*Review*

# Evolution of Three-Finger Toxins – a Versatile Mini Protein Scaffold

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***Dedicated to the memory of Professor Franc Gubenšek***

## Abstract

Among snake venom toxins, three-finger toxins – a superfamily of nonenzymatic proteins – are found in the venoms of all families of snakes. They share a common structure of three β-stranded loops extending from a central core containing all four conserved disulfide bonds. Despite the similar structural fold, they exhibit a wide variety of biological effects. This review describes briefly the structure-function relationships and evolution of this group of toxins. The functional sites in these 'sibling' toxins are located on various segments of the molecular surface. This group of mini proteins appears to evolve through a combination of accelerated rate of exchange of segments as well as point mutations in exons.

**Keywords:** Calciseptine, cardiotoxin, cytotoxin, fasciculin, functional site, muscarinic toxin, post-synaptic neurotoxin, protein–protein interaction, dimeric three-finger toxin, snake venom, toxin evolution.

## 1. Introduction

Snake venoms are complex mixtures of pharmacologically active enzymes and nonenzymatic proteins and polypeptides.[1,2] These toxins belong to a small number of superfamilies; the proteins within each family share remarkable similarities in their primary, secondary and tertiary structures, but they may differ from each other in their pharmacological effects. In general, enzymes contribute to both the immobilization and digestion of the preys, while the nonenzymatic proteins mostly contribute to the immobilization. These nonenzymatic proteins exhibit lethal and debilitating effects as a consequence of neurotoxic, cardiotoxic and tissue necrotizing effects, whereas others induce various pharmacological effects, but are of a lower order of toxicity. So far, at least 16 families of the nonenzymatic families of venom proteins/peptides have been characterized. They are: (i) three-finger toxins (3FTxs); (ii) proteinase inhibitors; (iii) snaclecs; (iv) nerve growth factors; (v) disintegrins; (vi) sarafotoxins; (vii) natruretic peptides; (viii) bradykinin potentiating peptides; (ix) cobra venom factors; (x) myotoxins; (xi) CRISPs (cysteine-rich secretory proteins); (xii) AWIT peptides; (xiii) vascular epithelial growth factors; (xiv) waprins; (xv) vespryns; and (xvi) veficolins.

The studies of snake venoms and toxins have focused on one or more of the following objectives: (i) to determine the mode and mechanism of action of the toxins; (ii) to find ways and means to neutralize the toxicity and adverse effects of snake bites; (iii) to develop specific research tools that are useful in understanding normal physiological processes at both cellular and molecular levels; and (iv) to develop prototypes of pharmaceutical agents based on the structure of toxins.[3] The structure–function relationships and the mechanisms of action of sibling toxins are intriguing and pose exciting challenges. Series of gene duplications followed by accelerated evolution play crucial roles in the evolution of simple molecular templates to form a wide arsenal of toxins with diverse biologic functions.

This family of 3FTxs contains nonenzymatic polypeptides with 60–74 amino acid residues.[4–6] They exhibit a distinct protein fold – three β-stranded loops extending from a small, globular, hydrophobic core that contains four conserved disulfide bridges.[7,8] The three loops extending from the core region resemble three outstretched fingers

(Figure 1) and hence the name. Despite the common scaffold, 3FTxs exhibit exciting array of potent toxic effects. They include α-neurotoxins, which bind to muscle nicotinic acetylcholine receptors (nAChRs);[4,8–10] κ-bungarotoxins, which bind to neuronal nAChRs;[11] muscarinic toxins, which bind to distinct types of muscarinic receptors;[12] fasciculins that inhibit acetylcholinesterase;[13] calciseptine and FS2 that block the L-type calcium channels;[14,15] cardiotoxins (cytotoxins) that interact with phospholipids;[16] dendroaspins, which interact with $\alpha_{IIb}\beta_3$ and inhibit platelet aggregation;[17] hemextin, which inhibits factor VIIa;[18] β-cardiotoxins, which bind to β1- and β2- adrenergic receptors,[19] and AdTx1, MTα and others, which are antagonists of α-adrenoceptors.[20–23] Some cardiotoxins also bind to heparin, potassium channel-interacting proteins or $\alpha_v\beta_3$ integrin.[24–26] Therefore, understanding their structure–function relationships and identifying their functional sites is a subtle, complicated and challenging task. Using a combination of theoretical and experimental approaches, structure–function relationships of some of 3FTxs have been delineated. In this review, I will describe the structure-function relationships and evolution of 3FTxs – the most versatile group of nonenzymatic sibling toxins.

## 1. 1. Structural Variations in 3FTxs

All 3FTxs have conserved residues which contribute to the characteristic folding. They include eight conserved cysteine residues found in the core region, conserved aromatic residue (Tyr25 or Phe27),[4,27,28] and charged amino acid residues (e.g., Arg39 in erabutoxin-a and Asp60 in α-cobratoxin) that stabilize the native conformation by forming a salt link with the C or N-terminus of the toxin.[5] In general, they are monomers and have minor differences in their loop length and conformation particularly turns and twists. There are some structural variations that may have significant impact on their functions. For example, some 3FTxs have an additional fifth disulfide bond either in loop I or loop II. The fifth disulfide in loop I in non-conventional toxins[29] twists and pushes the tip of loop in an orthogonal position, while the fifth disulfide bond in loop II in long-chain neurotoxins and κ-neurotoxins[11,30] introduces a turn and formation of a short helical segment at the tip of the loop (Figure 1). Some 3FTxs have long N-terminal and C-terminal extensions. All colubrid 3FTxs have an extended N-terminal segment with additional seven residues which is capped by a pyroglutamic acid.[31]

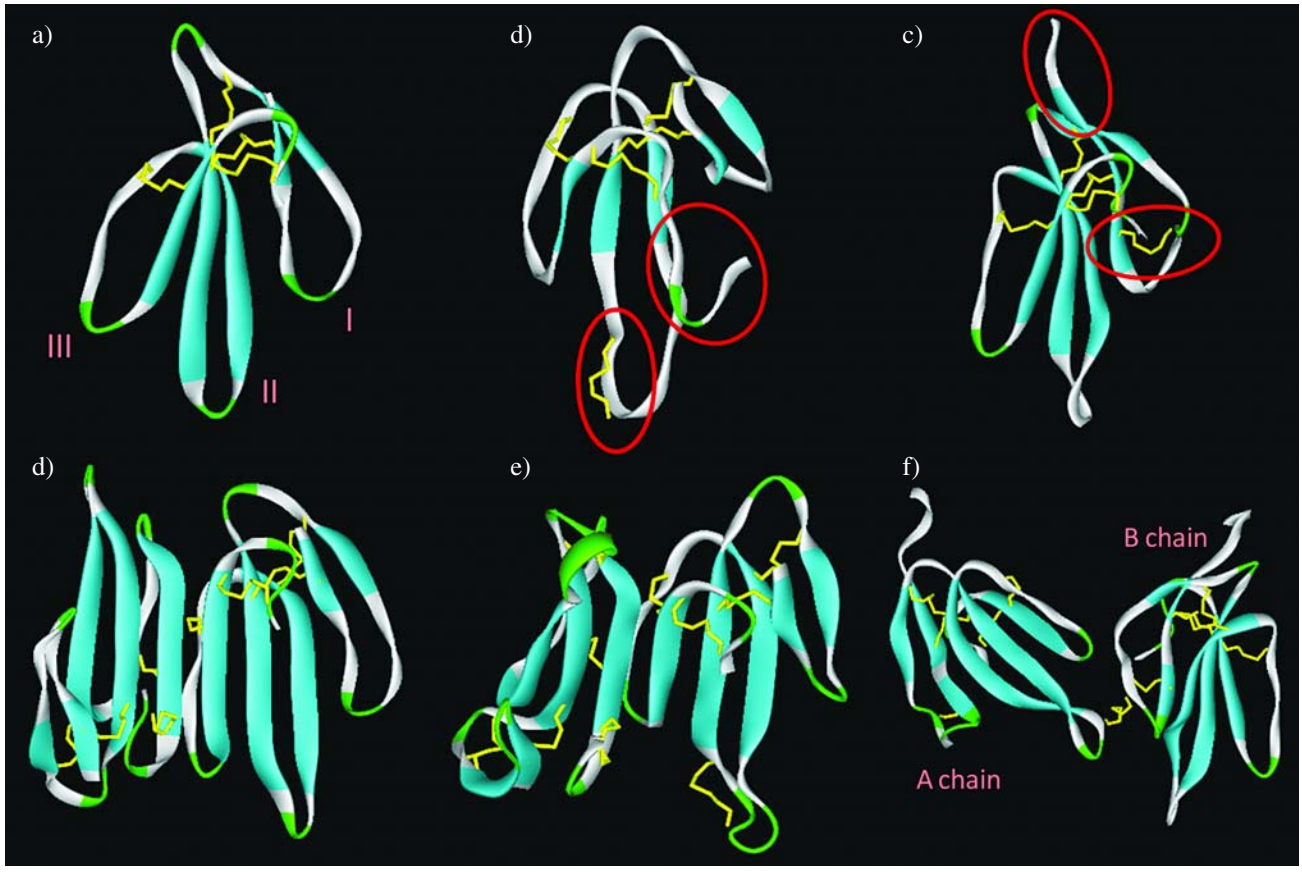


**Figure 1: Structural variations in three-finger toxins**. Monomeric toxins are shown in the top row. (a) Short-chain 3FTx (Erabutoxin a, 1QKD), (b) Long-chain 3FTx (α-bungarotoxin, 2ABX), (c) Non-conventional 3FTx (Denmotoxin, 2H5F). The differences between erabutoxin a and other toxins are shown in red circles. Dimeric toxins are shown in the bottom row. (d) Haditoxin (3HH7), (e) κ-Bungarotoxin (2ABX), and (f) Irditoxin (2H7Z). Haditoxin and κ-bungarotoxin are non-covalent homodimers of short- and long-chain 3FTx, respectively, whereas irditoxin is a covalent heterodimer.

The long-chain neurotoxins have additional 2–9 residues at the C-terminal end (Figure 1c). These segments flow down from the top of the molecule (Figure 1b).

Although most 3FTxs are monomers, a few of them exist as noncovalent homodimers in nature. The examples include κ-bungarotoxins and haditoxin.[32,33] The subunits are in antiparallel arrangement and the dimeric interface is formed between the outer strands of the third loops. In κ-toxins, the subunits are similar to long-chain α-neurotoxins whereas in haditoxin they are similar to short-chain α-neurotoxins. In contrast, irditoxin is a covalently-linked heterodimer.[34] Each subunit has an additional cysteine which is involved in the inter chain disulphide bridge. Covalently linked homodimer of α-cobrotoxin (αCT-αCT) and heterodimers of α-cobrotoxin and cytotoxins were reported from the venom of *Naja kaouthia*.[35] Additional structural variations in 3FTxs are reviewed recently.[6]

## 1. 2. Functional Sites in 3FTxs

### 1. 2. 1. Neurotoxins

A large number of members of 3FTxs are neurotoxins. They interfere with cholinergic transmission at postsynaptic sites in the peripheral and central nervous systems. Based on their receptor selectivity, they can be broadly classified as α-neurotoxins (curaremimetic), κ-neurotoxins and muscarinic toxins that target muscle nAChR, neuronal nAChR and various subtypes of muscarinic receptors, respectively. These toxins have contributed significantly to identification and characterization of respective subtypes of AChRs.[36]

*α-Neurotoxins* – These toxins bind to muscle (α1) nAChRs impairing neuromuscular transmission at the peripheral nervous system.[9,10] Thus, they imitate the effects of the alkaloid curare – the arrow poison (hence the name). They are further classified as short-chain neurotoxins (60–62 amino acid residues and four disulfides) and long-chain neurotoxins (66–74 amino acid residues and five disulfides).[5] The presence of a fifth disulfide bridge at the tip of the second loop and a longer carboxy terminal tail differentiate long-chain neurotoxins from short-chain neurotoxins.[5,7,8] However, both groups of neurotoxins bind to muscle nAChR with equal affinity and compete with each other for binding.[5] Interestingly, only long-chain neurotoxins bind to neuronal α7 nAChR with high affinity.[32] Thus, the long- and short-chain neurotoxins have differences in their targeting and this subtle difference has been correlated to the presence of the fifth disulphide in the second loop.[37]

The critical residues that are important for binding to the muscle (or *Torpedo*) nAChR were first identified based on the chemical modification of specific amino acid residues.[5,38] Subsequently, Menez and co-workers used systematic site-directed mutagenesis to delineate the functional sites of erabutoxin a and α-cobratoxin, classical examples of short- and long-chain neurotoxins respectively (Figure 2a).[27,39–41] These neurotoxins use a number of structurally equivalent residues, including Lys23/ Lys27, Asp27/Asp31, Arg33/Arg33 and Lys49/ Lys49, as well as Trp25/Trp29 and Phe29/Phe32, in binding to *Torpedo* receptor, respectively. In addition, the tip of the first loop in erabutoxin a and in contrast, the carboxy terminal tail in α-cobratoxin also play important roles in binding. Interestingly, α-cobratoxin binds to both *Torpedo* (α1) and neuronal α7 nAChRs using some common residues (Trp25, Asp27 and Arg33).[27] In addition, it also uses receptor-specific residues: Ala28, Lys35 and Cys26-Cys30 for recognition of the α7 receptor and Lys23 and Lys49 for the *Torpedo* receptor. Moreover, the cyclic structure formed by the fifth disulphide bridge at the tip of the second loop of α-cobratoxin has been reported to be essential for its binding to the α7 receptor (Antil-Delbeke et al., 2000).[41] Crystal structure of α-cobratoxin with acetylcholine-binding protein (AChBP) from *Lymnaea stagnalis* further reveals the details of interaction.[42] Therefore, neurotoxins appear to use a common core of critical residues for binding and additional residues to determine the specificity of their molecular target.

*κ-Neurotoxins* – These toxins bind specifically to neuronal (α3β2) nAChR.[11] Structurally they are similar to long-chain α-neurotoxins, with the fifth disulphide bridge located in the second loop. However, unlike long-chain neurotoxins, κ-neurotoxins exist as non-covalent homodimers.[11] Functionally κ-neurotoxins recognize α3β2 and α4β2 subtype but not to α1.[43] They also interact with α7 nAChRs.[35,37]

Interestingly, haditoxin from *Ophiophagus hannah* venom antagonizes muscle (αβγδ) and neuronal α7, α3β2 and α4β2) nAChRs with highest affinity towards α7 nAChRs. It is a homodimeric short-chain α-neurotoxin.[33]

*Muscarinic toxins* – Muscarinic toxins bind to muscarinic AChRs (mAChRs) and act as agonists or antagonists.[12,44–46] For example, MT1 and MT2 have facilitatory effect in the memory test suggesting agonist role of these toxins in rat model.[47] In contrast, MT3 from green mamba inhibits the binding of $^{[3H]}$NMS, a classical muscarinic radioligand, to native and cloned muscarinic receptors exhibiting the role of antagonist.[48] MT1 acts as an agonist at $M_1$ and an antagonist at $M_4$ receptors, with similar affinities for both receptors.[49] They bind specifically and with high selectivity to various subsets of mAChR (M1-M5).[50,51] The large size of the muscarinic toxins probably enables them to interact with the highly variable extracellular loop regions of the GPCRs.[51] This has made them invaluable research and diagnostic tools for biomedical applications.[12,50–53] Functionally these toxins differ in their pharmacological properties with short-chain neurotoxins however structurally they are related (Figure 1a). For details, refer to a recent review.[53]

### 1. 2. 2. Acetylcholinesterase Inhibitors (Fasciculins)

This class of 3FTxs interferes with neuromuscular transmission by inhibiting the enzyme acetylcholinesterase (AChE) present at the neuromuscular junction. Thus, due to accumulation of acetylcholine at the synapse, they induce fasciculations in muscle and are aptly named as fasciculins.[13,54] Fasciculins have been isolated only from mamba (*Dendroaspis*) snake venoms. They are structurally similar to short-chain neurotoxins (Figure 1a) and bind to the peripheral site of AChE and block the entry of acetylcholine into the active site of the enzyme, thereby preventing its breakdown.[55]

The functional site of fasciculin was identified almost simultaneously by two different approaches, namely the generation of synthetic peptides and site-directed mutagenesis.[56,57] Molecular models of the fasciculin-AChE complex and chemical modification studies suggested a role for the second loop. A cyclic peptide based on the second loop inhibited AChE at 15–20 μmol/L compared with 300 pmol/L native toxin.[54,56] Marchot *et al.* using 14 mutants encompassing 16 amino acid residues located in all three loops of fasciculin, identified Thr8, Thr9, Gln11, Arg24, Arg27, His29, Pro30, Pro31 and Met33, which are located in the first and second loops, as being functionally important for interaction with AChE (Figure 2b).[57] Interestingly, this interaction site is located on the opposite surface of the molecule as compared to the nAChR recognition site in α-neurotoxins.[39,40,58] A chimera in which the entire first loop and the tip of the second loop of toxin-α (a short-chain neurotoxin) was replaced by that of fasciculin 2 significantly inhibited AChE.[59] Recently, Sharabi et al. designed mutants by modifying 5 out of 13 interfacial residues on fasciculin to optimize its interaction with AChE and some of the mutants showed enhanced affinity to AChE.[60]

### 1. 2. 3. Cardiotoxins

This is the second-largest group of 3FTxs and found only in cobra venoms. Structurally, cardiotoxins (CTxs) resemble short-chain neurotoxins (59–62 amino acid residues and 4 disulphide bonds) (Figure 1a).[4,16] At lower concentrations, they increase heart rate and, at higher concentrations, kill the animal by cardiac arrest.[61] However,

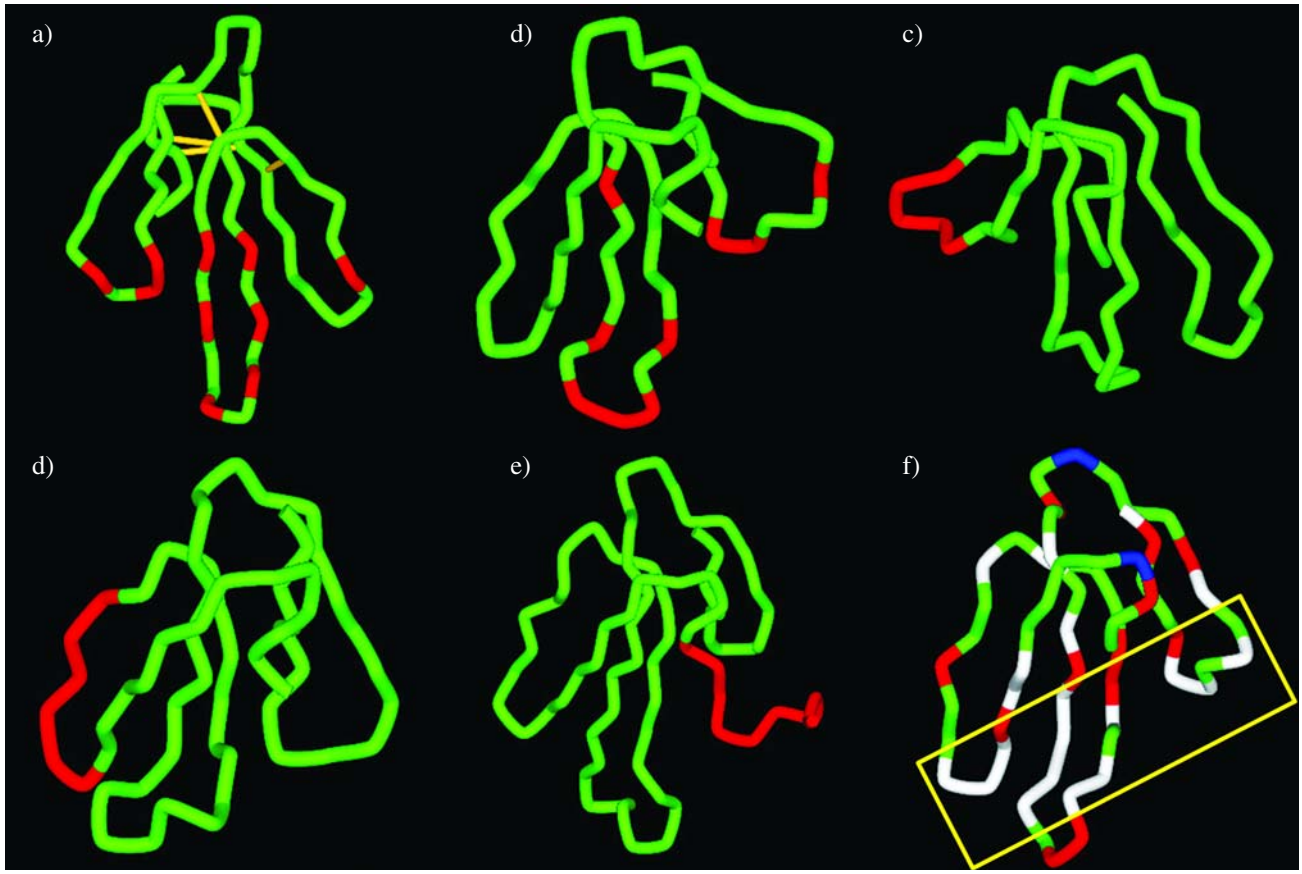


**Figure 2: Functional sites in three-finger toxins**. The residues involved in the binding of erabutoxin a (1QKD) to muscle nAChR (a), fasciculin (1DRS) to AChE (b), mambin (or dendroaspin, 1DRS) to platelet receptor $\alpha_{IIb}\beta_3$ (c), and FS2 to L-type calcium channel (d) are shown in red. Only in erabutoxin a conserved disulfide bridges are shown. Analgesic site of hannalgesin (e) is also shown in red. Cytolytic site of CTx has a hydrophobic region (white residues highlighted in yellow box) and cationic residues (shown in red). Acidic residues in CTx are shown in blue. All structures are in the same orientation as shown in Figure 1a.

the protein target of CTxs in cardiac myocytes has not yet been identified. A large number of this group of toxins also exhibits general cytolytic effects (i.e. form ion pores in the lipid membranes) and, therefore, they are also referred to as cytolysins or cytotoxins.[61,62]

The cytolytic site of CTxs was identified by a combination of theoretical and chemical modification methods.[63–66] We showed that all cytolytic proteins, independent of their target cells, contained hydrophobic and cationic sites flanking each other either in the primary, secondary or tertiary structure (Figure 2f).[63] The cytolytic region in CTxs is spread on all three loops: there is a significant hydrophobic patch extending from the middle to the bottom end of all loops and a row of positively charged lysine residues is located at the top of the hydrophobic patch. The modification of the positive charges on lysine residues to negative and neutral charges using succinylation and carbamylation, respectively, led to the loss of cytolytic activity.[64] But the native and guanidinated derivative showed cytolytic activity. All derivatives, however, retained similar protein folding and their ability to bind to phospholipids.[64] These experiments clearly showed the importance of cationic residues for cytolytic activity. Our results were corroborated by the chemical modifications of methionine, lysine and aromatic residues.[65–68] Interaction of cardiotoxins with phospholipids and penetration of loops into the membranes has also been characterized.[68–72]

### 1. 2. 4. β-Cardiotoxin and Related Toxins

β-Cardiotoxin is a new class of 3FTx isolated from the venom of *Ophiophagus hannah* which show β-blocking activity.[19] Structurally it shares about 55% identity but functionally it is different from the classical CTxs. It does not show lethality to mouse up to 10 mg/kg body weight whereas classical CTxs are potent toxins with $LD_{50}$ values ranging between 1.48 to 2.8 mg/kg.[73] β-Cardiotoxin decreases the heart rate in dose-dependent manner in anesthetized rats, whereas classical CTXs increase the heart rate. In Langendorff preparation of isolated rat heart, β-cardiotoxin decreases the heart rate. β-Cardiotoxin binds to β1– and β2–adrenergic receptors.[19] A number of toxins (CTX-9, CTX14, CTX15, CTX21 and CTX23) from *O. hannah* venom that were misidentified as classical CTxs or α-neurotoxins.[74] These toxins have only 1–4 residue substitutions (some are even conserved substitutions) compared to β-cardiotoxin. We believe that all these members are β-blockers and they might bind to β–adrenergic receptors. The functional site of this unique group of 3FTxs is not known.

### 1. 2. 5. L-Type Calcium Channel Blockers

There is a group of 3FTxs, which include calciseptine and FS2, that specifically block L-type calcium channels.[14,15] These polypeptides are structurally similar to short-chain neurotoxins. They bind to the 1,4-dihydropyridine binding site of the L-type calcium channels and physically block the calcium currents.[75] By a systematic survey of over 1600 protein–protein interaction sites, we showed that proline residues are most commonly found in the flanking segments of the interaction sites.[76,77] Based on this observation, we developed a simple method for the identification of protein–protein interaction sites directly from the amino acid sequence of a protein.[78] Using this approach, we predicted that the segment between Pro42 and Pro47 is the potential interaction site of calciseptine and FS2.[79] The predicted functional site of L-type calcium channel blockers is located on the outer strand of the third loop of the three-finger scaffold (Figure 2d). A synthetic 8-mer peptide designed based on this putative interaction site, namely L-calchin, showed negative inotropic effects in rat atrium. L-Calchin also showed dose-dependent and voltage-independent inhibition of L-type calcium channels in rabbit cardiac myocytes. It did not affect the opening and closing kinetics indicating that it only blocks the L-type calcium channel in a similar manner as the parent toxin.[69] This segment, particularly Met45-Trp46-*cis*Pro47-Tyr48, displayed similar hydrophobic and hydrogen bond-forming properties as nifedipine, a 1,4-dihydropyridine derivative.[80] Based on the presence of identical functional sites, we hypothesize that two other toxins, namely $C_{10}S_2C_2$ from *Dendroaspis angusticeps* and $S_4C_8$ from *D. jamesoni kaimose* venoms, might also block the L-type calcium channels.

### 1. 2. 6. Platelet Aggregation Inhibitors

There are two 3FTxs which inhibit platelet aggregation. Dendroaspin (or mambin) isolated from *D. jamesoni* venom is a potent inhibitor of platelet aggregation.[17] It contains an Arg-Gly-Asp tripeptide sequence, which is involved in the adhesive function of several proteins (Figure 2c). As expected, dendroaspin interferes with the interaction between fibrinogen and its receptor glycoprotein IIB-IIIa ($\alpha_{IIb}\beta_3$) complex and, hence, platelet aggregation. Lu et al. evaluated the role of the two flanking prolines by substituting both with alanine, with a resulting five- to eight- fold loss in the ability to inhibit platelet aggregation.[81] The replacement of the Arg-Gly-Asp sequence by Arg-Tyr-Asp and Arg-Cys-Asp tripeptide sequences promote selective inhibition of $\beta_1$ and $\beta_3$ integrins, respectively.[82] Substitution of Arg with other residues such as Lys, His, Gln and Ala residues, also alters its integrin specificity.[81] Thus, the functional site of dendroaspin is located at the tip of its third loop (Figure 2c). The second 3FTx with RGD sequence was isolated from *Bungarus multicinctus* venom.[83] But this RGD sequence is found in the second loop and not accessible for interaction and hence has poor ability to inhibit platelet aggregation ($IC_{50}$ of 34 mM).

### 1. 2. 7. Orphan Toxins

There are 3FTxs isolated from snake venom whose function is not yet been determined and they are referred to as "orphan toxins". They are grouped into 20 different clades (I-XX).[84] These observations indicate the presence of 3FTxs with distinct pharmacological potencies. It will be interesting to elucidate the function of these and other newer orphan toxins. Gene duplication and accelerated evolution of 3FTx gene accounts for such large number of diverse toxins.

## 1. 3. Origin and Evolution of 3FTxs

Several other non-venom proteins and polypeptides including Ly-6 alloantigens, trout toxin-1, SLURP, lynx1, urokinase-type plasminogen activator receptor and the complement regulatory protein CD59 use similar three-finger scaffold.[85–90] Thus venom 3FTxs have most likely evolved from some of these non-toxic ancestral proteins. Studies on gene structure and evolution of 3FTxs show that they have evolved through gene duplication and accelerated evolution similar to other toxin superfamilies.[91–95] Accelerated non-synonymous substitutions of nucleotides occur in the protein coding region of 3FTxs genes. Thus, similar to other toxins, adaptive evolution and positive Darwinism plays important roles in functional and structural diversification.[94,96–98] These accelerated point mutations, however, affect small areas on the surface and may not be sufficient to explain distinct pharmacological activities exhibited by 3FTxs. Therefore, some alternative mechanisms probably play important role in their evolution (see below).

### 1. 3. 1. Insertion of Fifth Disulfide in Second Loop

Unlike most 3FTxs, long-chain α-neurotoxins and κ-neurotoxins have a fifth disulphide in the second loop (Figure 1). Comparison of the nucleotide sequence of the short-chain and long-chain neurotoxin genes reveals that the boundary of third exon has shifted 12 bases upstream due to the creation of a new splicing site by the insertion of "A" in the intron of the long-chain neurotoxin.[99,100] This change in intron-exon boundary results in the addition of a few more residues including a Cys which forms the fifth disulfide bridge. This fifth disulfide bridge introduces a turn and formation of a short helical segment at the tip of the loop, and accounts for their ability to bind to α7 nAChRs.[37] Thus structural and functional diversification occurs in 3FTxs due to change in the intron-exon boundary.

### 1. 3. 2. Extension at N-terminal End

Colubrid 3FTxs have an extended N-terminal segment compared to elapid and viperid 3FTxs.[31,34,101] Colubrid toxin (denmotoxin) gene has four exons, unlike the genes of elapid and viperid 3FTxs, which have three exons and the longer N-terminal segment is coded by a newly inserted exon 2.[100,102,103] Although the functional implications of this extension is not clear, 3FTxs also appear to evolve through insertion of new exons.[102]

### 1. 3. 3. ASSET in the Evolution of 3FTxs

Recently we showed that in *Sistrurus* 3FTx genes undergo rapid changes through accelerated segment switching in exons.[103] Such an accelerated segment switch in exons can lead to change in the surface properties and hence functional diversification. We named this phenomenon as Accelerated segment switch in exons to alter targeting (ASSET) and analyzed in 3FTxs of elapid snake venoms.[103] ASSET seems to occur more often in 3FTx family compared to other toxin families.[104] Thus this family of proteins appears to have evolved through several mechanisms to form one of the most functionally diverse groups of snake venom toxins with a conserved structural scaffold.

# 2. Conclusions

The compact structure of 3FTxs has been exploited by nature for developing ligands that perform a wide variety of functions. In snakes, the ancestral gene(s) encoding for 3FTx(s) have duplicated several times and a wide array of toxins have evolved through accelerated evolution. Protein folding and structural integrity of these 'sibling' toxins is conserved during evolution. The studies revealing structure-function relationships and delineation of functional sites clearly indicate that there is no single designated location for the functional sites. As with other superfamilies of toxins, the robust and highly versatile three-finger protein scaffold has generated a group of toxins with wide variations in function involving subtle changes in the functional sites in snake venoms. Thus, 3FTxs provide us with ample challenging opportunities to decipher the subtleties in their functional sites and to understand the plasticity of protein structure and function in this mini protein scaffold.

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## Povzetek

Med toksini iz strupov kač so triprstni toksini – naddružina beljakovin brez encimske aktivnosti – tisti, ki jih srečamo v strupih prav vseh družin kač. Tem beljakovinam je skupna struktura treh zank, ki v obliki β-trakov izhajajo iz osrednjega dela molekule, ki ga povezujejo štiri ohranjene disulfidne vezi. Kljub podobnemu strukturnemu zvitju, lahko te molekule izzovejo zelo raznolike biološke učinke. Pregledni članek na kratko opisuje odnos med strukturo in funkcijo pri tej skupini toksinov. Funkcijska mesta v teh žsestrskih'toksinih se nahajajo na različnih predelih molekulske površine. Ugotovljeno je bilo, da se je ta skupina mini proteinov razvila s kombinacijo povečane hitrosti izmenjave celih predelov molekule in točkovnih mutacij na področju eksonov.