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Review

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NrTP - from snake venom to drug delivery system and beyond

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Dedicated to the retirement of Professor Hans-Günther (Hagga) Schmalz, an exceptional scientist and a valued representative of the German School of Chemistry

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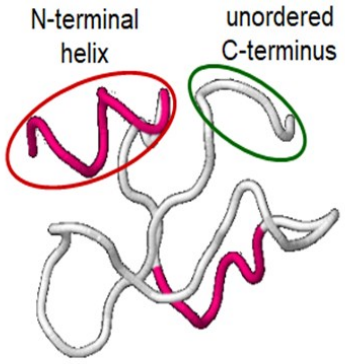
Abstract

Snake venoms are a rich source of structurally diverse bioactive peptides with significant biomedical potential. Among them, crotamine, a cysteine-rich, cationic peptide from the venom of *Crotalus durissus terrificus*, has attracted particular attention due to its unique ability to selectively penetrate eukaryotic cells and accumulate in the nucleus. Structure-guided deconstruction of crotamine led to the identification of short nucleolar-targeting motifs, giving rise to the NrTP (Nucleolar Targeting Peptide) family of cell-penetrating peptides (CPP). These peptides retain efficient cellular uptake, exhibit low cytotoxicity, and display broad applicability across multiple cell types, including primary human immune cells.



Keywords: Antimicrobial activity, cell penetrating peptides, drug delivery, nanostructures, nucleolar-targeting peptides

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1. Introduction

This mini-review summarizes the discovery, structural features, cellular uptake mechanisms, and biological performance of NrTP peptides, with particular emphasis on NrTP6 as a versatile intracellular delivery vector. Key studies demonstrating the successful transport of large and biologically active cargos, such as enzymes, polymers, and therapeutic peptides, are discussed, highlighting the superior performance of NrTP6 relative to classical CPPs. Recent advances in NrTP6 lipidation are also reviewed, showing how fatty acid modification enables controlled self-assembly into nanostructures with tunable morphology and secondary structure. These lipidated constructs function as efficient nanocarriers for sustained and targeted nuclear drug delivery and additionally display antimicrobial activity with synergistic effects when combined with conventional antibiotics. Overall, NrTP peptides represent a promising multifunctional platform at the interface of peptide-based delivery, nanotechnology, and antimicrobial therapy, illustrating how venom-derived peptides can be repurposed into advanced drug delivery systems and beyond.

2. Snake Venom and Crotamine

Snake venoms are complex mixtures, primarily composed of proteins and enzymes, which exert biological and toxic effects with the purpose of weakening, paralysing, killing and digesting prey.[1, 2]

The venom of *Crotalus durissus* contains four principal toxins: crotoxin, convulxin, gyroxin and crotamine.[3] Unlike crotoxin, which is the major venom component across all species, crotamine is produced only by a subspecies of *Crotalus durissus terrificus* in select regions of South America. Crotamine was first isolated by Gonçalves and Polson in 1947.[4] However, it was not until 28 years later, in 1975, that its primary structure was elucidated using chemoenzymatic methods.[5] It is a 42-amino-acid polypeptide containing six cysteine, nine lysine, two arginine, two histidine, and two tryptophan residues, with the sequence:

H-YKQCHKKGGHCFPKEKICLPPSSDFGKMDCRWRWKCCKKGSG-OH

It has a molecular weight of 4880. The connectivity of the six Cys residues was later established as Cys4-Cys36, Cys11-Cys30, and Cys18-Cys37; this is a 1-5, 2-4, 3-6 pattern, similar to β-defensins.[6] The secondary structure comprises a short N-terminal α-helix and three small antiparallel β-sheets (Figure 1).[7-9]

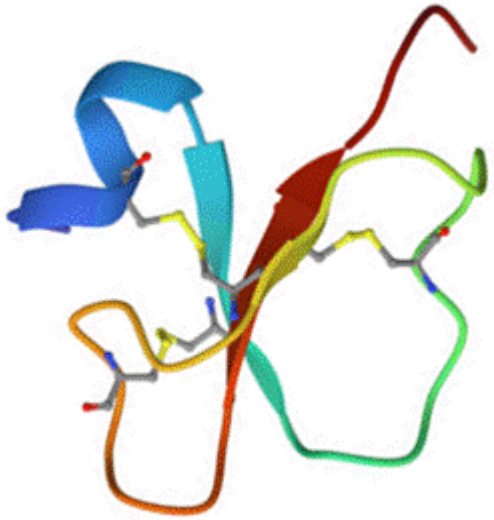


Figure 1. 3D structure of crotamine (PDB entry no. 1h5o).

Furthermore, crotamine is a myotoxin that induces severe muscle necrosis via a non-enzymatic mechanism. Notably, it was the first snake venom peptide shown to selectively penetrate eukaryotic cells.[10] Its ability to penetrate and accumulate in the nucleus makes crotamine a versatile tool for DNA delivery, cell cycle monitoring, and intracellular vesicle tracking. Additionally, it exhibits antimicrobial activity and potential selective antitumor effects.[11]

3. Nucleolar-targeting Motifs

Precedent for nucleolar-targeting motifs was established in cell-penetrating proteins such as the Trans-Activator of Transcription (Tat) from HIV-1[12] and Drosophila *Antennapedia* (Antp) homeodomain from *Drosophila melanogaster*,[13] where short fragments, Tat(47-57) or Tat(48-60)[14] and penetratin [Antp(43-58)],[15] were sufficient to cross cellular membranes.

These findings prompted researchers to identify analogous motifs within crotamine responsible for nucleolar targeting. In highly folded peptides, the standard procedure is to follow a structure-guided deconstruction, which involves breaking them down into their structural components or fragments and analyzing the biological activity of these individual parts. The most direct structural dissection of crotamine could be to synthesize the fragments linked by the disulfide bonds and substitute the other Cys residues embedded in the sequence with serine or 2-aminobutyric acid (Abu), which are isosteric analogues. As shown in Figure 2A, this strategy would result in three different peptides: one consisting of 33 amino acids and the other two consisting of 20 amino acids.

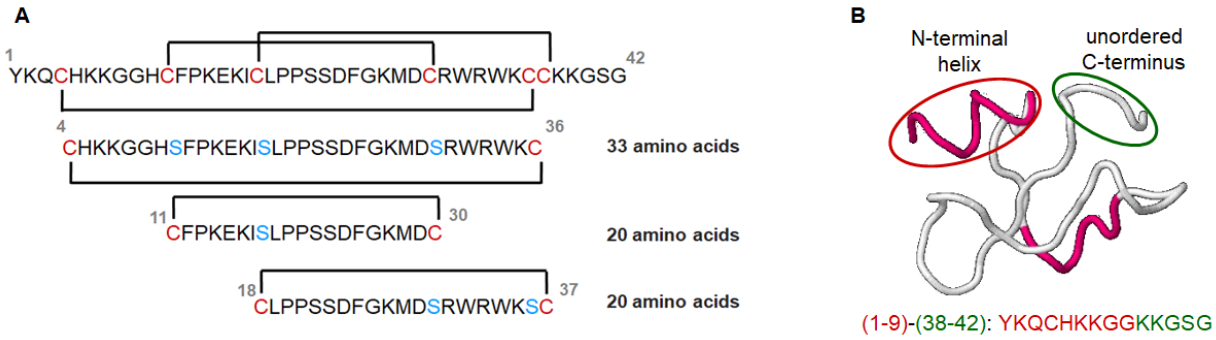
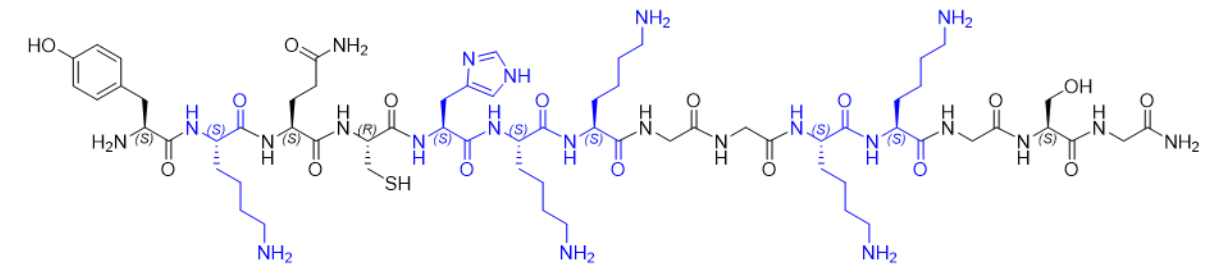


Figure 2. **(A)** Structural dissection of crotamine showing its peptide fragments linked via disulfide bonds, with non-bridging cysteine residues substituted by serine. **(B)** 3D structure of crotamine highlighting the close spatial proximity of the N- and C-termini, resulting from its folded conformation.

4. NrTPs as Cell-penetrating Peptides

Radis-Baptista *et al*. [16] analyzed the 3-D structure of the protein, and observed that the folded structure brings the C- and N-termini were close. Thus, splicing the fragments 1-9 and 38-42 would result in a 14-amino acid peptide rich in basic residues (5 Lys and 1 His) that is also a common characteristic found in cell-penetrating peptides (CPP) sequences (Figure 2B). The authors hypothesized that this sequence might produce a peptide with translocating properties. Initially, the spliced peptide was prepared as H-YKQCHKKGGKKGSG-NH_2 (NrTP1) (Figure 3A), and the peptide in which the two fragments were bridged by using a flexible spacer, 6-aminohexanoic acid (Ahx) H-YKQCHKKGGAhxKKGSG-NH_2 (NrTP2). The peptides were assayed in HeLa cells showing their ability to translocate the cell membrane and accumulate in the nucleus, specifically in the nucleolus, thus, they were named Nucleolar Targeting Peptides (NrTP). Figure 3B shows the structure of the NrTP analogues discussed in this work.

A



Chemical Formula: $C_{64}H_{109}N_{23}O_{17}S$
Molecular Weight: 1504,78

B

	Sequence	Modification
NrTP1	H-YKQCHKKGGKKGSG-NH_2	NA
NrTP2	H-YKQCHKKGGAhxKKGSG-NH_2	Ahx between fragments
NrTP3	H-GSGKKGGKKHCQKY-NH_2	Inverted sequence order
NrTP4	H-YKQCIKKGGKKGSG-NH_2	His substituted by Ile
NrTP5	H-ykqchkkGGkkGsG-NH_2	All D-amino acids
NrTP6	H-YKQSHKKGGKKGSG-NH_2	Cys substituted by Ser
NrTP7	H-YRQSHRRGGRRGSG-NH_2	NrTP6 + Lys substituted by Arg
NrTP8	H-WKQSHKKGGKKGSG-NH_2	NrTP6 + Tyr substituted by Trp

Figure 3. **(A)** Chemical structure of the splicing peptide NrTP1. **(B)** NrTP analogues discussed in this work.

In the same study, the enantiomeric (enantio) version in which the L-amino acids were replaced by D (H-ykqchkkGGkkGsG-NH_2, NrTP5) and retro sequence consisting of the same sequence inverting C and N-termini (H-GSGKKGGKKHCQKY-NH_2, NrTP3) were synthesized and tested. In the case of the retro peptide, higher concentrations were needed to observe translocation and nucleolus localisation, while in the case of the enantio version (NrTP5), no translocation was observed. This result suggests that receptor-mediated endocytosis may be the mechanism of translocation. Further studies in that sense confirmed the uptake mechanism by an endocytic pathway dependent on the formation of clathrin-coated vesicles, probably, through a receptor.[17]

Later studies aimed to demonstrate the ability of NrTP peptides to translocate large biomolecules into cells with full preservation of activity, a necessary condition for delivering therapeutically relevant cargos into cells and tissues. To this end, the NrTP1 was first modified by substituting the Cys residue in position 4 with Ser (NrTP6), the isosteric analogue as pointed above. This replacement would allow the addition of one extra Cys to the C-terminal to facilitate subsequent cargo conjugation. The resulting analogue (YKQSHKKGGKKGSG, NrTP6) was initially tested and confirmed not to be detrimental to the CPP properties. Thus, in the work by Rodrigues *el al*.[18] β-Galactosidase (β-gal) was chosen as cargo. It is a homotetrameric protein of 465 kDa mass which is above of many important cargoes as for example antibodies that are around 150 kDa.[19] The Lys residues of the enzyme where derivatized as maleimide and make react with NrTP6-Cys. The resulting conjugate showed the

ability to internalize β-gal into HeLa cells in an active form. Thus, NrTP6 was envisioned as a strong candidate for transporting drugs and nanoparticles intracellularly.

The same authors also investigated the potential of several NrTP analogues to translocate human primary immune cells using peripheral blood mononucleated cells (PBMCs), which contain mainly lymphocytes and monocytes.[20] The study included the previously mentioned analogues NrTP1, NrTP2, NrTP5, and NrTP6, as well as to new ones that in addition of Cys4-Ser modification, in one, all Lys residues were replaced by Arg (NrTP7) and in the other Tyr1 was replaced by Trp (NrTP8). The six analogues were tested and in all the cases the compounds penetrated both lymphocytes and monocytes, although in different extents and kinetics, being localized in the cytoplasm of these cells. Comparing the results, the authors concluded that NrTP6 was the best candidate to be used as a delivery agent for pathologies associated with human primary immune cells because of its good penetrability and low toxicity. Moreover, NrTP6 even displayed better efficiency uptake and lower toxicity than Tat 48-60, which is the main CPP model. In general, it has been demonstrated that the NrTP family can penetrate all type of cells, at exception of erythrocytes, having different localizations. The second important feature lies in the fact that they did not show toxicity in a wide range of concentrations.[21]

As an example of the carrier ability of NrTP6, it is worth mentioning the work by Xie *et al.* [22], in which, using the same chemistry described above for the conjugation to β-gal, NrTP6-Cys peptide was attached to a modified and enzyme-responsive polymeric conjugate platform containing a peptide designed to inhibit the function of the c-Myc protein that lacks cell permeability. The full conjugate showed the better results of toxicity against tumoral cell over, i) the simple conjugation of NrTP6 to the c-Myc inhibitor, thus the polymer play also a role in the internalization and the polymeric construct; and ii) the polymeric construct without NrTP6, meaning that the polymer and NrTP6 had an additive-synergistic effect in the penetrability.

NrTP1 had also been used to generate more efficient CPP with preferential location in subnuclear organelles.[23] To this end, it was fused to a 12 amino acid peptide named sC18* in the C-terminus. The fused NrTP1-sC18* peptide showed a tendency to form an amphipathic α-helix, which is a factor for efficient membrane interaction, key to initiating the translocation. In fact, the NrTP1-sC18* uptake by cancer cells was proven to be more efficient than each peptide separately and also the accumulation in the nucleolus was higher than that of NrTP1 by itself. Finally, treating cancer cells with doxorubicin in the presence of the chimeric peptide, the viability was reduced to a significant extent.

The above-described research identified NrTP peptides as promising carriers able to deliver drugs into cells by specifically targeting the nucleolus. Building on this and on earlier findings that modifying the N-terminus of the well-known 47–60 TAT cell-penetrating peptide with different numbers of fatty acid (FA) chains promotes the formation of nanostructures capable of encapsulating therapeutic agents and enabling their controlled intracellular release,[24] Phungula et al. investigated the self-assembly properties of NrTP6 after lipidation with 1, 2, and 4 chains of twelve-carbon saturated fatty acid, lauric acid (Figure 4). The nanostructure-forming capabilities of each construct were investigated and characterized using various physicochemical techniques.[25] Not only did the number of lipid chains strongly influence the physicochemical properties of the lipopeptides, but also the conditions under which they assembled, including peptide concentration, pH, and medium composition (water vs. phosphate buffer). Together, these parameters determined the critical aggregation concentration (CAC), morphology, and secondary structure of the assemblies, resulting in distinct nanostructures.

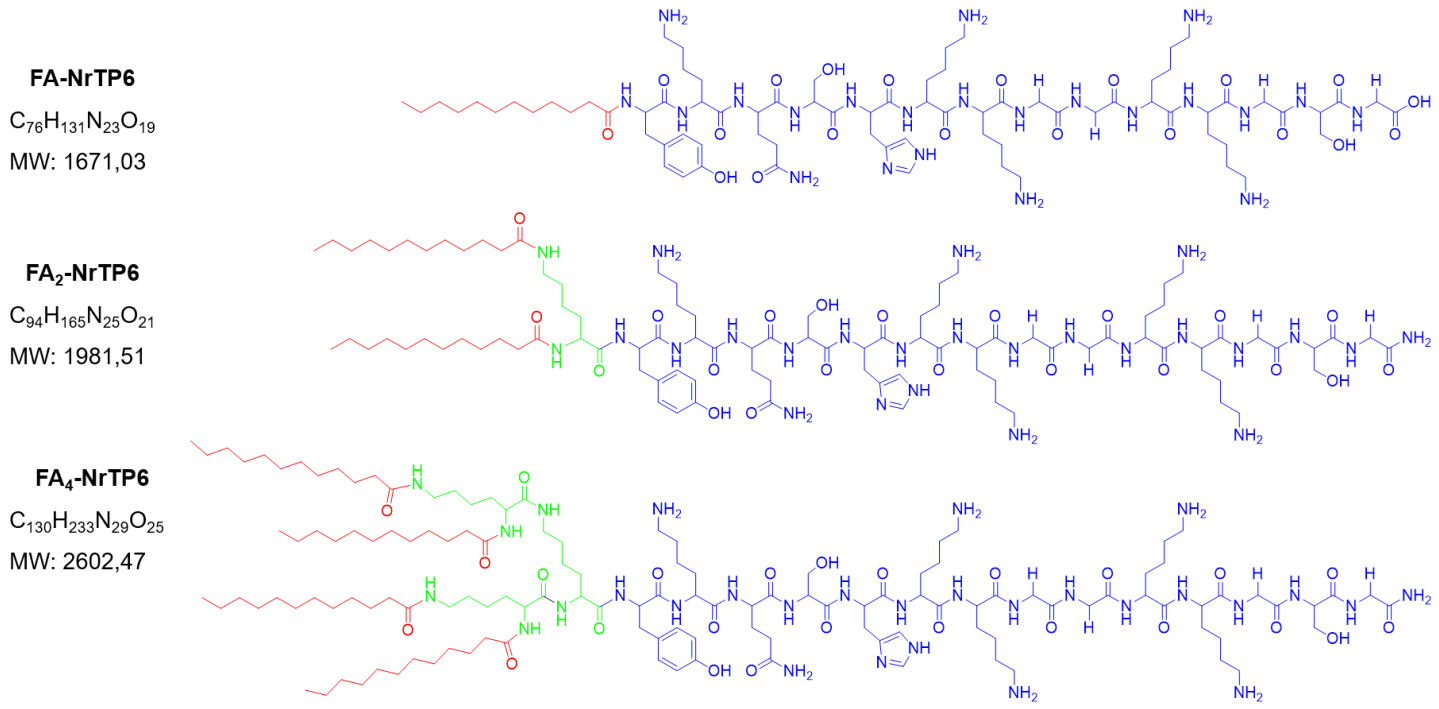


Figure 4. Lipidated analogues of NrTP6. Blue denotes the peptide; green the Lys residues used for branching; red the lipid tails.

NrTP6 modified with a single lipid chain formed mainly spherical structures in both water and phosphate buffer. In contrast, the construct bearing two lipid chains produced spherical assemblies in water but long fibers in phosphate buffer. The lipopeptide with four aliphatic chains showed the most complex behavior, forming spheres, rods, or fibers depending on the concentration and medium. Increasing the number of fatty acid chains also promoted β-sheet formation: this secondary structure appeared in phosphate buffer for the two-chain construct and dominated for the four-chain construct in both water and phosphate media.

Furthermore, the capacity of the three lipopeptides to encapsulate a model drug (doxorubicin, DX) and release it efficiently into the cell nucleus was assessed.[26] The two-chain construct showed the best performance, achieving successful and complete encapsulation of protonated DX. Moreover, TEM images revealed self-assembly, specifically in the form of multilamellar nanorods ~150 nm long and 20 nm in diameter. Cellular uptake studies in A549 cells showed rapid nuclear accumulation of free DX, while encapsulated DX exhibited slower but progressive nuclear delivery. In a colony formation assay, the authors demonstrated that encapsulated DX fully suppressed long-term proliferation at subtoxic doses, whereas free DX did so only partially. These results indicate that the formulation offered sustained drug release, reduced side effects, and improved long-term efficacy, making it a strong candidate for targeted nuclear drug delivery.

5. Conclusions

Snake venom–derived peptides have emerged as a rich source of bioactive molecules with unique structural and functional properties. Among them, crotamine stands out not only for its toxicological relevance but also for its remarkable ability to selectively penetrate eukaryotic cells and accumulate in the nucleus. Structural deconstruction of crotamine led to the identification of short nucleolar-targeting motifs, giving rise to the NrTP

family of peptides, which retain efficient cellular uptake while exhibiting low cytotoxicity and broad applicability across multiple cell types.

Extensive studies have established NrTP peptides, particularly NrTP6 (where cysteine was replaced by serine), as robust and versatile cell-penetrating peptides capable of delivering large and biologically active cargos, including enzymes, polymers, and therapeutic peptides, into both immortalized and primary human cells. Their favourable uptake profiles, minimal toxicity, and superior performance relative to classical CPPs such as Tat underscore their potential as next-generation intracellular delivery vectors. Importantly, mechanistic investigations have demonstrated that NrTP internalization occurs predominantly via receptor-mediated, clathrin-dependent endocytosis, providing valuable insight for rational optimization and targeted delivery strategies.

Building upon these properties, lipidation of NrTP6 has further expanded its functional scope by enabling controlled self-assembly into nanostructures with tuneable morphology and secondary structure. The number of lipid chains, together with environmental conditions, governs aggregation behaviour, resulting in diverse supramolecular architectures. These lipidated constructs effectively encapsulate small-molecule drugs such as doxorubicin and facilitate sustained, targeted nuclear delivery, leading to enhanced long-term therapeutic efficacy at reduced toxicity.

Furthermore, lipidation confers antibacterial activity, with FA1-NrTP6 (just one fatty acid unit) displaying broad-spectrum efficacy and synergistic potential when combined with conventional antibiotics. Notably, the antibacterial activity appears to be governed primarily by molecular composition rather than nanostructure, providing valuable insight for the rational design of next-generation antimicrobial agents. Furthermore, studies of FA1-NrTP6 in combination with standard antibiotics suggest that the peptide primarily compromises bacterial membrane integrity, facilitating antibiotic entry without inducing extensive membrane disruption or complete cell lysis.

Overall, the body of work reviewed here positions NrTP peptides, especially lipidated FA1-NrTP6, as highly promising multifunctional platforms that bridge peptide-based delivery, nanotechnology, and therapeutic intervention. Their capacity to combine nucleolar targeting, efficient intracellular transport, and tuneable self-assembly opens new avenues for the development of advanced drug delivery systems and highlights the continued potential of venom-derived peptides as inspiration for innovative biomedical applications.

6. Acknowledgements

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Amanda Phungula obtained her Master and PhD at the University of KwaZulu-Natal in the Peptide Science Laboratory under the supervision of Beatriz and Fernando. During this time, she spent almost one year at CIC biomaGUNE (Donostia, Basque Country, Spain) under the supervision of Dr. Sergio Moya. She is currently a post-doctoral fellow at the University of the Witwatersrand in the group of Professor Maya Makatini

Fernando Albericio is a Research Professor at the University of KwaZulu-Natal and an Emeritus Professor of Organic Chemistry at the University of Barcelona (Spain), with over 50 years of experience in peptide chemistry. Among other recognitions, he was awarded the Gold Medal of the South African Chemistry Institute (SACI) in 2022.

Beatriz G. de la Torre is a Research Professor at the University of KwaZulu-Natal. She has been working extensively on glyco, nucleo-, and lipopeptides. Her scientific interests are primarily focused on the discovery of new antimicrobial peptides, including those for combating tuberculosis, peptide-based vaccines, and peptide-based drug-delivery systems.