Clinical and Experimental Pharmacology and Physiology (2002) **29**, 815–822

Invited Paper: Animal Toxins of Asia and Australia

MOLECULAR MOULDS WITH MULTIPLE MISSIONS: FUNCTIONAL SITES IN THREE-FINGER TOXINS

R Manjunatha Kini

Department of Biological Sciences, Faculty of Science, National University of Singapore, Singapore and Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia, USA

SUMMARY

1. Snake venoms are complex mixtures of pharmacologically active peptides and proteins.

2. These protein toxins belong to a small number of superfamilies of proteins. The present review describes structure–function relationships of three-finger toxins.

3. All toxins share a common structure of three β-stranded loops extending from a central core. However, they bind to different receptors/acceptors and exhibit a wide variety of biological effects.

4. Thus, the structure–function relationships of this group of toxins are complicated and challenging.

5. Studies have shown that the functional sites in these 'sibling' toxins are located on various segments of the molecular surface.

Key words: calciseptine, cardiotoxin, cytotoxin, fasciculin, functional site, muscarinic toxin, post-synaptic neurotoxin, protein–protein interaction, snake venom, toxin evolution.

INTRODUCTION

Snake venoms are complex mixtures of pharmacologically active proteins and polypeptides. Some of these 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. All these protein toxins attack various physiological processes at specific sites. The study of snake venoms and toxins by scientists with diverse backgrounds and expertise has 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. Important lessons can be learnt, particularly from the latter two objectives, as to how simple molecular templates have been used in nature to design a wide arsenal of proteins that exhibit diverse (toxic) functions.

A large number of protein toxins has been purified and characterized from snake venoms.[1–5] Many early efforts were directed towards the isolation and characterization of either proteins that are found in abundance or the most toxic components of the venom. The advent of more sophisticated purification techniques has resulted in the study of more interesting proteins found in smaller quantities. It is now known that snake venoms contain over 100 protein toxins. However, these toxins belong to a very small number of superfamilies of proteins. Some of the well-recognized families of venom proteins are: (i) three-finger toxin family; (ii) proteinase inhibitor family; (iii) lectin family; (iv) phospholipase A_2 (PLA_2) family; (v) serine proteinase family; and (vi) metalloproteinase family. The members in a single family show remarkable similarities in their primary, secondary and tertiary structures. At times, however, they differ from each other in their biological targeting and, hence, their pharmacological effects. That is, each family of protein toxins has a similar molecular scaffold but exhibits multiple functions. Thus, structure–function relationships and the mechanisms of action of snake venom proteins are intriguing and pose exciting challenges to scientists. So far, we have understood structure–function relationships of only a small number of toxins in some of these families. The theme of 'molecular moulds with multiple missions' is well illustrated by the structure–function relationships of the family of three-finger toxins, which is concisely covered in the present review.

THREE-FINGER TOXIN FAMILY

This is a family of non-enzymatic polypeptides containing 60–74 amino acid residues.[2,6] This family of proteins is found only in the venoms of elapids (cobras, kraits and mambas) and hydrophid (sea snakes) and not those of vipers and crotalids (rattlesnakes). Similar to other snake venom proteins, three-finger toxins are also rich in disulphide bonds. They contain four or five disulphide bridges, of which four are conserved in all members.[2] Consequently, all proteins of this family show a similar pattern of protein folding: three β-stranded loops extending from a central core containing the

Correspondence: R Manjunatha Kini, Department of Biological Sciences, Faculty of Science, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260. Email: dbskinim@nus.edu.sg

This is an invited paper, subjected to peer review.

Received 17 April 2002; accepted 19 April 2002.

four conserved disulphide bridges.[7, 8] Because of this appearance, this family of proteins is called the three-finger toxin family.

DIVERSITY IN BIOLOGICAL PROPERTIES

Despite the overall similarity in structure, these polypeptides differ from each other in their biological activities. Members of this family include α-neurotoxins, which antagonize muscle nicotinic acetylcholine receptors (nAChR),[8–9] κ-bungarotoxins, which recognize neuronal nicotinic receptors,[10] muscarinic toxins with selectivity towards distinct types of muscarinic receptors,[11] fasciculins that inhibit acetylcholinesterase,[2] calciseptins that block the L-type calcium channels,[12,13] cardiotoxins (cytotoxins) that exert their toxicity by forming pores in cell membranes[17] and dendroaspins, which are antagonists of various cell-adhesion processes.[15] Interestingly, the three-fingered fold is not restricted to snake venom toxins because several other non-venom proteins and polypeptides also belong to this superfamily of proteins.[16–20]

Neurotoxins

A large number of members of this family of toxins are neurotoxins. These neurotoxins interfere with cholinergic transmission at various post-synaptic sites in the peripheral and central nervous systems.[9] Based on their receptor selectivity, they can be broadly classified as curaremimetic or α-neurotoxins, κ-toxins and muscarinic toxins that target muscle nAChR, neuronal nAChR and various subtypes of muscarinic receptors, respectively. Over the years, curaremimetic toxins have contributed significantly to isolation and characterization of muscle (α1) AChR, making it one of the best characterized receptors today.[21] Similarly, muscarinic and κ-toxins have also helped us understand molecular details of various muscarinic and neuronal AChR subtypes and their role in neurotransmission.

Curaremimetic toxins

Curaremimetic toxins (or α-neurotoxins) bind to muscle (α1) nAChR and inhibit acetylcholine from binding to the receptor,

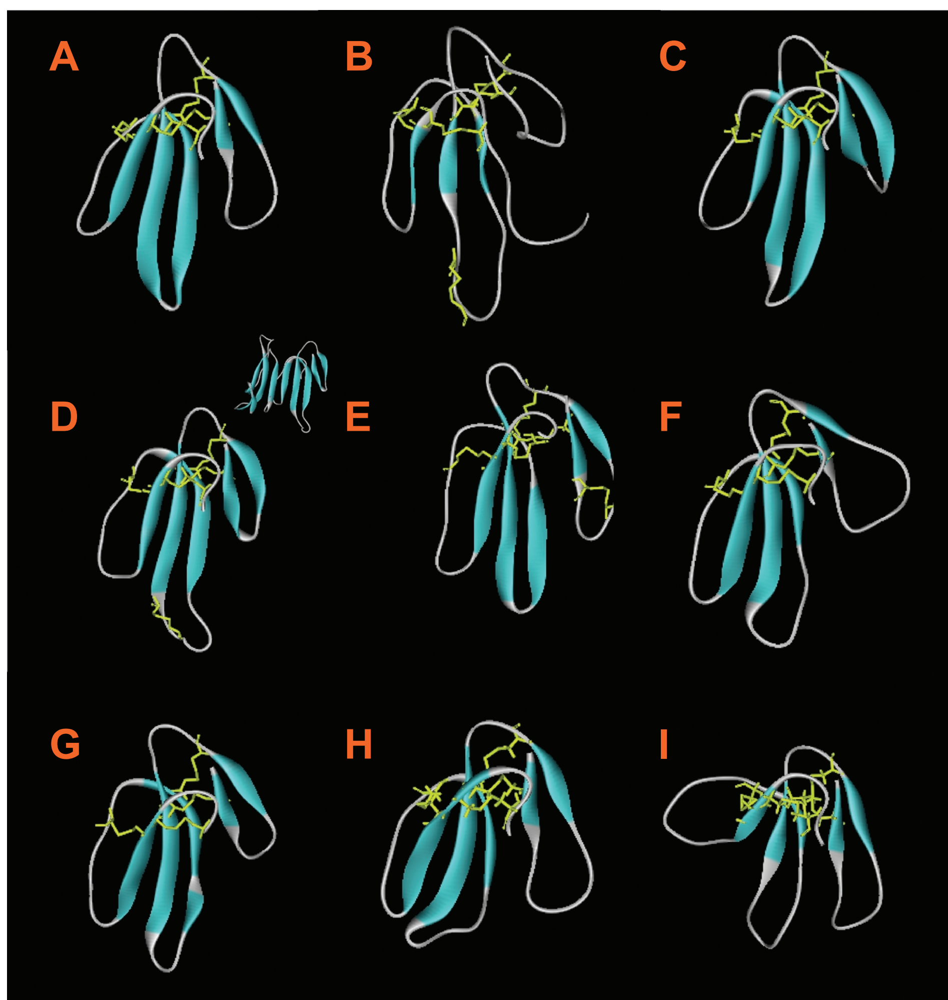

Fig. 1 Three-dimensional structural similarity among three-finger toxins from snake venoms. (a) Erabutoxin a (1QKD); (b) α-bungarotoxin (2ABX); (c) cardiotoxin V4 (1CDT); (d) κ-bungarotoxin (1KBA), inset, dimer; (e) candoxin (1JGK); (f) fasciculin 2 (1FAS); (g) muscarinic toxin MT-2 (1FF4; R Menez *et al.*, pers. comm., 2002); (h) FS2 toxin (1TFS); (i) dendroaspin (1DRS). Note all these 'sibling' toxins share a similar structural fold; the core at the top of the molecules contains all four conserved disulphide bridges and three β-sheeted 'fingers' start from the core. These β-sheeted loops are numbered right to left as loop I, II and III, respectively. Some toxins, such as α-bungarotoxin (b) and κ-bungarotoxin (d) have the fifth disulphide bridge in loop II. In contrast, candoxin (e) has the fifth disulphide bridge in loop I. However, these toxins differ from each other in their biological activities.

thereby impairing neuromuscular transmission.[1,9] In this respect, they imitate the effects of the alkaloid curare (hence the name). They are further classified as short-chain neurotoxins (60–62 amino acid residues and four disulphides) and long-chain neurotoxins (66–74 amino acid residues and five disulphides). Despite their difference in size, they share similarity in structural folding (Fig. 1a,b). However, the presence of a fifth unconserved disulphide bridge at the tip of the second loop, as well as a longer carboxy terminal tail, constitute two significant differences seen in long-chain neurotoxins.[2,7,8] These differences notwithstanding, both long- and short-chain neurotoxins bind to the same site on the muscle nAChR with equal affinity, competing with each other for binding.[2] In addition, long-chain but not short-chain neurotoxins bind to neuronal α7 nAChR with high affinity.[22–24] Thus, the long- and short-chain neurotoxins appear to have differences in their targeting and this subtle difference has been correlated to the presence of the fifth disulphide in the second loop.[22]

Muscarinic toxins

Muscarinic toxins bind specifically and with high selectivity to various subsets of muscarinic AChR.[25] These toxins are structurally related to short-chain neurotoxins (Fig. 1c). However, unlike α-neurotoxins, some muscarinic toxins act as agonists, whereas others act as antagonists on muscarinic AChR.

κ-Toxins

κ-Toxins bind specifically to neuronal (α3β4) nAChR.[10] Like long-chain α-neurotoxins, κ-toxins have five disulphide bridges, with the fifth disulphide bridge located in the second loop (Fig. 1d). However, unlike any other member of the three-finger toxin family, κ-toxins exist as dimmers.[10] Short- and long-chain neurotoxins have poor affinity for neuronal (α3β4) nAChR. Similarly, κ-toxins do not bind to α1 nAChR.

Other toxins

Other toxins with a unique cysteine scaffold different from those of short- and long-chain neurotoxins have recently been characterized. These toxins, in addition to the four conserved disulphide bridges, have a fifth disulphide bond in the first loop.[8,26–28] Based on their toxicity, they were classified as weak neurotoxins.[29,30] One of the members, WTX from cobra (*Naja kaouthia*) venom, has been shown to bind to both muscle (α1) and neuronal (α7) AChR, albeit at micromolar concentrations.[30] In contrast, candoxin from the venom of the Malayan krait (*Bungarus candidus*; Fig. 1e) binds to muscle (α1) and neuronal (α7) AChR with nanomolar affinity. Interestingly, its binding to muscle (α1) nAChR is easily reversible, in contrast with its binding to neuronal α7 nAChR, which is only partially reversible.[31]

Acetylcholinesterase inhibitors

This class of three-finger toxins interferes with neuromuscular transmission by inhibiting the enzyme acetylcholinesterase (AChE) present at the neuromuscular junction. Thus, these toxins induce fasciculation in muscle due to accumulation of acetylcholine at the synapse and are aptly named as fasciculins.[2] Fasciculins have been isolated from mamba (*Dendroaspis*) snake venoms. They are structurally similar to short-chain neurotoxins (Fig. 1f). They bind to the peripheral site of AChE and block the entry of acetylcholine into the active site of the enzyme, thereby preventing its breakdown.[32]

Cardiotoxins

This group of polypeptides is found only in cobra venoms and is the second-largest group of three-finger toxins. Structurally, cardiotoxins resemble short-chain neurotoxins: they have 59–62 amino acid residues and four conserved disulphide bonds (Fig. 1g).[6,14] At lower concentrations, cardiotoxins increase heart rate and, at higher concentrations, kill the animal by cardiac arrest.[6] However, the protein target of cardiotoxins 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.[6, 33] In addition, poorly characterized cardiotoxin-like basic polypeptides (CLBP) have also been described.[34,35] These polypeptides do not act as cardiotoxins or cytotoxins, their nomenclature notwithstanding, and may have entirely different biological activity.

Other three-finger toxins

A group of three-finger toxins, such as calciseptine and FS2 (Fig. 1h), specifically block L-type calcium channels.[12,13] These polypeptides are structurally similar to short-chain neurotoxins, with 60 amino acid residues and four conserved disulphide bridges. They bind to the 1,4-dihydropyridine binding site of the L-type calcium channels and physically block the calcium currents.[36] Another toxin, named dendroaspin (or mambin) and isolated from *Dendroaspis jamesoni* venom, is a potent inhibitor of platelet aggregation.[15] This protein is also structurally similar to short-chain neurotoxins, with 60 amino acid residues and four conserved disulphide bridges (Fig. 1i). Dendroaspin contains an Arg-Gly-Asp tripeptide sequence, which is involved in the adhesive function of several proteins. As expected, dendroaspin interferes with the interaction between fibrinogen and its receptor glycoprotein IIB-IIIa ($\alpha_{IIb}\beta_3$) complex and, hence, platelet aggregation. In addition, there are several other three-finger toxins, including synergistic toxins and angusticeps toxins,[2] that have not been well characterized functionally and, hence, will not be dealt in any further detail in the present review.

FUNCTIONAL SITES

As is clearly evident, three-finger toxins share similar protein folds and three-dimensional structures, but exhibit diverse biological properties. 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, we and others have successfully identified some of the functional sites in a number of three-finger toxins.

Neurotoxins

Earlier studies on neurotoxins were based on the chemical modification of specific amino acid residues from which some critical residues have been identified to be important for binding to the

muscle (or *Torpedo*) nAChR. More recently, Menez *et al.*, using systematic and well-targeted site-directed mutagenesis, have delineated the functional sites of erabutoxin a,[37,38] a short-chain neurotoxin, and α-cobratoxin,[24,39] a long-chain neurotoxin (Fig. 2). These studies reveal that both short- and long-chain neurotoxins use a number of structurally equivalent residues, including Lys23/Lys27, Asp27/Asp31, Arg33/Arg33 and Lys49/Lys4, as well as Trp25/Trp29 and Phe29/Phe32, in binding to *Torpedo* receptor. In addition, the tip of the first loop in erabutoxin a and, in contrast, the carboxy terminal tail in α-cobratoxin also play important binding roles. Antil-Delbeke *et al.*[24] and Antil *et al.*[39] have identified the residues in α-cobratoxin that are involved in the recognition of and binding to neuronal α7 nAChR. Interestingly, α-cobratoxin binds to both *Torpedo* (α1) and α7 receptors using some common residues (Trp25, Asp27 and Arg33). 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.[24] Therefore, neurotoxins appear to use a common core of critical residues for binding and additional residues to determine the specificity of their molecular target.

Cardiotoxins

The cytolytic site of cardiotoxins was identified in our laboratory, essentially by a combination of theoretical[44] and chemical modification[45] methods. We had previously predicted the neurotoxic[46] and myotoxic[47] sites in venom phospholipases. Of significance was the finding that the myotoxic site of venom phospholipases and the non-enzymatic myotoxins contained a cationic segment flanking a hydrophobic region.[43] Because myotoxic activity is a reflection of the cytolytic effects of these proteins, we extended this observation to cytolytic regions of proteins from other sources, including bacteria, plants, insects, amphibians, snakes and even humans. Interestingly, all cytolytic proteins, independent of their target cells, showed the presence of hydrophobic and cationic sites flanking each other either in the primary, secondary or tertiary structure.[40] Using this approach, we showed that the cytolytic region in cardiotoxins is spread on all three loops: there is a significant hydrophobic patch extending from the middle to the bottom end of all loops, whereas there is a row of positively charged lysine residues located at the top end (Fig. 3). We modified the positive charges on lysine residues to negative, neutral and positive charges using chemical methods of succinylation, carbamylation and guanidination, respectively.[41] The native and guanidinated derivative showed cytolytic activity, whereas the succinylated and carbamylated derivatives did not. However, all derivatives showed similar protein folding, as shown by circular dichroism, and retained their ability to bind to phospholipids.[41] These experiments clearly showed the importance of cationic residues for cytolytic activity. In addition, we also oxidized the two methionine residues present in the middle of the hydrophobic segment to methionine sulphoxide, thereby disrupting the hydrophobic site. This derivative failed to show any cytolytic activity and firmly supported the importance of the hydrophobic site in cytolytic region of cardiotoxins (RM Kini and HJ Evans, unpubl. obs., 1989). Our results were corroborated by the demonstration of the role of lysine[48] and methionine[49] residues by monoacetylation and oxidation, respectively. Nuclear magnetic resonance studies indicate that cardiotoxin interacts with phospholipid membranes through its hydrophobic face.[46]

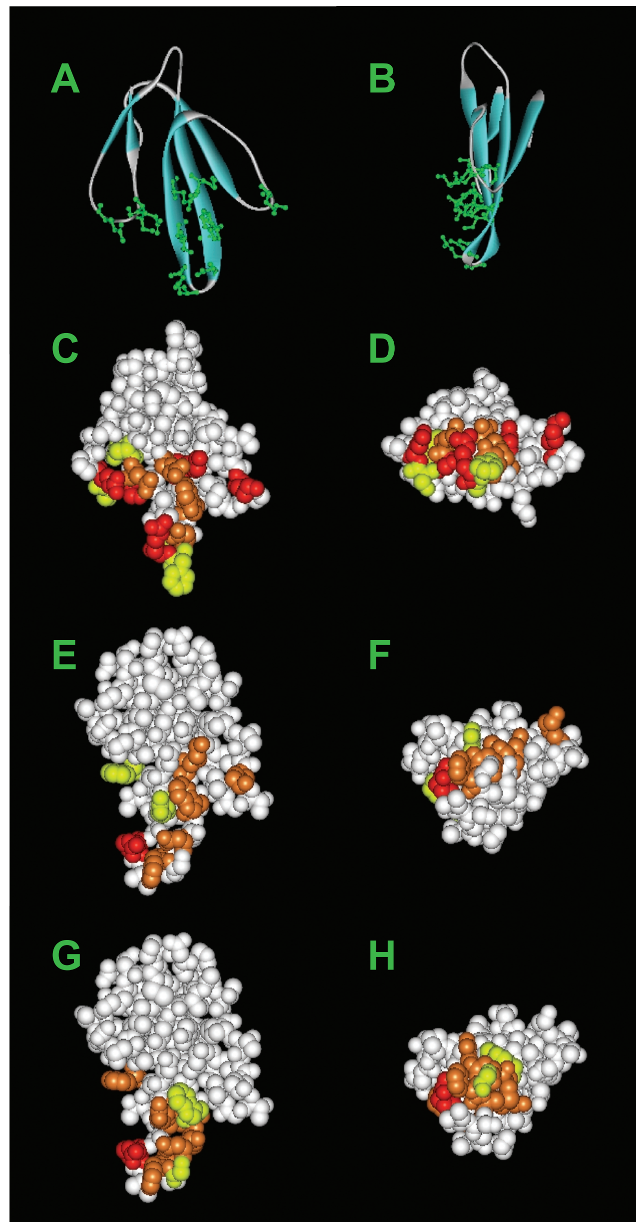

Fig. 2 Molecular determinants of neurotoxins. Interaction site residues of (a–d) erabutoxin a involved in binding to muscle nicotinic acetylcholine receptors (nAChR) and (e–h) α-cobratoxin involved in binding to muscle nAChR (e,f) and neuronal α7 nAChR (g,h). Space-filling models of toxins are shown. The residues on the 'concave' surface (a,b) that interacts with nAChR are shown. Corey–Pauling–Koltun (CPK) models (c–h) show the functionally important residues. The molecules are rotated 90° in (d,f,h) to show the surface as seen by the receptor. Site-directed mutation studies were used to determine the residues involved in interaction. The residues shown in white are either not mutated or do not affect the affinity of interaction. The residues in yellow, brown and red, when mutated, decrease the affinity by at least five-, 10- or 100-fold, respectively. For details see Servent *et al.*,[22,23] Antil-Delbeke *et al.*,[24] Harvey,[2] Pillet *et al.*[37] and Tremaeau *et al.*[38]

Acetylcholinesterase inhibitors

The residues constituting the functional site of fasciculin were identified almost simultaneously by two different approaches, namely the generation of synthetic peptides[51] and site-directed mutagenesis.[48] Molecular models of the fasciculin–AChE complex and chemical modification studies suggested a role for the second loop in the interaction of fasciculin with AChE. This was confirmed by a cyclic peptide derivative based on the second loop of fasciculin that inhibited AChE at 15–20 μmol/L compared with 300 pmol/L native toxin.[47,49] Marchot *et al.*,[48] using 14 mutants targeting 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 (Fig. 4a,b). Interestingly, however, this interaction site on fasciculin is located on the opposite surface of the molecule as opposed to the nAChR recognition site in α-neurotoxins.[37,38] More recently, Ricciardi *et al.*[50] engineered 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, together with an Asn61Tyr mutation. This chimera inhibited AChE with a K_i of 680 pmol/L, which was only approximately 15-fold less active than native fasciculin, further highlighting the importance of these segments for the AChE activity.

L-Type calcium channel blockers

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.[51–53] We proposed that the flanking proline residues protected the integrity and conformation of the interaction site, as well helped in the presentation of the interaction site to the complementary protein.[52,53] 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.[54] Using this approach, we predicted that the segment between Pro42 and Pro47 was the potential interaction site of calciseptine, FS2 and related toxins[59] (Fig. 4c,d). A synthetic 8 mer peptide, namely L-calchin, designed based on this putative interaction site, showed dose-dependent and voltage-independent inhibition of L-type calcium channels in rabbit cardiac myocytes. L-Calchin 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.[55] Schleifer,[56] using molecular modelling, also showed that this segment, particularly Met45-Trp46-*cis*Pro46-Tyr47, displayed similar hydrophobic and hydrogen bond-forming properties as nifedipine, a 1,4-dihydropyridine derivative. Therefore, the functional site of L-type calcium channel blockers is located on the outer strand of the third loop of the three-finger scaffold.

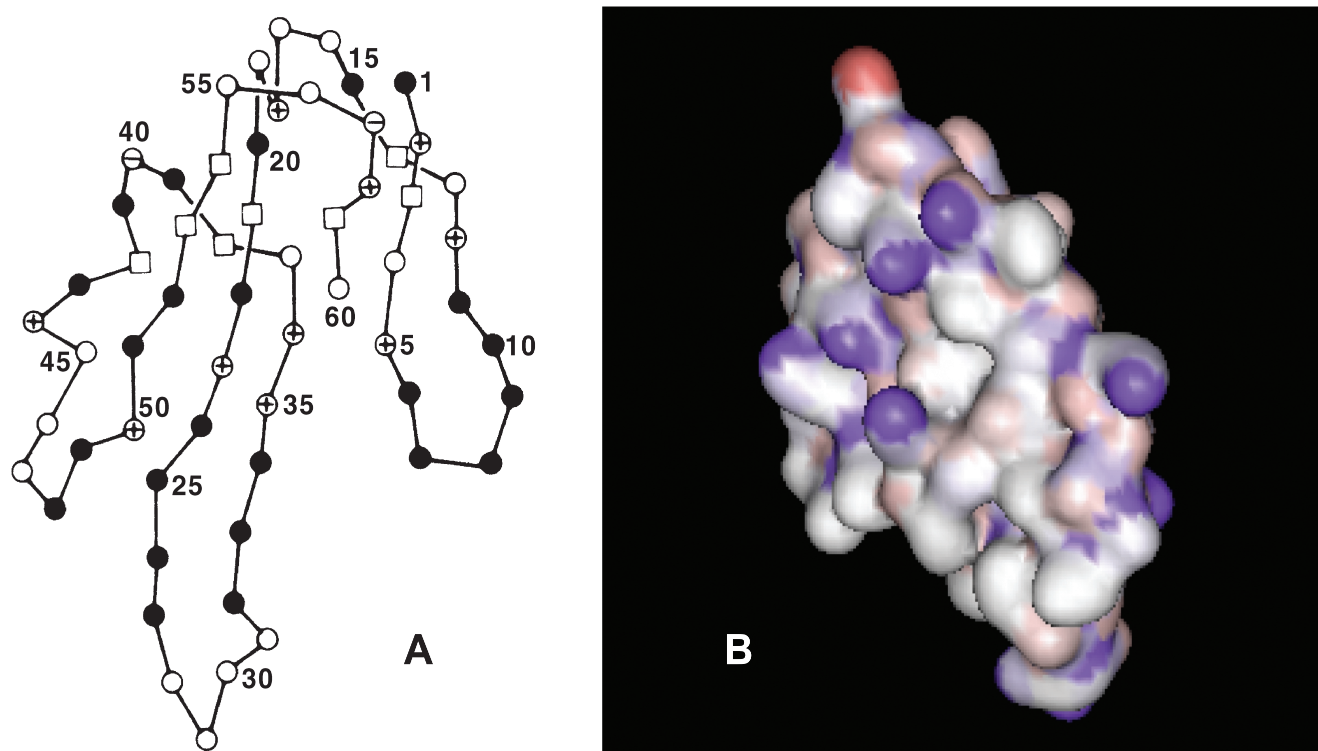

Fig. 3 Cytolytic site of cardiotoxins. (a) The schematic diagram shows the distribution of charged and hydrophobic residues in cardiotoxins. The composite was obtained by studying the structure of over 40 cardiotoxin/cytotoxin sequences. Residues are determined as hydrophobic (?) and positively (+) or negatively (–) charged, respectively, if they are found in at least 75% of proteins. Hydrophobic residues form a continuous surface at the tips of loops I, II and III. There are several positively charged residues flanking this hydrophobic segment. The hydrophobic segment is missing in neurotoxins. (b) Surface model of cardiotoxin showing hydrophobic (white) and positively (blue) or negatively (red) charged residues. For details, see Kini and Iwanaga[47] and Gatineau *et al.*[44]

Platelet aggregation inhibitor

As described previously, the amino acid sequence of dendroaspin (or mambin) contains the Arg-Gly-Asp tripeptide sequence.[15] This tripeptide sequence is flanked by two proline residues and is involved in adhesive function (Fig. 4e,f). Recently, Lu *et al*.[57] evaluated the role of the two flanking prolines by substituting both with alanine, with a resulting five- to eightfold loss in the ability to inhibit platelet aggregation. Moreover, Wattam *et al*.[58] have also replaced the Arg-Gly-Asp sequence by Arg-Tyr-Asp and Arg-Cys-Asp tripeptide sequences and have demonstrated that these mutations promote selective inhibition of β_1 and β_3 integrins, respectively. Thus, the functional site of dendroaspin is located at the tip of its third loop.

Hannalgesin

Hannalgesin, isolated from *Ophiophagus hannah* (King cobra) venom by our group, exhibited potent analgesic as well as neurotoxic effects in mice.[59] Using the proline bracket method, we identified the functional site of this protein to be located at the carboxy terminal end of the toxin (RM Kini and P Gopalakrishnakone, unpubl. obs., 1996). A short peptide synthesized on the basis of the predicted site showed significant selective analgesic effect *in vivo* in the absence of neurotoxicity.[60] Thereby, we were able to identify the analgesic site of hannalgesin and isolate and develop the beneficial effect of this toxin.

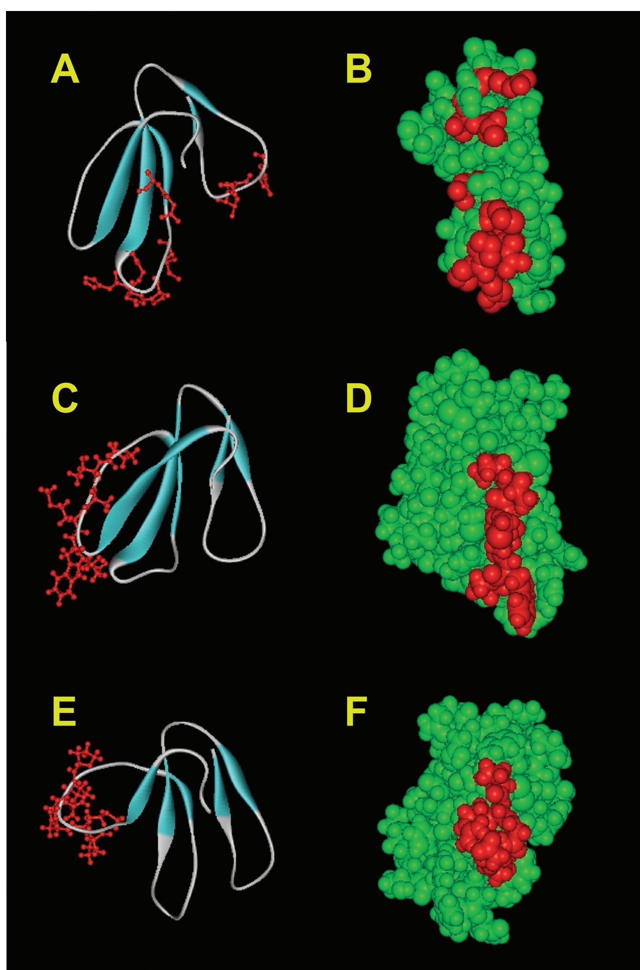

Fig. 4 Functional sites of fasciculin (a,b), calciseptine (c,d) and dendroaspin (e,f). The interaction site of fasciculin is found in loops I and II, whereas that of calciseptine and dendroaspin is found in loop III. The residues involved in interaction are shown as stick models (red) in (a,c,e). Space-filling models of the interaction residues (red) are shown in (b,d,f).

CONCLUSIONS AND FUTURE PROSPECTS

The compact structure of three-finger proteins has been exploited by nature for developing ligands that perform a wide variety of functions. In elapid and hydrophid snakes, the ancestral gene(s) encoding for three-finger protein(s) were duplicated several times and a wide array of offensive weapons in their armamentarium resulted through accelerated evolution in their exons.[61–63] During evolution, most features that are essential for protein folding and structural integrity were preserved, leading to 'sibling' toxins that resembled each other in their overall structural features but differed in their missions of targeting various vital physiological processes. This is clearly evident in three-finger toxins, because the core, consisting of the four disulphide bridges, is highly conserved and the functional sites are located on either surface of the molecule on the different loops and/or the carboxy terminal tail. In fact, there is no single designated location for the functional sites. As is the case with other superfamilies of toxins, snakes have used the robust three-finger protein mould to construct a group of toxins with wide variations in function involving just a few subtle changes in the functional sites. This capacity of adaptation no doubt offers them the flexibility to effectively capture any species of prey at their disposal. Their intended interests notwithstanding, snake venom toxins provide us with ample challenging opportunities to decipher the subtleties in their functional sites such that we may better understand the plasticity of protein structure and function. Moreover, they will also tremendously enhance our potential to use their molecular architecture to design and develop mini proteins with novel functions of scientific and therapeutic interest.

ACKNOWLEDGEMENTS

I thank Professors André Ménez and Renée Menez (Department of Protein Engineering, CEA Saclay, France) for providing the crystal structure data of muscarinic toxin before its publication. I also thank my colleagues Drs Bryan Fry, S Nirthanan and T Sivaraman (Department of Biological Sciences, National University of Singapore, Singapore) for their critical reading of the manuscript and suggestions. This work was supported by several research grants from the Academic Research Fund of the National University of Singapore and a research grant from Defense Science and Technology Agency of Singapore.

REFERENCES

1. Lee CY. *Snake Venoms. Handbook of Experimental Pharmacology*, **Vol. 52**. Springer-Verlag, Berlin. 1979.
2. Harvey AL. *Snake Toxins*. Pergamon Press, New York. 1991.
3. Bailey GS. *Enzymes from Snake Venom.* Alaken, Fort Collins. 1998.
4. Tu AT. *Reptile Venoms and Toxins.* Marcel Decker, New York. 1991.
5. Kini RM. *Venom Phospholipase A_2 Enzymes: Structure, Function and Mechanism*. John Wiley and Sons, Chichester. 1997.

6. Dufton MT, Hider RC. Structure and pharmacology of elapid cytotoxins. *Pharmacol. Ther.* 1988; **36**: 1–40.
7. Menez A. Functional architectures of animal toxins: A clue to drug design? *Toxicon* 1998; **36**: 1557–72.
8. Tsetlin V. Snake venom alpha-neurotoxins and other ‘three-finger’ proteins. *Eur. J. Biochem.* 1999; **264**: 281–6.
9. Changeux JP. The TiPS Lecture. The nicotinic acetylcholine receptor. An allosteric protein prototype of ligand-gated ion channels. *Trends Pharmacol. Sci.* 1990; **11**: 485–92.
10. Grant GA, Chiappinelli VA. κ-Bungarotoxin: Complete amino acid sequence of a neuronal nicotinic receptor probe. *Biochemistry* 1985; **24**: 1532–7.
11. Jerusalinsky D, Harvey AL. Toxins from mamba venoms: Small proteins with selectivities for different subtypes of muscarinic acetylcholine receptors. *Trends Pharmacol. Sci.* 1994; **15**: 424–30.
12. De Weille JR, Schweitz H, Maes P, Tartar A, Lazdunski M. Calciseptine, a peptide isolated from black mamba venom, is a specific blocker of the L-type calcium channel. *Proc. Natl Acad. Sci. USA* 1991; **88**: 2437–40.
13. Albrand JP, Blackledge MJ, Pascaud F, Hollecker M, Marion D. NMR and restrained molecular dynamics study of the three-dimensional solution structure of toxin FS2, a specific blocker of the L-type calcium channel, isolated from black mamba venom. *Biochemistry* 1995; **34**: 5923–37.
14. Bilwes A, Rees B, Moras D, Menez R, Menez A. X-Ray structure at 1.55 A of toxin gamma, a cardiotoxin from *Naja nigricollis* venom. Crystal packing reveals a model for insertion into membranes. *J. Mol. Biol.* 1994; **239**: 122–36.
15. McDowell RS, Dennis MS, Louie A, Shuster M, Mulkerrin MG, Lazarus RA. Mambin, a potent glycoprotein IIb–IIIa antagonist and platelet aggregation inhibitor structurally related to the short neurotoxins. *Biochemistry* 1992; **31**: 4766–72.
16. Fleming TJ, O’hUigin C, Malek TR. Characterization of two novel Ly-6 genes. Protein sequence and potential structural similarity to alpha-bungarotoxin and other neurotoxins. *J. Immunol.* 1993; **150**: 5379–90.
17. Ploug M, Ellis V. Structure–function relationships in the receptor for urokinase-type plasminogen activator. Comparison to other members of the Ly-6 family and snake venom alpha-neurotoxins. *FEBS Lett.* 1994; **349**: 163–8.
18. Gumley TP, McKenzie IFC, Sandrin MS. Tissue expression, structure and function of the murine Ly-6 family of molecules. *Immunol. Cell Biol.* 1995; **73**: 277–96.
19. Miwa JM, Ibanez-Tallon I, Crabtree GW *et al. lynx1*, an endogenous toxin-like modulator of nicotinic acetylcholine receptors in the mammalian CNS. *Neuron* 1999; **23**: 105–14.
20. Cordero-Erausquin M, Marubio LM, Klink R, Changeux JP. Nicotinic receptor function: New perspectives from knockout mice. *Trends Pharmacol. Sci.* 2000; **21**: 211–17.
21. Unwin N. Nicotinic acetylcholine receptor at 9 Å resolution. *J. Mol. Biol.* 1993; **229**: 1101–24.
22. Servent D, Winckler-Dietrich V, Hu HY *et al.* Only snake curaremimetic toxins with a fifth disulfide bond have high affinity for the neuronal alpha7 nicotinic receptor. *J. Biol. Chem.* 1997; **272**: 24 279–86.
23. Servent D, Antil-Delbeke S, Corringer PJ, Changeux JP, Ménez A. Molecular characterization of the specificity of interactions of various neurotoxins on two distinct nicotinic acetylcholine receptors. *Eur. J. Pharmacol.* 2000; **393**: 197–204.
24. Antil-Delbeke S, Gaillard C, Tamiya T *et al.* Molecular determinants by which a long chain toxin from snake venom interacts with the neuronal alpha 7-nicotinic acetylcholine receptor. *J. Biol. Chem.* 2000; **275**: 29 594–601.
25. Karlsson E, Jolkkonen M, Mulugeta E, Onali P, Adem A. Snake toxins with high selectivity for subtypes of muscarinic acetylcholine receptors. *Biochimie* 2000; **82**: 793–806.
26. Carlsson FHH. Snake venom toxins. The primary structure of protein S4C11. A neurotoxin homologue from the venom of forest cobra (*Naja melanoleuca*). *Biochim. Biophys. Acta* 1975; **400**: 310–21.
27. Joubert FJ. The purification and amino acid sequence of toxin CM-13b from *Naja haje annulifera* (Egyptian cobra) venom. *Hoppe-Seylers Z. Physiol. Chem.* 1975; **356**: 1901–8.
28. Joubert FJ, Taljaard N. Snake venoms. The amino acid sequences of two Melanoleuca-type toxins. *Hoppe-Seylers Z. Physiol. Chem.* 1980; **361**: 425–36.
29. Utkin YN, Kukhtina VV, Maslennikov IV *et al.* First tryptophan-containing weak neurotoxin from cobra venom. *Toxicon* 2001; **39**: 921–7.
30. Utkin YN, Kukhtina VV, Kryukova EV *et al.* ‘Weak toxin’ from *Naja kaouthia* is a nontoxic antagonist of alpha 7 and muscle-type nicotinic acetylcholine receptors. *J. Biol. Chem.* 2001; **276**: 15 810–15.
31. Nirthanan S, Charpantier E, Gopalakrishnakone P *et al.* Candoxin, a novel toxin from *Bungarus candidus* is a reversible antagonist of muscle (αβγδ) but a poorly reversible antagonist of neuronal 7 nicotinic acetylcholine receptors. *J. Biol. Chem.* 2002; **277**: 17 811–20.
32. Eastman J, Wilson EJ, Cervanansky C, Rosenberry TL. Fasciculin 2 binds to the peripheral site on acetylcholinesterase and inhibits substrate hydrolysis by slowing a step involving proton transfer during enzyme acylation. *J. Biol. Chem.* 1995; **270**: 19 694–701.
33. Condrea E. Hemolytic disorders associated with a primary red cell membrane defect. *Experentia* 1975; **32**: 537–42.
34. Takechi M, Tanaka Y, Hayashi K. Amino acid sequence of a cardiotoxin-like basic polypeptide (CLBP) with low cytotoxic activity isolated from the venom of the Formosan cobra (*Naja naja atra*). *Biochem. Int.* 1985; **11**: 795–802.
35. Sivaraman T, Kumar TK, Yang PW, Yu C. Cardiotoxin-like basic protein (CLBP) from *Naja naja atra* is not a cardiotoxin. *Toxicon* 1997; **35**: 1367–71.
36. Yasuda O, Morimoto S, Chen Y *et al.* Calciseptine binding to a 1,4-dihydropyridine recognition site of the L-type calcium channel of rat synaptosomal membranes. *Biochem. Biophys. Res. Commun.* 1993; **194**: 587–94.
37. Pillet L, Tremeau O, Ducancel F *et al.* Genetic engineering of snake toxins. Role of invariant residues in the structural and functional properties of a curaremimetic toxin, as probed by site-directed mutagenesis. *J. Biol. Chem.* 1993; **268**: 909–16.
38. Tremeau O, Lemaire C, Drevet P *et al.* Genetic engineering of snake toxins. The functional site of Erabutoxin a, as delineated by site-directed mutagenesis, includes variant residues. *J. Biol. Chem.* 1995; **270**: 9362–9.
39. Antil S, Servent D, Ménez A. Variability among the sites by which curaremimetic toxins bind to torpedo acetylcholine receptor, as revealed by identification of the functional residues of α-cobratoxin. *J. Biol. Chem.* 1999; **274**: 34 851–8.
40. Kini RM, Evans HJ. A common cytolytic region in myotoxins, hemolysins, cardiotoxins and antibacterial peptides. *Int. J. Peptide Protein Res.* 1989; **34**: 277–86.
41. Kini RM, Evans HJ. Role of cationic amino acid residues in cytolytic activity. Modifications of lysine residues in the cardiotoxin from *Naja nigricollis* venom and correlation between cytolytic and antiplatelet activities. *Biochemistry* 1989; **28**: 9209–15.
42. Kini RM, Iwanaga S. Structure–function relationships of phospholipases I. Prediction of presynaptic neurotoxicity. *Toxicon* 1986; **24**: 527–41.
43. Kini RM, Iwanaga S. Structure–function relationships of phospholipases II. Charge density distribution and the myotoxicity of presynaptically neurotoxic phospholipases. *Toxicon* 1986; **24**: 895–905.
44. Gatineau E, Takechi M, Bouet F *et al.* Delineation of the functional site of a snake venom cardiotoxin: Preparation, structure, and function of monoacetylated derivatives. *Biochemistry* 1990; **29**: 6480–9.
45. Carlsson FHH, Louw AI. The oxidation of methionine and its effect of the properties of cardiotoxin V 1 II from *Naja melanoleuca* venom. *Biochim. Biophys. Acta* 1978; **534**: 322–30.

46. Dauplais M, Neumann JM, Pinkasfeld S, Ménez A, Roumestand C. An NMR study of the interaction of cardiotoxin γ from *Naja nigricollis* with perdeuterated dodecylphosphocholine micelles. *Eur. J. Biochem.* 1995; **230**: 213–20.
47. Falkenstein RJ, Pena C. Synthetic peptides derived from the central loop of fasciculin: Structural analysis and evaluation as acetylcholinesterase inhibitors. *Biochim. Biophys. Acta* 1997; **1340**: 143–51.
48. Marchot P, Prowse CN, Kanter J *et al.* Expression and activity of mutants of fasciculin, a peptidic acetylcholinesterase inhibitor from mamba venom. *J. Biol. Chem.* 1997; **272**: 3502–10.
49. Karlsson E, Mbugua PM, Rodriguez-Ithurralde D. Fasciculins, anticholinesterase toxins from the venom of the green mamba *Dendroaspis angusticeps*. *J. Physiol.* 1984; **79**: 232–40.
50. Ricciardi A, Le Du MH, Khayati M *et al.* Do structural deviations between toxins adopting the same fold reflect functional differences? *J. Biol. Chem.* 2000; **275**: 18 302–10.
51. Kini RM, Evans HJ. A common structural feature enclosing interaction sites: Prediction of protein–protein interaction sites and development of potent bioactive peptides. In: Menon J (ed.). *Current Topics in Peptide and Protein Research*, **Vol. 1**. Council of Scientific Information, Trivandrum. 1994; 297–311.
52. Kini RM, Evans HJ. A hypothetical structural role for proline residues in the flanking segments of protein–protein interaction sites. *Biochem. Biophys. Res. Commun.* 1995; **212**: 1115–24.
53. Kini RM. Proline brackets and identification of potential functional sites in proteins: Toxins to therapeutics. *Toxicon* 1998; **36**: 1659–70.
54. Kini RM, Evans HJ. Prediction of potential protein–protein interaction sites from amino acid sequence. Identification of a fibrin polymerization site. *FEBS Lett.* 1996; **385**: 81–6.
55. Kini RM, Caldwell RA, Wu QY, Baumgarten CM, Feher JJ, Evans HJ. Flanking proline residues identify the L-type Ca^{2+} channel binding site of calciseptine and FS2. *Biochemistry* 1998; **37**: 9058–63.
56. Schleifer KJ. Comparative molecular modelling study of the calcium channel blockers nifedipine and black mamba toxin FS2. *J. Comput. Aided Mol. Des.* 1997; **11**: 491–501.
57. Lu X, Sun Y, Shang D *et al.* Evaluation of the role of proline residues flanking the RGD motif of dendroaspin, an inhibitor of platelet aggregation and cell adhesion. *Biochem. J.* 2001; **355**: 633–8.
58. Wattam B, Shang D, Rahman S *et al.* Arg-Tyr-Asp (RYD) and Arg-Cys-Asp (RCD) motifs in dendroaspin promote selective inhibition of β_1 and β_3 integrins. *Biochem. J.* 2001; **356**: 11–17.
59. Pu XC, Wong PT, Gopalakrishnakone P. A novel analgesic toxin (Hannalgesin) from the venom of king cobra (*Ophiophagus hannah*). *Toxicon* 1995; **33**: 1425–31.
60. Gopalakrishnakone P, Wong P, Gwee MCE, Kini RM, inventors. National University of Singapore, assignee. *Therapeutic molecules.* Singapore patent pending. 20 September 1996.
61. Nakashima K, Nobuhisa I, Deshimaru M *et al.* Accelerated evolution in the protein-coding regions is universal in crotalinae snake venom gland phospholipase A_2 isozyme genes. *Proc. Natl Acad. Sci. USA* 1995; **92**: 5605–9.
62. Deshimaru M, Ogawa T, Nakashima K *et al.* Accelerated evolution of crotalinae snake venom gland serine proteases. *FEBS Lett.* 1996; **397**: 83–8.
63. Ohno M, Ménez R, Ogawa T *et al.* Molecular evolution of snake toxins: Is the functional diversity of snake toxins associated with a mechanism of accelerated evolution? *Prog. Nucleic Acids Res. Mol. Biol.* 1998; **59**: 307–64.