few years ago, the peptide showed toxicity to *Escherichia coli*, with minimum inhibitory concentrations (MICs) ranging between 25 and 100 μg/ml depending on the evaluated strain, but did not inhibit growth of other Gram-negative (e.g. *Pseudomonas aeruginosa*) and Gram-positive (e.g. *Staphylococcus aureus*) strains up to 200 μg/mL. Furthermore, it was pointed out in two separate studies that crotamine tertiary structure was not mandatory for that modest antibacterial activity. In the first study, slightly enhanced anti-*E. coli* effects were observed with crotamine after it was reduced with 5 mM of dithiothreitol (DTT) at 100 °C [61]. This unusual cytotoxic improvement after disulfide bridges reduction could also be found with human β-defensin 1 in colonic mucosa, where there is a low oxygen partial pressure, against anaerobic microorganisms [62]. In the second study, crotamine was obtained from genetically modified bacteria and, when compared with the peptide either isolated from the rattlesnake venom or chemically synthesized, presented somewhat better antibacterial effects than the analogues even though circular dichroism (CD) spectra revealed a disordered structure for the recombinant crotamine [55]. More recently, however, native crotamine inhibited the growth of not only *E. coli* but also *S. aureus* and oxacilin-resistant *S. aureus* (ORSA) with MICs at 16 μg/mL, which was almost half of the control (levofloxacin at 30 μg/mL) [63].

The interaction of crotamine with model membranes containing negatively charged phospholipids to imitate bacterial cell membranes has also been investigated. However, as it has happened with the antibacterial evaluations, the presented results from two separate studies have showed some divergences too, which can be related to the utilized methodologies. In one of them, Sieber and co-workers noticed that the peptide was able to not only be incorporated in lipid ("Langmuir") monolayers but also to form monolayers by itself, due to its amphipathic nature, on air-water interfaces. Moreover, when planar lipid bilayers were produced, crotamine was capable of assembling in pores within these membranes, and the authors suggested that those pores could be oligomeric aggregates of the peptide after mass spectrometry (MS) observations of crotamine in solution [64]. In the other study, Costa and collaborators employed large and giant unilamelar vesicles (LUVs and GUVs) and found that fluorescent-labeled crotamine initially had a uniform distribution on those vesicles and later accumulated in a single region producing a macropore that lead to membrane instability [65].

Additional antimicrobial properties of crotamine were discovered that involved immunomodulation. *In vitro*, the peptide enhanced

macrophage phagocytic activities in a dose-dependent manner by increased expression of the inducible nitric oxide synthase (iNOS) enzyme and the TNF-α, through p38 phosphorylation and NF-κB [66]. *In vivo*, 200 − 800 μg of intradermally applied crotamine increased the levels of both anti- (interleukin 10, IL-10) and pro-inflammatory (TNF-α) cytokines, which suggested a cautious usage of the peptide as an AMP without further modifications [67].

2.3. Crotamine anticancer properties

Crotamine anti-tumor activities have also been studied during the last ten years. The peptide has showed *in vitro* toxicity to cancer cells from epithelial origins such as murine (B16(F10)) and human (SK-Mel-28) melanomas as well as human pancreatic (Mia PaCa-2) carcinoma. At a concentration of 5 μg/mL, the peptide was lethal to those cells while it was innocuous to non-tumor cells [68]. The found mechanisms underlying that cancer cell death by crotamine were the permeabilization of lysosomes [69], similarly to the cytotoxicity to *C. elegans* [57] and *P. falciparum* [58], the intracellular calcium release from the endoplasmic reticulum, and the loss of mitochondrial membrane potential [70]. *In vivo*, mouse models of melanoma have been treated with the peptide administered either through parenteral or oral routes. When B16(F10) cells were injected in mice for tumor growth, subcutaneous daily injection of 1 μg of crotamine per animal, which started the next day after cancer cell inoculation, for 21 days retarded tumor growth and significantly increased the survival rate of treated mice in comparison to the control (saline-treated) group [68]. In other set of experiments, the same type of tumor-bearing mice were treated with an intraperitoneal injection of Cy3-labeled crotamine and fluorescence was detected within these cancer cells in tumor slices [70]. These later *in vivo* experiments demonstrated the promising actions of crotamine as an anti-cancer drug and as a cancer biomarker. Recently, another set of B16(F10) tumor-bearing mice were treated with 10 μg of the peptide given orally and crotamine could cross the gastrointestinal tract and significantly inhibit tumor growth and increase survival rate compared to the control group. Noteworthy, the oral administration of crotamine in mice did not cause toxicity to the organs that mostly accumulated the peptide (i.e., liver and kidney) and, in mice without tumors, crotamine was able to increase blood glucose clearance and high density lipoproteins as well as decrease total cholesterol and low density lipoproteins [71].

2.4. Crotamine is also a CPP

Crotamine cell penetrating properties have been evaluated along with its anticancer actions. In fact, the findings that the peptide quickly internalized in different cell types (in approximate 5 min) and preferentially accumulated in actively proliferating (AP) cells such as mouse embryonic stem [72] and Chinese Hamster Ovary (CHO-K1) [69] cells firstly triggered cell penetration and, later, anticancer studies. At non-toxic concentrations, crotamine could enter these cells especially during their S/G2 period, and label centrosomes and, in the nucleus, chromosomes. Thus, the peptide could be proposed as a cell cycle and/or a centriole biomarker [72]. The determined mechanism of crotamine cell entrance was endocytosis after the utilization of low temperature conditions and chloroquine and chlorpromazine, pharmacological inhibitors of endosome acidification and clathrin-coated pits, respectively [73].

That ability to escape the low pH of the lysosome/late endosome compartment motivated advanced studies about crotamine cargo delivery capabilities. Since the peptide has a highly cationic charge, it was investigated whether crotamine could bind DNA and deliver it intracellularly. The peptide-DNA complexes were then obtained and characterized with agarose gel electrophoresis and fluorescence and UV–vis spectroscopy methodologies. The peptide-DNA weight ratio of 10:1 was sufficient to form stable complexes at 37 °C and 150 mM NaCl that could resist proteolytic degradation [74]. Furthermore, it was found that each crotamine molecule could non-cooperatively bind around 5 DNA base pairs through ionic and non-ionic interactions [75]. Next, the transfection of a plasmid (pEGFP-N1) complexed with the peptide was verified *in vitro* and *in vivo*. Employing AP cells, it was found that DNA-bound crotamine interacted with heparan sulfate proteoglycans before endocytosis and delivered the functional reporter gene, which was confirmed by the green fluorescent protein (GFP) signal in 98% of the cells after incubation for 24 h. *In vivo*, crotamine-DNA complexes were injected intraperitoneally and, after 24 h, green fluorescence signal was detected in bone marrow cells, liver and lung bronchioles, and there were no signs of toxicity in the treated mice [73].

That crotamine gene delivery ability has been explored for possible biotechnological applications such as transgenesis. *In vitro* fertilized embryos from cattle were obtained and exposed to the peptide at different concentrations and incubations periods. Rhodamine-labeled crotamine at 10 μM entered the cells and did not interfere, up to 24 h of treatment, with embryo development [76]. Later, crotamine-GFP plasmid complexes were obtained that entered the embryos but overall could not enhance gene expression [77], which have suggested the need for additional improvements.

2.5. The nucleolar-targeting peptides (NrTPs)

In the search for smaller structures from crotamine that could maintain and/or improve its anticancer and, especially, cell-penetrating properties, some interesting peptides were obtained [78–80]. One group of such fragments is called the Nucleolar-Targeting Peptides (NrTPs) (Fig. 1; Table 1) that were designed based on crotamine tertiary structure where its N- and C-termini have a spatially close proximity (5.9 Å between the alpha carbon of Gly^{9} and Lys^{38}, indicated by the dashed arrow in Fig. 1-B). Since both termini also have prevalence of Lys and His (basic) residues, it was verified if a linear peptide sequence (dark gray domains in crotamine secondary structure – Fig. 1-B) derived from the combination of those termini was capable of translocating cell membranes. The peptides NrTP1 (YKQCHKKGGKKGSG- Fig. 1-C), and NrTP2 (YKQCHKKGG-Ahx-KKGSG, where “Ahx” (6-aminoheaxanoic acid) is a chemical spacer between the termini), and their retro- (NrTPs 3 and 4) and D- (NrTP5) analogues were then synthesized and evaluated *in vitro* (Table 1).

The fluorescent analogues of NrTP1 and NrTP2 could enter HeLa cervical cancer cells at concentrations as low as 15 μM in 45 − 60 min and localize in their nuclei and, more specifically, in their nucleoli, which are sub-nuclear compartments responsible for rRNA and ribosome biogenesis. These peptides were non-toxic to those cells at least up to 100 μM, and their retro – and D- analogues showed low percentage (retro-) or no (D-) internalization, which suggested a receptor mediated uptake [81]. Later, that NrTP1 could also penetrate and show nucleolar localization in other cancer cells such as human pancreatic (BxPC-3) and breast (BT-474) carcinomas. Moreover, confocal microscopy and flow cytometry illustrated that the peptide entrance was independent of the cell cycle period, but it was reliant on clathrin-mediated endocytosis since the same pharmacological inhibitors and low temperature conditions used for crotamine also hindered NrTP1 uptake by those cells [82].

The interactions of NrTPs with model membranes were next evaluated. In addition to the later peptides, three more structures were added to the subsequent studies: NrTP6 (similar to NrTP1, with the Cys residue replaced by Ser), NrTP7 (~NrTP6, with the substitution of all Lys residues by Arg), and NrTP8 (~NrTP6, with the exchange of Tyr for Trp). Employing molecular partition assays, Rodrigues and collaborators found that increases in the percentage of anionic phospholipids raised the interaction of NrTPs with lipid membranes. Furthermore, CD analyses revealed that the unstructured NrTPs in solution tended to adopt an α-helical like conformation when in contact with phospholipid

Table 1
Crotamine and peptide derivatives with distinct antiproliferative and cell-penetrating bioactivities.

Name	Sequence[a]	Fragments[b]	Ref.	Bioactivity and/or hallmarks[c]
crotamine	YKQCHKKGGHCFPKEKICLPPSSDFGKMDCRWRWKCCKKGSG	–	[50]	Ion-channel modulator; myotoxin; antitumor; CPP; centriole marker
NrTP1	YKQCHKKGGKKGSG	[1–9] ~ [38–42]	[81,82]	CPP; nucleolar marker
NrTP2	YKQCHKKGG-Ahx-KKGSG	[1–9] ~ [38–42]	[81,82]	CPP; nucleolar marker
NrTP3	GSGKKGGKKHCQKY	[42–38] ~ [9–1]	[81]	CPP with low uptake efficiency;
NrTP4	GSGKKGGKKICQKY	[42–38] ~ [9–1]	[81]	CPP with low uptake efficiency;
NrTP5	ykqchkkGGkkGsG	[1–9] ~ [38–42]	[85]	Receptor-independent uptake
NrTP6	YKQSHKKGGKKGSG	[1–9] ~ [38–42]	[85]	Efficient CPP; cellular-delivery of large cargo; low cytotoxicity
NrTp7	YRQSHRRGGRRGSG	[1–9] ~ [38–42]	[85]	CPP; cytotoxicity equivalent to TAT_{48-60}
NrTP8	WKQSHKKGGKKGSG	[1–9] ~ [38–42]	[85]	Low efficient CPP; highly cytotoxic
DY676-NrTP6	YKQSHKKGGKKGSG	[1–9] ~ [38–42]	[87]	Selective CPP of tumor heterogeneity
CyLop-1	CRWRWKCCKK	30–39	[79,80]	CPP; cytosolic-homing and -delivery
C1	KQCHKKGGHCFPKEKIC	2–18	[50]	Candicidal; non-membranolytic
C2	KMDCRWRWKCCKK	27–39	[81,82]	Candicidal; non-membranolytic
P1	KQSHKKGGHSFPKEKIS	2–18	[81,82]	Devoid of activity
P2	KMDSRWRWKSSKK	27–39	[81]	Devoid of activity

[a] Crotamine disulfide bonding, Cys^4-Cys^{36}, Cys^{11}-Cys^{30} and Cys^{18}-Cys^{37}; "Ahx" in NrTP2 sequence is the chemical spacer 6-aminoheaxanoic acid; Cys(DY676) NrTP-6 has the near infra-red dye DY676 covalently attached to a Cys residue in the N-terminal of NrTP-6.

[b] NrTP-1 is a spliced derivative of crotamine, comprising residues 1–9 and 38–42 ([1–9] ~ [38–42]); NrTP-2, a NrTP-1 analog with Ahx as a chemical spacer; NrTP-3, inverted version of NrTP-1; NrTP-4, an analog of NrTP-3 in which His residue was replaced for a Ile; NrTP5, an all *D*-enantiomer of NrTP1; NrTP6, a NrTP1 analog in which Cys was replaced for Ser (Cys-Ser replacement); NrTP7, a NrTP6 analog in which Lys was replaced for Arg; NrTP8, a NrTP6 with a Tyr→Trp replacement; DY676-NrTP-6, named originally by the authors, Cys(DY676)-[4-Ser-crotamine(Δ10-37)]; Cys-NrTP-6 and NrTP-6-Cys have also been also prepared for functional studies of gene delivery or delivery of large cargoes (see refs. 85 and 87); NrTP analogs with carboxyfluorescein and rhodamine-B, conjugated directly in the N-terminal amino acid residue were synthesized for several studies of function (see ref. 103).

[c] CPP, cell-penetrating peptide.

vesicles. Also, the same research group could demonstrate the translocation of a fluorescently-labeled NrTP through these membranes with the utilization of giant multivesicular liposomes [83].

Additional studies involved the uptake profile assessments of the above mentioned NrTPs at different concentrations and with diverse cell types. In peripheral blood mononuclear cells (PBMCs) that comprise lymphocytes and monocytes, all NrTPs were internalized and accumulated mainly in the cytosol, rather than the nuclei and nucleoli. However, distinct fluorescence intensities among the NrTPs were observed which were correlated to their primary amino acid sequences. Overall, in lymphocytes, the fluorescence intensity/uptake trend was: NrTP6 > NrTP7 > NrTP2 > NrTP8 > NrTP5 > NrTP1 > Tat_{48-60} (control). The peptides had higher entry rates (i.e., faster uptake) in lymphocytes than monocytes, but, in both cells, these rates were not concentration-dependent [84]. The NrTPs were also evaluated in other healthy cells, such as BHK-21 kidney fibroblasts and RBCs. Whereas the peptides did not penetrate erythrocytes, BHK-21 cells showed a similar cytosolic accumulation of NrTPs as PBMCs. NrTPs entrance in cancer cells (HeLa, MOLT-4 and BV-173) were also tested, and nucleolar targeting was observed at higher concentrations. Among these later peptides, NrTP7 and NrTP8 were the most cytotoxic, along with Tat_{48-60} (arginine-rich peptide), while NrTP6 had the highest cell uptake and the lowest toxicity [85].

As a proof-of-concept, the NrTP6 ability to deliver biologically active molecules intracellularly was verified. The peptide was prepared with an extra cysteine residue at either terminus to achieve chemical conjugation with the β-galactosidase (β-gal) enzyme through a maleimide group. The NrTP6/β-gal conjugate maintained comparable enzyme activity as the free enzyme in solution on the same substrate. Moreover, equivalent β-gal activity was found in HeLa cell-free extracts after those cells were previously incubated with the conjugate, which confirmed the intracellular delivery of a functional enzyme by NrTP6 [86]. Finally, Tansi and collaborators [87] coupled the near-infrared dye DY676 to NrTP6, also via maleimide chemistry and a N-terminal cysteine residue, and they named this analogue Cys(DY676)-[4-Ser-crotamine(Δ10-37)] or mini-crotamine, for simple (Table 1). Such DY676-NrTP6 peptide penetrated and accumulated in certain lines and populations of cancer cells, which lead the authors to consider the use of that NrTP6 analogue as a potent theranostic probe for imaging of cancer heterogeneity and delivery of chemotherapeutics straight into subpopulations of tumor cells.

3. Crotalicidin and fragments Ctn[1–14] and Ctn[15–34]

3.1. Crotalicidin

Crotalicidin (KRFKKFFKKVKKSVKKRLKKIFKKPMVIGVTIPF – Fig. 2; Table 2) is a recently discovered 34-residue AMP from the venom gland of *Crotalus durissus terrificus*, which was found along with other cathelicidin-related peptides from diverse South American pit viper snakes. From the venom glands of each studied species, individual cDNA

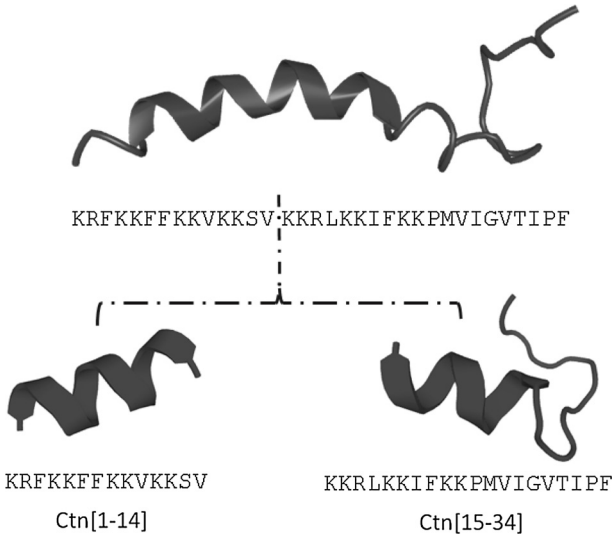

Fig. 2. The primary sequences and 3-D structures of crotalicidin (Ctn) (top) and designed fragments Ctn[1–14] (bottom – left) and Ctn[15–34 (bottom – right). Peptides Ctn[1–14] and Ctn[15–34] were designed taking into account the putative elastase proteolytic cleavage site (after Val^{14}, dashed line). Ctn[1–14] maintained the α-helical conformation of Ctn but lost AMP activity. On the other hand, the unstructured Ctn[15–34] preserved the antibacterial and anticancer properties of the parental peptide with greater selectivity than Ctn.

Table 2
Crotalicidin, peptide derivatives and related sequences with distinct bioactivities.

Name	Sequence[a]	Fragments[b]	Ref.	Bioactivity and/or hallmark[c]
Crotalicidin	KRFKKFFKKVKKSVKKRLKKIFKKPMVIGVTIPF	–	[90,100]	Anti-infective; antiproliferative; relatively cytotoxic and hemolytic
Ctn[1–14]	KRFKKFFKKVKK	1–14	[100]	Inactive; low cytotoxic
Ctn[15–34]	KKRLKKIFKKPMVIGVTIPF	15–34	[100]	Antibacterial; antitumoral; Non-hemolytic; serum-stable;
Ctn[15–34]-c	KKRLKKIFKKPMVIGVTIPF-COOH	15–34	[101]	Less stable in serum
Batroxicidin	KRFKKFFKKLKNSVKKRVKKFFRKPRVIGVTFPF	–	[90,93]	Anti-infective; antiproliferative
EVP50	KRFKKFFKK	1-9	[99]	Inactive; cytotoxic and toxic
ACP1b	PVGGVEEEEEDEEEQKAEVEKDEEKEDEEKDRPKRVKRFKKFFKK	(−)36−(+)9	[99]	Toxic to zebrafish
ACP1l	PVGGVEEEEEEEEEEQKAEAENDEEVEKEKEDEEKDQPKRVKRFKKFFKK	(−)41−(+)9	[99]	Toxic to zebrafish

[a] Peptides are in their amidated form, except Ctn[15–34]-c (Ctn[15–34]-carboxyl), carboxylated at the C-terminal, as well as EVP50, ACp1b and ACP1l.
[b] Fluorescent-labeled, covalently conjugated derivatives have also been prepared and studied; The encrypted vipericidin peptide (EVP) sequences are underlined in batroxicidin derivatives; In ACP (acidic connecting peptide) sequences (1b and 1 l) the acidic moiety preceding the vipericidin (antimicrobial peptide) sequences were maintained in the synthetic peptides.
[c] Anti-infective activity comprises mainly antibacterial, antifungal and anti-parasitic activity; antiproliferative means active against several lines of cancer cells; EVP50, ACP1b and ACP1l are toxic to zebrafish when conjugated with fluorescent dye rhodamine-B; rhodamine B-EVP50 is also cytotoxic to cancel cells at low micromolar concentrations.

libraries were prepared and checked for the presence of such AMPs, with the utilization of primers that were developed based on earlier findings from Asian elapid snakes [88,89]. After PCR screening and the Sanger (dideoxy chain termination method) sequencing of positive amplicons, cathelicidin precursors (mRNAs) were revealed and their sequences deposited in the GenBank nucleotide database. Next, expression of these peptide precursors was confirmed with quantitative PCR (qPCR), and their amino acid sequences were deduced. When these AMP primary structures were aligned with the Asian elapid homologues, the mature sequences could be determined and they were collectively named vipericidins.

Crotalicidin and batroxicidin (KRFKKFFKKLKNSVKKRVKKFFRKPRVIGVTFPF, Table 2), vipericidins from *Crotalus durissus terrificus* and *Bothrops atrox*, respectively, were then synthesized, each with a C-terminal amide group, for evaluation of their biological properties. Crotalicidin and batroxicidin showed antimicrobial activities against all tested bacteria, either from standard or clinical strains, principally against the Gram-negative species with lower MICs than the control drug gentamicin by at least 2-fold. Moreover, those MICs were more than an order of magnitude lower than concentrations that caused noticeable hemolysis (~20%) [90].

Later, those antimicrobial activities were confirmed by Oliveira-Junior and co-workers that also analyzed the immunomodulatory effects of those vipericidins in macrophages. Overall, the peptides especially crotalicidin demonstrated anti-inflammatory properties, similarly to LL-37 [24][25], because there were down-regulation of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), and up-regulation of the anti-inflammatory cytokine IL-10, even in the presence of some inflammatory stimuli, such as interferon-gamma (IFN-γ) and heat-killed bacteria [91].

In addition, crotalicidin and batroxicidin presented cytotoxic effects against all morphological forms of *Trypanosoma cruzi*, the causative agent of Chagas disease. These peptides were able to reduce parasitemia, with low toxicity to the host cells (i.e., high selectivity index), through the disruption of *T. cruzi* cell membranes that was visualized by scanning electron microscopy [92,93]. Such cell membrane rupture was previously observed when *E. coli* was treated with the cathelicidin BF-30, from the Asian snake *Bungarus fasciatus* (banded krait), which is highly homologous to crotalicidin and batroxicidin [94,85].

3.2. Crotalicidin peptide fragments Ctn[1–14] and Ctn[15–34]

Furthermore, as it has happened with other cathelicidins such as LL-37 [95–97] and BF-30 [98], crotalicidin and batroxicidin have been subjected to structural dissection studies to look for smaller fragments that can preserve and/or enhance their antimicrobial properties.

In one of these studies, it was noticed that there is a nine-residue amino acid sequence that is conserved among them, also present in the non-synthesized peptides, and is essentially repeated (i.e., with very few conservative mutations) in tandem in each vipericidin primary structure. That oligopeptide was named EVP50 (KRFKKFFKK) and chemically obtained, but did not present any cytotoxicity (Table 2). However, when rhodamine B was conjugated to EVP50 at its N-terminal, the fluorescent-labeled analogue (Rho-EVP50) showed activity against MCF-7 and MDA-MB-231 breast cancer cells [99].

In the following study, crotalicidin (Ctn – Fig. 2) was submitted to an *in silico* proteolysis that simulated a possible defense mechanism of a host skin in a snake bite site. Employing neutrophil elastase as a model enzyme, the two generated fragments with the highest molecular weights were selected and obtained for further physicochemical and pharmacological evaluations: Ctn[1–14] and Ctn[15–34], the N- and C-termini of Ctn, respectively (Fig. 2, Table 2). CD and NMR analyses revealed that Ctn[1–14] (KRFKKFFKKVKKSV) maintained the α-helical conformation of Ctn but that fragment lost the antibacterial and anticancer activities of crotalicidin, which agreed with the highly homologous EVP50 mentioned above. In contrast, Ctn[15–34] (KKRLKKIFKKPMVIGVTIPF) could maintain the antibacterial and the cytotoxic actions of Ctn, especially against the most susceptible hematological cancer cells to the parental peptide, even though that later fragment had a random coil structure. In addition, the fragments were much less toxic to human fibroblasts and RBCs than Ctn, which confered to Ctn [15–34] the highest selectivity among the peptides (Fig. 2). Remarkably, it was also found that while Ctn and Ctn[1–14] had half-lives below 30 min in a 50% human serum solution at 37 °C, Ctn[15–34] had a half-life of approximately 12 h in the same medium and incubation conditions [100].

That extended half-life was further investigated in two subsequent studies by Perez–Peinado and collaborators. In one of them, Ctn[15–34] and some analogues were obtained to investigate what were the structural components of the peptide involved in that long-lasting properties. It was found that the α-helical and hydrophilic N-terminal (high homology with Ctn[1–14] and EVP50) followed by the unstructured hydrophobic domain and the amide group at the C-terminal were all necessary since any changes in the analogues drastically reduced their half-lives in comparison to Ctn[15–34] [101]. In the other evaluation, affinity chromatography coupled with MS utilizing N-terrminal biotinylated Ctn[15–34] captured and identified glycoproteins and lipoproteins from serum that interacted with the peptide. Binding of Ctn[15–34] to serum proteins with known carrier properties was then confirmed through Surface Plasmon Resonance (SPR), which can explain the peptide ability to escape proteolytic degradation and, therefore, to last longer in blood serum [102].

The mechanisms involved in Ctn and Ctn[15–34] antimicrobial activities were also thoroughly investigated. As the peptide concentrations increased on the surface of bacteria, observed by confocal microscopy, their cell membrane ζ-potentials increased and were neutralized below the MICs and minimal bactericidal concentrations (MBCs) of both Ctn and Ctn[15–34]. Those increases in peptide concentrations also allowed the entrance of a cell-impermeable nucleic acid fluorophore, which characterized membrane permeabilization by the peptides and consequent bacterial cell death. Time-resolved flow cytometry revealed that Ctn [15–34] entered the cell membranes as soon as the peptide was added to the bacteria while Ctn had a delay before such cell permeabilizations [103].

Further antimicrobial studies involved the evaluation of Ctn and fragments against fungi and a virus. Among the peptides, Ctn was the most active against dermatophytes while Ctn[15–34] and even Ctn [1–14] had lower MICs against yeasts, including standard and clinical strains from diverse *Candida* species, than Ctn. Noteworthy, when either of the peptides was combined with amphotericin B (AMB), there were decreases in both drug and peptide MICs against clinical strains of *C. albicans* by at least 4-fold. These peptide and drug associations were considered synergistic, which can be highly useful in clinical applications to avoid yeast resistance to AMB and to reduce that drug toxic renal effects [104]. Later, separate flow cytometry assays confirmed that synergism between Ctn[15–34] and AMB and indicated a necrosis-like cell death pathway after interaction of Ctn[15–34] with a *C. albicans* clinical strain [105]. That necrosis-like cell death pathway could also be observed with *T. cruzi* after Ctn or batroxicidin treatments, as previously mentioned [92,93]. Ctn[15–34] was also able to neutralize the toxicity promoted by the Infectious Myonecrosis Virus (IMNV) at loads able to cause 50% cell death in shrimp primary hemocyte cultures in 24 h. Interestingly, the peptide retained that protective effect against IMNV for at least up to 48 h in a medium with 20% serum and high salt concentrations, which were necessary conditions to have the same osmolarity as the hemocytes. Therefore, Ctn[15–34] has also potential utilization in aquaculture against shrimp viral diseases [106].

4. Conclusions and perspectives

Although the antibacterial actions of crotamine [61,63] need to be revisited, the peptide has been an effective molecule against eukaryotic pathogens [54,55,58–60]. In cancer therapy, crotamine can be utilized as a biomarker of the cell cycle and actively proliferating cells. The peptide has also been considered a potential lead for anticancer drug development since it has shown antitumoral activities *in vitro* and *in vivo* [68–71]. Furthermore, crotamine cell-penetrating properties were elucidated and its gene delivery abilities can be further improved as tools for biotechnological applications such as transgenesis, RNA interference and gene editing purposes [32,72,73,76,77]. In the search for smaller structures from crotamine that could maintain and/or enhance most of its biological properties, the NrTPs were designed and evaluated. They were able to enter cells and to work as biomarkers of nucleoli, which are dynamic intranuclear compartments that could be compromised in some disease outcomes. Moreover, that cell-penetrating capacity has been explored for antitumor therapy and the intracellular delivery of biologically active molecules [81,82,85,86]. Crotalicidin is a recently found AMP that has shown antimicrobial activities against Gram-negative bacteria, fungi, protozoa, as well as anticancer actions [90,92,93,100,103,104]. Ctn[15–34], the C-terminal fragment of Ctn, retained those anti-proliferative properties of the parental peptide and had greater selectivity than Ctn [100,103–106]. In addition, Ctn[15–34] extended longevity in blood serum can make this peptide a prospective anti-infective lead [101,102]. Taken together, crotamine and the NrTPs, and crotalicidin and Ctn[15–34] have shown promising pharmacological activities that can have potential biomedical applications.

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Declaration of Competing Interest

None.

CRediT authorship contribution statement

Claudio Borges Falcao: Conceptualization, Writing - review & editing. **Gandhi Radis-Baptista:** Conceptualization, Writing - review & editing, Funding acquisition, Project administration.

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