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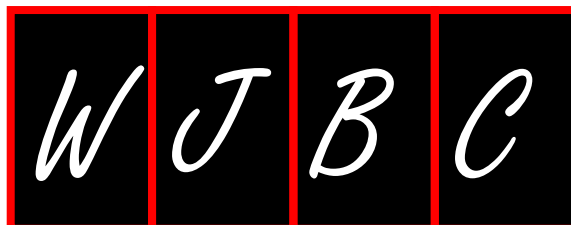
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## Last decade update for three-finger toxins: Newly emerging structures and biological activities

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### Abstract

Three-finger toxins (TFTs) comprise one of largest families of snake venom toxins. While they are principal to and the most toxic components of the venoms of the *Elapidae* snake family, their presence has also been detected in the venoms of snakes from other families. The first TFT,  $\alpha$ -bungarotoxin, was discovered almost 50 years ago and has since been used widely as a specific marker of the  $\alpha 7$  and muscle-type nicotinic acetylcholine receptors. To date, the number of TFT amino acid sequences deposited in the UniProt Knowledgebase free-access database is more than 700, and new members are being added constantly. Although structural variations among the TFTs are not numerous, several new structures have been discovered recently; these include the disulfide-bound dimers of TFTs and toxins with nonstandard pairing of disulfide bonds. New types of biological activities have also been demonstrated for the well-known TFTs, and research on this topic has become a hot topic of TFT studies. The classic TFTs  $\alpha$ -bungarotoxin and  $\alpha$ -cobratoxin, for example, have now been shown to inhibit ionotropic receptors of  $\gamma$ -aminobutyric acid, and some muscarinic toxins have been shown to interact with adrenoceptors. New, unexpected activities have been demonstrated for some TFTs as well, such as toxin interaction with interleukin or insulin receptors and even TFT-activated motility of sperm. This minireview provides a summarization of the data that has emerged in the last decade on the TFTs and their activities.

**Key words:** Three-finger toxins; Snake; Venom; Structure; Biological activity

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**Core tip:** The three-finger toxins (TFTs) of snake venoms are principal to and the most toxic components of elapid venoms. Over 700 TFT amino acid sequences are listed in the UniProt Knowledgebase currently, with new members added constantly. The past decade has also seen multitudinous new discoveries, including structural variations in

TFTs (*i.e.* disulfide-bound dimers), new types of biological activities for the well-known TFTs (*e.g.*,  $\alpha$ -bungarotoxin's inhibition of ionotropic receptors of  $\gamma$ -aminobutyric acid), and other new, unexpected activities for the TFTs (*i.e.* interaction with interleukin or insulin receptors and activation of sperm motility). This minireview provides an up-to-date overview of these data.

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## INTRODUCTION

Three-finger toxins (TFTs) form an abundant family of nonenzymatic proteins found in snake venoms<sup>[1]</sup>. The TFTs were so-named according to their characteristic spatial structure, in which three loops (fingers) protrude from the central core, stabilized by four conserved disulfide bonds. The TFTs contain from 57 to 82 amino acid residues, with some toxin types having an extra fifth disulfide bond, located in either their central loop II or N-terminal loop I. The position of the bond affects the toxin's biological activity.

The TFTs manifest a wide array of biological activities, ranging from selective interaction with certain receptor types to nonselective cell lysis<sup>[2]</sup>. Typically, the TFTs represent the main components of elapid venoms<sup>[3]</sup>. Thus, in the venom of the desert coral snake *Micrurus tschudii*, 95% of toxins are represented by TFTs<sup>[4]</sup>. Their presence has also been detected in the venoms of snakes from other families. For example, TFTs were found in different snake genera from colubrid family<sup>[5,6]</sup>. The TFTs also represent one of the largest families of snake toxins, having more than 700 TFT amino acid sequences deposited in the UniProt Knowledgebase free-access database. Intriguingly, nontoxic structural counterparts of the TFTs have been found in animal organisms, namely the lymphocyte antigen 6 (Ly6) proteins. Based on their commonality of three-finger folding, the TFTs and Ly6 proteins are combined into one Ly6/neurotoxin family<sup>[7]</sup>.

The first TFT discovered,  $\alpha$ -bungarotoxin ( $\alpha$ -Bgt), was published almost 50 years ago<sup>[8]</sup>. Since then,  $\alpha$ -Bgt has become a widely used specific marker of the  $\alpha 7$  and muscle-type nicotinic acetylcholine receptors (nAChRs). In addition, a tremendous number of other TFTs have been discovered, and new members of this family possessing original structures and biological activities are emerging constantly. Moreover, new activities have been recognized for the well-known TFTs, and the discovery of new activities for both the new and the well-known TFTs may be regarded as a recent trend in toxinology.

This minireview briefly summarizes the data obtained for TFTs during the last decade (Table 1). The toxins with new structural features appearing in the recent literature are considered herein; those that have garnered the most interest is the covalently-bound TFT dimers. In addition, the recently discovered uncommon biological activities of some TFTs are discussed; these include the so-called mambalgins that exert a potent analgesic effect upon central and peripheral injection and represent the most remarkable discovery of late.

## RECENTLY-DISCOVERED TFTS WITH NEW STRUCTURAL FEATURES

Ten years ago, the first data revealing covalently-bound TFT dimers were published<sup>[9]</sup>. Disulfide-bound dimers of TFTs, including heterodimers formed by  $\alpha$ -cobratoxin ( $\alpha$ -CTX; a long-chain  $\alpha$ -neurotoxin) with different cytotoxins and the homodimer of  $\alpha$ -CTX were isolated from the *Naja kaouthia* cobra venom. Determination of the homodimer crystal structure allowed identification of the intermolecular disulfides formed by Cys3 in the protomer one and Cys20 in the protomer two, and other way round (Figure 1A). All other disulfides in protomers had the same pairing as in natural  $\alpha$ -CTX<sup>[10]</sup>.

The dimerization itself strongly affected the biological activity of the original

Table 1 Novel three-finger toxin biological effects and their potential applications

	Toxin	Effect/Target	Potential application
Impact on signal transduction	Mambalgins	Inhibitors of ASICs	Analgesics
	Micrurotoxins	Modulators of GABAA receptor	Biochemical instruments for the GABAA receptor study
	$\alpha$ -Neurotoxins	Inhibitors of GABAA receptor	
	Muscarinic toxin MT $\alpha$	Antagonist of $\alpha$ 2B-adrenoceptor	Treatment of blood pressure disorders
	Toxin CM-3	Interaction with $\alpha$ 1A-adrenoceptor	
	Toxin AdTx1 (p-Da1a)	Specific and selective inhibitor for the $\alpha$ 1A-adrenoceptor	
	Toxin p-Da1b	Antagonist of $\alpha$ 2A-adrenoceptors	
	Toxin Tx7335	Potassium channel activator	Biochemical instrument for the study of potassium channels
Impact on blood coagulation	Calliotoxin ( $\delta$ -elapitoxin-Cb1a)	Activator of voltage-gated sodium channel	Biochemical instrument for the study of sodium channels
	Toxin KT-6.9	Inhibitor of platelet aggregation	Treatment of blood coagulation disorders
	Ringhalexin	Inhibitor of FX activation	
	Exactin	Inhibitor of FX activation	
Insulinotropic activity	Cardiotoxin-I	Induction of insulin secretion from $\beta$ -cells	Treatment of type 2 diabetes
Impact on sperm motility	Actiflagelin	Activator of sperm motility in vitro	Infertility treatment

ASIC: Acid sensing ion channel; GABAA: Type A receptor of gamma-amino butyric acid; FX: Factor X.

toxins, with the cytotoxic activity of cytotoxins within dimers being completely abolished. However, the dimers were found to retain most of the  $\alpha$ -CTX capacity to interact with *Torpedo* and  $\alpha$ 7 nAChRs as well as with the *Lymnea stagnalis* acetylcholine-binding protein. Moreover, in contrast to the  $\alpha$ -CTX monomer, the  $\alpha$ -CTX dimer acquired the capacity to interact with  $\alpha$ 3 $\beta$ 2 nAChR, similar to that seen with  $\kappa$ -bungarotoxin ( $\kappa$ -Bgt), a dimer with no disulfides between its monomers (Figure 1D). Collectively, these data show that dimerization of three-fingered neurotoxins is essential for binding to heteromeric  $\alpha$ 3 $\beta$ 2 nAChRs.

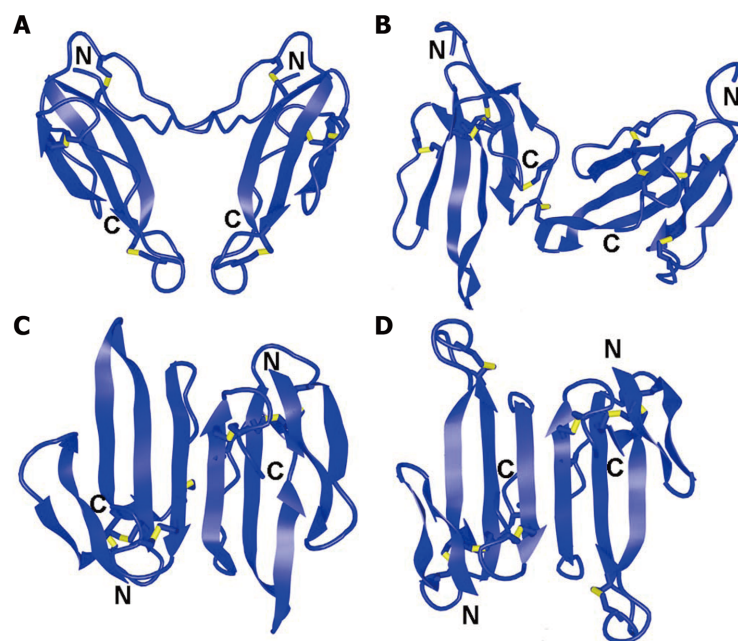
In 2009, from venom of the brown cat snake *Boiga irregularis* a new heterodimeric TFT, irditoxin, was isolated<sup>[11]</sup>. Irditoxin spatial structure determined by X-ray analysis displayed two subunits possessing a three-finger fold, characteristic for nonconventional toxins (Figure 1B). The subunits in the irditoxin dimer are connected by an interchain disulfide bond, formed by extra cysteine residues present in each subunit. In contrast to the dimeric toxins discussed above, irditoxin does not inhibit mouse neuronal  $\alpha$ 3 $\beta$ 2 and  $\alpha$ 7 nAChRs. However, irditoxin is a bird- and reptile-specific postsynaptic neurotoxin which inhibits the chick muscle nAChR three orders of magnitude more efficiently than the mouse receptor. *In vivo*, it was lethal to birds and lizards and was nontoxic toward mice<sup>[11]</sup>.

Covalently-bound dimers are undoubtedly the most interesting TFT posttranslational modification recently found. A new TFT forming a noncovalent dimer was discovered recently as well; this neurotoxin, haditoxin, was isolated from the venom of king cobra *Ophiophagus hannah*<sup>[12]</sup>. The high-resolution X-ray analysis revealed that haditoxin is a homodimer (Figure 1C), with a structure very similar to that of  $\kappa$ -Bgt (Figure 1D). However, in contrast to  $\kappa$ -Bgt, the amino acid sequences of the monomeric subunits of haditoxin correspond to those of  $\alpha$ -neurotoxins of the short-chain type. It should be noted that  $\kappa$ -Bgt targets the neuronal  $\alpha$ 3 $\beta$ 2 and  $\alpha$ 4 $\beta$ 2 nAChRs and  $\alpha$ -neurotoxins of the short-chain type block the muscle-type nAChRs only, while haditoxin demonstrated new pharmacological features, being antagonist toward muscle ( $\alpha$ 3 $\beta$ 2, and  $\alpha$ 4 $\beta$ 2) nAChRs, and having the highest affinity (IC<sub>50</sub> 180 nmol/L) for  $\alpha$ 7 nAChRs<sup>[12]</sup>.

The above data indicate that TFT dimerization may underlie the capacity for interaction with neuronal nAChRs.

The disulfide bonds play an essential role in maintaining the spatial structure of TFTs, and their pairing pattern is largely conserved. However, an unusual disulfide bond scaffold was found in a TFT isolated from the venom of eastern green mamba *Dendroaspis angusticeps*<sup>[13]</sup>. This toxin, named Tx7335, has the highest amino acid sequence similarity to the TFTs of nonconventional type, but differs from them in the number and positions of cysteines (Figure 2). Similar to  $\alpha$ -neurotoxins of the short-chain type, Tx7335 has only eight cysteines, while nonconventional toxins have ten





**Figure 1 Spatial structures of dimeric three-finger toxins.** A: Homodimer of  $\alpha$ -cobratoxin, Protein Data Bank Identification code (PDB ID): 4AEA; B: Irditoxin, PDB ID: 2H7Z; C: Haditoxin, PDB ID: 3HH7; D:  $\kappa$ -bungarotoxin, PDB ID: 1KBA. Disulfide bonds are shown in yellow. N and C indicate N- and C-terminus, respectively.

cysteines. Furthermore, Tx7335 contains a tyrosine residue at position 43, which is occupied by a cysteine in all TFTs, while a cysteine is found at position 25. In most other TFTs this position is occupied by a tyrosine (Figure 2). According to structure modeling, Cys55 may make a disulfide bond with Cys25; due to the spatial proximity of these residues no major distortion in the three-finger structure occurs. This novel arrangement of disulfides may explain the unique functional effects observed for Tx7335. Indeed, it has been shown to activate the bacterial pH-gated potassium channel KcsA by a dose-dependent increase in both mean open time and open probability. Moreover, Tx7335 binds at the KcsA extracellular domain at a site probably different from that of channel inhibitors<sup>[11]</sup>.

Several new TFTs retaining the classical arrangement of disulfide bonds but possessing novel structural features have been identified recently. So, from the venom of black mamba *Dendroaspis polylepis polylepis*, a non-typical long-chain TFT,  $\alpha$ -elapitoxin-Dpp2d ( $\alpha$ -EPTX-Dpp2d), was isolated<sup>[14]</sup>.  $\alpha$ -EPTX-Dpp2d contains an amidated C-terminal arginine, a posttranslational modification that had not been observed before in snake TFTs. Biological activity studies showed that, at a 1  $\mu\text{mol/L}$  concentration, the  $\alpha$ -EPTX-Dpp2d potently inhibited neuronal  $\alpha 7$  ( $\text{IC}_{50}$  58 nmol/L) and muscle-type nAChRs ( $\text{IC}_{50}$  114 nmol/L) however showed no effect on  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  receptors. Therefore, this amidation was deemed to have no significant effect on the toxin's selectivity, as the activity profile is fairly similar to that of the classic long-chain TFTs with a free carboxyl termini<sup>[14]</sup>.

It was shown that the earlier characterized TFT Oh9-1<sup>[15]</sup> from *Ophiophagus hannah* venom may represent a new group of competitive nAChR antagonists, known as the  $\Omega$ -neurotoxins<sup>[16]</sup>. Electrophysiology experiments on *Xenopus* oocytes showed that Oh9-1 inhibited rat muscle-type  $\alpha 1\beta 1\epsilon\delta$  (adult,  $\text{IC}_{50}$  3.1  $\mu\text{mol/L}$ ) and  $\alpha 1\beta 1\gamma\delta$  (fetal,  $\text{IC}_{50}$  5.6  $\mu\text{mol/L}$ ) and rat neuronal  $\alpha 3\beta 2$  nAChRs ( $\text{IC}_{50}$  50.2  $\mu\text{mol/L}$ ), but manifested low or no affinity for other human and rat neuronal subtypes. Interestingly, Oh9-1 potentiated the human glycine receptor (homopentamer composed of  $\alpha 1$  subunits), with activity increase by about 2-fold. Alanine-scan mutagenesis showed a novel mode of interaction with the ACh binding pocket of nAChRs via a set of functional amino acid residues that are different from those in the classical  $\alpha$ -neurotoxins. Herewith, the central loop of Oh9-1 interacts with  $\alpha 1\beta 1\epsilon\delta$  nAChR by both sides of the  $\beta$ -strand, while only one side of the  $\beta$ -strand interacts with the  $\alpha 3\beta 2$  receptor<sup>[16]</sup>.

The taxon-specific dimeric TFT irditoxin, isolated from a rear-fanged snake, was discussed above. Another taxon-specific TFT, fulgimotxin, was isolated from the venom of the rear-fanged green vine snake *Oxybelis fulgidus*<sup>[17]</sup>. This toxin is a monomer and contains five disulfides, typical for the nonconventional TFTs. It is highly neurotoxic to lizards; however, mice are unaffected. Similar to other colubrid TFTs, fulgimotxin has an extended N-terminal amino acid sequence and a



**Figure 2** Alignment of amino acid sequence of toxin Tx7335 with those of nonconventional toxins. Cysteine residues are marked in yellow. Black lines indicate the locations of the typical disulfide bond in nonconventional toxins; red line indicates the unusual disulfide bond 25-55 in Tx7335. 3NOJ\_DENAN: Toxin Tx7335 from *Dendroaspis angusticeps* (Eastern green mamba); 3NOJ\_BUNCA: Bucandin from *Bungarus candidus* (Malayan krait); 3NOJ6\_DENJA: Toxin S6C6 from *Dendroaspis jamesoni kaimosae* (Eastern Jameson's mamba); 3NOJ\_WALAE: Actiflagelin from *Walterinnesia aegyptia* (desert black snake).

pyroglutamic acid at the N-terminus.

The longest TFT, the nonconventional toxin BMLCL, consisting of 82 amino acid residues and five disulfide bridges, was identified in *Bungarus multicinctus* venom<sup>[18]</sup>. Earlier studies of biological activity revealed no interaction with the muscarinic acetylcholine receptors (mAChRs) M1 and M2 nor with the muscle-type nAChR<sup>[19]</sup>. However, recent studies showed that BMLCL interacted efficiently with both  $\alpha 7$  ( $IC_{50}$  43 nmol/L) and muscle-type nAChR ( $IC_{50}$  31 nmol/L)<sup>[19]</sup>. Thus, the longest TFT functions as an antagonist of nAChRs.

It should be noted that so far, no TFTs have been found in the venoms of snakes from the *Viperidae* family; however, transcripts encoding these toxins were identified in venom gland transcriptomes of several *Viperidae* species. To address the question of biological activity of *Viperidae* TFTs, two toxins were obtained by heterologous expression in *Escherichia coli*. Based on the nucleotide sequences of cDNA encoding TFTs in the venom glands of vipers *Azemiops feae* and *Vipera nikolskii*, the corresponding genes optimized for bacterial expression were synthesized<sup>[20]</sup>. Expressed *A. feae* TFT (TFT-AF) and *V. nikolskii* TFT (VN-TFT), both of the nonconventional type, were refolded under the conditions elaborated on earlier for cobra TFTs. The biological activity of the toxins obtained was studied by electrophysiological techniques, calcium imaging, and radioligand analysis. Both toxins inhibited neuronal  $\alpha 3$ -containing and muscle-type nAChRs in the micromolar concentration range, but they were each very weak antagonists of neuronal  $\alpha 7$  nAChRs. Thus, viper TFTs can function as antagonists of nAChRs of neuronal and muscle-type<sup>[20]</sup>.

## RECENTLY DISCOVERED TFTS WITH NEW BIOLOGICAL ACTIVITIES

### Novel TFTs affecting signal transduction

The most fascinating biological activity of TFTs discovered during the last decade is their capacity to interact with acid-sensing ion channels (ASICs). ASICs are proton activated and  $Na^+$ -selective ion channels, widely distributed throughout the peripheral and central nervous systems (CNS) in vertebrates. ASICs take part in an array of physiological processes, from synaptic plasticity and neurodegeneration to pain sensation. Therefore, the finding of new regulatory modes for these proteins opens up a new avenue of research for pain management, as well as for addiction or fear.

The new mambalgins class of TFTs has been characterized as potent, rapid and reversible inhibitors of ASICs, based on studies with the protein from African black mamba (*Dendroaspis polylepis*) venom<sup>[21]</sup>. The mambalgins are composed of 57 amino acids and eight cysteine residues, and have about 50% amino acid sequence identity to other snake TFTs. While mambalgins were found to be nontoxic in mice, they were found to exert a potent analgesic effect, as strong as that of morphine but causing much less tolerance than morphine and no respiratory distress. Pharmacological studies showed that mambalgins produce their analgesic effect through the blockade of heteromeric channels containing ASIC1a and ASIC2a subunits in CNS and of channels including ASIC1b subunit in nociceptors. Mambalgins were also shown to inhibit heteromeric channels including ASIC1a and ASIC1b subunits, homomeric rodent and human ASIC1a channels and homomeric rodent ASIC1b channels, the  $IC_{50}$ s being in the range from 11 nmol/L to 252 nmol/L<sup>[22,23]</sup>.

The structure of an ASIC1a-mambalgin-1 complex was determined by cryoelectron microscopy at a resolution of 5.4 Å<sup>[24]</sup>. The data obtained showed that mambalgin-1



binds precisely to the thumb domain of ASIC1a but not to the acid-sensing pocket, as suggested earlier<sup>[25]</sup>. However, mambalgins-1 binding induced conformational changes in the thumb domain of the channel, which may disturb the sensing of an acidity in ASIC1a<sup>[24]</sup>. The structural data obtained might provide a structural basis for further development of ASIC modulators.

No less significant than the discovery of mambalgins was the finding of TFTs that interact with ionotropic GABA receptors (GABA<sub>A</sub>). Almost simultaneously, three research groups found that snake TFTs were able to bind GABA<sub>A</sub> receptors<sup>[26–28]</sup>. Thus, two TFTs, called micrurus toxin 1 (MmTX1) and 2 (MmTX2), were isolated from Costa Rican coral snake (*Micrurus mipartitus*) venom and sequenced<sup>[26]</sup>. It was shown that at subnanomolar concentrations MmTX1 and MmTX2 increased receptor affinity for the agonist by binding to allosteric site, and thus potentiated opening and macroscopic desensitization of the receptor. The authors suggested that at the molecular level, the  $\alpha + \beta$ - subunit interface might be involved in toxin action. When injected into mouse brain, both toxins evoked seizures against the background of reduced basal activity<sup>[26]</sup>. The discovery of toxins enhancing GABA<sub>A</sub> receptor sensitivity to agonist established a new class of ligands for this receptor family.

In 2006, it was shown that  $\alpha$ -Bgt, a classical blocker of  $\alpha 7$  and muscle-type nAChRs, binds to and blocks GABA<sub>A</sub> receptors containing the interface of  $\beta 3/\beta 3$  subunit<sup>[29]</sup>. No effects were observed for  $\alpha$ -Bgt on heterooligomeric GABA<sub>A</sub> receptors which contain  $\alpha$ -,  $\beta$ - and  $\gamma$ -subunits or  $\alpha$ -,  $\beta$ - and  $\delta$ -subunits. However, recently, two research groups independently showed that  $\alpha$ -Bgt and some other TFTs could bind to recombinant and native GABA<sub>A</sub> receptors<sup>[27,28]</sup>. Both electrophysiology experiments and fluorescent measurements with  $\alpha$ -Bgt coupled to Alexa-Fluor 555 revealed the highest toxin affinity to  $\alpha 2\beta 2\gamma 2$  receptor subtype<sup>[27]</sup>. GABA reduced fluorescent labeling by  $\alpha$ -Bgt, suggesting that the  $\alpha$ -Bgt binding site overlaps the GABA binding site at the interface of  $\beta/\alpha$  subunits<sup>[27]</sup>.

Binding at the  $\beta/\alpha$  subunit interface was demonstrated for the long-chain  $\alpha$ -neurotoxin  $\alpha$ -CTX<sup>[28]</sup>, and this toxin interacted more efficiently with the GABA<sub>A</sub> receptor than  $\alpha$ -Bgt. Electrophysiology experiments showed mixed competitive and noncompetitive  $\alpha$ -CTX action, with highest affinity of this toxin being to the  $\alpha 1\beta 3\gamma 2$  receptor ( $IC_{50}$  236 nmol/L). Other receptor subtypes were inhibited less potently, as follows:  $\alpha 1\beta 2\gamma 2 \approx \alpha 2\beta 2\gamma 2 > \alpha 5\beta 2\gamma 2 > \alpha 2\beta 3\gamma 2$  and  $\alpha 1\beta 3\delta$ . Among the several TFTs studied, the long  $\alpha$ -neurotoxins Ls III (*Laticauda semifasciata*) and neurotoxin I (*Naja oxiana*) as well as the nonconventional toxin WTX (*Naja kaouthia*) interacted with the GABA<sub>A</sub> receptor. These data demonstrate that GABA<sub>A</sub> receptors are a target for diverse TFTs, including the very well-studied  $\alpha$ -Bgt and  $\alpha$ -CTX.

Among the vast variety of TFTs there is a class of toxins that interact with mAChRs, which are G-protein coupled receptors (GPCRs)<sup>[30]</sup>. For many years, the mAChRs were thought to be the only GPCRs affected by TFTs; however, over the last decade, several TFTs capable of interacting with other GPCRs, namely adrenoceptors of different types, were reported.

It was shown that muscarinic toxin  $\alpha$  (MT $\alpha$ ) was a more potent antagonist for the  $\alpha_{2B}$ -adrenoceptor than for mAChR<sup>[31]</sup>. MT $\alpha$  inhibited the  $\alpha_{2B}$ -adrenoceptor, but did not affect the  $\alpha_{2A}$ -,  $\alpha_{2C}$ -,  $\alpha_{1A}$ - or  $\alpha_{1B}$ -adrenoceptors. In ligand binding experiments, MT $\alpha$  superseded the radioligand efficiently ( $IC_{50}$  3.2 nmol/L) and decreased the maximum binding without any influence on the radioligand affinity, demonstrating a noncompetitive inhibition mode<sup>[31]</sup>. The study of another MT, MT $\beta$ , showed nonselective low affinity interaction with the five muscarinic receptor subtypes<sup>[32]</sup>. Study of the toxin CM-3 (having undefined biological function to date) and MT $\beta$  demonstrated high efficacy for  $\alpha$ -adrenoceptors and particularly a subnanomolar affinity for the receptor of  $\alpha_{1A}$ -subtype<sup>[32]</sup>. Both toxins were isolated more than 20 years ago from the venom of the African mamba *Dendroaspis polylepis*<sup>[33,34]</sup>. No or very weak affinity of these toxins were found for muscarinic receptors in the work of Blanchet *et al*<sup>[32]</sup>.

Targeted searches for toxins interacting with  $\alpha$ -adrenoceptors have yielded novel information in the last decade. The interactions of fractions obtained from green mamba (*Dendroaspis angusticeps*) venom with  $\alpha_1$ -adrenoceptors were tested in binding experiments using <sup>3</sup>H-prazosin as a radioligand<sup>[35]</sup>. A new TFT inhibitor, AdTx1 (renamed later as  $\rho$ -Da1a<sup>[36]</sup>), comprising 65 amino acid residues with four disulfide bridges, was found.  $\rho$ -Da1a showed subnanomolar affinity with  $K_i$  of 0.35 nmol/L and demonstrated high specificity for the human adrenoceptor of  $\alpha_{1A}$ -subtype. Interestingly, the biological activity profile of  $\rho$ -Da1a appeared very similar to those of MT $\beta$  and CM-3; however, these latter two toxins interacted more potently than  $\rho$ -Da1a with  $\alpha_{1B}$ - and  $\alpha_{1D}$ -adrenoceptor subtypes<sup>[32]</sup>.  $\rho$ -Da1a was, thus, characterized as a specific and selective peptide inhibitor for the  $\alpha_{1A}$ -adrenoceptor, acting as a potent relaxant of smooth muscle<sup>[35]</sup>.

Using a similar targeted screening approach, but with application of <sup>3</sup>H-

rauwolscine as a radioligand, the effects of venom fractions obtained from green mamba on  $\alpha_2$ -adrenoceptors from rat brain synaptosomes were studied<sup>[37]</sup>. A novel TFT,  $\rho$ -Da1b, comprising 66 amino acid residues with four disulfide bridges was isolated. It inhibited binding of  $^3\text{H}$ -rauwolscine to the three  $\alpha_2$ -adrenoceptor subtypes by 80% with affinity in the range of 14-73 nmol/L and with Hill coefficient of about unity. Furthermore, calcium imaging experiments on human  $\alpha_{2A}$ -adrenoceptors expressed in mammalian cells showed that  $\rho$ -Da1b was an antagonist of this adrenoceptor type<sup>[37]</sup>.

The structural scaffold of aminergic TFTs that are known to interact with various  $\alpha$ -adrenergic, muscarinic and dopaminergic receptors was used to generate experimental toxins with new functions. Specifically, the ancestral protein resurrection methodology was applied to identify the functional substitutions that might happen during evolution, and then utilize them for molecular design<sup>[38]</sup>. Six variants of ancestral toxin (AncTx, 1-6) were generated, and their biological activity was studied. AncTx1 was found to be the toxin possessing to date the highest selectivity to  $\alpha_{1A}$ -adrenoceptor. AncTx5 was the strongest inhibitor for  $\alpha_2$ -adrenoceptor of the three subtypes. The toxin  $\rho$ -Da1a affinities for the  $\alpha_1$ - and  $\alpha_{2C}$ -adrenoceptor subtypes were modulated most strongly by amino acids at positions 28, 38 and 43 in the evolutionary pathway<sup>[38]</sup>. Thus, this molecular engineering study represents the first successful attempt to engineer more potent aminergic TFTs.

Among the snake venoms, the mamba ones are unique in their variety of toxins affecting signal transductions<sup>[39]</sup>. A multitude of toxins capable of disturbing the different stages of cholinergic and adrenergic (see above paragraphs) transmission have been isolated from these venoms. Several toxins affecting voltage-gated ion channels have been isolated as well. The very recently discovered TFT Tx7335, in eastern green mamba *Dendroaspis angusticeps* venom, interacts with the KcsA potassium channel<sup>[43]</sup>. The unusual structure of this toxin was discussed above. Interestingly, Tx7335 is a channel activator but not an inhibitor, as evidenced by its ability to increase in a dose-dependent mode both mean open times and open probabilities of KcsA incorporated in artificial bilayers; yet, the Tx7335 binding site on KcsA is distinct from that of the canonical pore-blocker toxins. The authors of this study suggested that the toxin allosterically reduced inactivation of KcsA that results in increase of potassium flow through the channel<sup>[43]</sup>.

Blue coral snake *Calliophis bivirgatus* belong to the Elapidae family of snakes the neurotoxic venoms of which typically produce the flaccid paralysis. However it was shown that the *C. bivirgatus* venom uniquely produced spastic paralysis<sup>[40]</sup>. The toxin producing this paralysis was isolated and called calliotoxin (protein name:  $\delta$ -elapitoxin-Cb1a). Although calliotoxin is a TFT, it has low amino acid sequence similarity to the other known toxins. It comprises 57 amino acid residues with four disulfide bridges in the classical scaffold. Biological activity studies using HEK293 cells heterologously expressing  $\text{Na}_v1.4$  showed that the voltage-dependence of channel activation was shifted to more hyperpolarized potentials by calliotoxin. It inhibited inactivation and produced significant ramp currents. These data conformed with profound effects of calliotoxin on contractile force in preparation of isolated skeletal muscle. Thus, calliotoxin represents a functionally novel class of TFTs and is the first activator of voltage-gated sodium channel purified from snake venoms<sup>[40]</sup>.

### Novel TFTs affecting blood coagulation

TFTs affecting blood coagulation are not so numerous as those affecting signal transduction. Nevertheless, a new member of the TFT family that is capable of influencing different stages of blood coagulation appeared recently. TFTs inhibiting both primary and secondary hemostasis have been reported.

Primary hemostasis involves platelets, which immediately form a plug at the site of injury. A novel TFT which inhibits the human platelet aggregation process in a dose-dependent manner was purified from cobra *Naja kaouthia* venom and named KT-6.9<sup>[41]</sup>. KT-6.9 was shown to inhibit platelet aggregation induced by adenosine diphosphate (ADP), thrombin and arachidonic acid but not by collagen and ristocetin. It was 25-times more active than the antiplatelet drug clopidogrel. Based on the data showing significant inhibition (70%) of the platelet aggregation induced by ADP, the authors suggested toxin binding to ADP receptors located on the platelet surface<sup>[41]</sup>.

As for secondary hemostasis, two TFTs capable of inhibiting the extrinsic tenase complex (ETC) were purified from the venom of African ringhals cobra *Hemachatus haemachatus*<sup>[42,43]</sup>. ETC activates conversion of factor X (FX) to factor Xa (FXa) and represents an important target for the development of novel anticoagulants. A novel TFT anticoagulant, ringhalexin (the ringhals extrinsic tenase complex inhibitor) was shown to inhibit FX activation with an  $\text{IC}_{50}$  of 123.8 nmol/L<sup>[42]</sup>. As an inhibitor of mixed type, on chick biventer cervicis muscle preparations ringhalexin manifested an irreversible weak neurotoxicity. The amino acid sequence of ringhalexin is 94%

identical to that of NTL2, an uncharacterized neurotoxin-like protein from *Naja atra*. X-ray crystallography of ringhalexin revealed a typical three-finger structure stabilized by four conserved disulfide bridges<sup>[42]</sup>.

Another novel anticoagulant TFT from *Hemachatus haemachatus* venom, called exactin, can specifically and potently inhibit the activation of FX by ETC (IC<sub>50</sub> 116.49 nmol/L), similar to ringhalexin<sup>[43]</sup>. It is also a mixed-type inhibitor of ETC and weakly inhibits FX activation by intrinsic tenase complex (IC<sub>50</sub> 4.05 μmol/L) and prothrombin activation by prothrombinase complex (IC<sub>50</sub> 17.66 μmol/L). In contrast to other TFT anticoagulants that are structurally similar to snake cytotoxins, exactin manifests structural similarity to postsynaptic neurotoxins. It also has 82% identity to the weak toxin CM1b from *H. haemachatus* venom and 58% identity to a number of *Ophiophagus hannah* neurotoxins, including the Ω-neurotoxin Oh9-1 discussed above.

### Novel TFTs with unexpected biological activities

The last decade has also seen the discovery of several new TFTs possessing quite unusual biological activities.

TFTs, being structurally well defined, thermally stable and resistant to proteolysis, are very good subjects for directed evolution. When a randomization scheme was applied to α-neurotoxin amino acid residues in the loops involved in binding with nAChRs, followed by the cDNA display screening method, new modulators of the interleukin-6 receptor (IL-6R) were obtained<sup>[44]</sup>. The proteins obtained possessed nanomolar affinity and high specificity for IL-6R. The IL-6-dependent cell proliferation assay revealed both antagonists and agonists in the protein pool. Application of the size minimization procedure resulted in proteins with the molecular mass of about one-third of the original toxin; no significant loss of activities was observed. Moreover, the loops important for function were identified<sup>[44]</sup>. In another work by the same group, directed evolution was applied to produce a trypsin inhibitor based on the TFT scaffold<sup>[45]</sup>. The DNA sequences converged after seven rounds of selection. The recombinant proteins obtained were good inhibitors of trypsin (K<sub>i</sub> of 33-450 nmol/L). Three groups of proteins had K<sub>i</sub> values close to those of soybean trypsin inhibitor and bovine pancreatic trypsin inhibitor. Two proteins inhibited chymotrypsin and kallikrein as well. The authors suggested that the technique developed may be widely applied for the targeted generation of different regulatory molecules based on the TFT motif<sup>[45]</sup>.

Studies of a TFT cardiotoxin showed a quite unexpected effect on insulin secretion. The fractions of cobra *Naja kaouthia* venom obtained by combination of ultrafiltration and reversed-phase high-performance liquid chromatography were screened for insulinotropic activity using the rat INS-1E β-cell line<sup>[46]</sup>. Only one fraction of the total 22 obtained induced secretion of insulin from the INS-1E cells with no influence on cell integrity and viability. Liquid chromatography-tandem mass spectrometry analysis revealed that this fraction represented the cardiotoxin-I (CTX-I) isolated earlier from *Naja kaouthia* venom. Analysis of the isolated CTX-I toxin in INS-1E cells showed that its insulin stimulation ability persisted even in the absence of glucose. In contrast to typical cobra cardiotoxin, CTX-I did not induce direct hemolysis of human erythrocytes and showed no potent vasoconstriction capability. Based upon this toxin, a truncated analogue [Lys(52)CTX-I(41-60)] was obtained by structure-guided modification<sup>[47]</sup>. This analogue showed insulinotropic activity similar to CTX-I and appeared to exert its action through K<sub>v</sub> channels<sup>[47]</sup>. As such, it may serve as a basis for the design of new therapeutic agents for the treatment of type 2 diabetes (Table 1).

A new paradoxical TFT, nakoroxin, was isolated from the cobra *Naja kaouthia* venom<sup>[48]</sup>. Nakoroxin belongs to the group of orphan TFTs (group "XX"), the biological activities of which are practically unknown. Nakoroxin was not cytotoxic to rat pheochromocytoma PC12 cells nor to human lung carcinoma HT1080 cells. It did not inhibit the binding of α-Bgt to α7 or muscle-type nAChRs, but potentiated the binding of α-Bgt to the acetylcholine-binding protein from *Lymnaea stagnalis*. The reason for this unusual property of nakoroxin is not clear.

Another quite interesting TFT, actiflagelin, was isolated from cobra *Walterinnesia aegyptia* venom by combination of reverse-phase and ion-exchange chromatography<sup>[49]</sup>. Actiflagelin activated *in vitro* motility of sperm from OF1 male mice. The amino acid sequence established by Edman sequencing combined with tandem mass spectrometry analyses showed that the protein comprised 63 amino acid residues with five disulfide bonds, the pattern of which corresponded to that of nonconventional toxins. Actiflagelin had a noticeable homology to buccandin, a nonconventional toxin from *Bungarus candidus* venom<sup>[49]</sup>. The authors suggested that the protein found may have therapeutic potential for cases of infertility when the problem is related to the sperm motility.

## CONCLUSION

TFTs were among the first toxins isolated from snake venoms. At present, they form one of the largest toxin families and their number is increasing constantly. Several TFTs are used as sophisticated pharmacological tools to study the function and structure of their molecular targets. The new TFTs that have emerged recently possess both novel structural and functional characteristics, expanding the possibilities for their future applications. The TFTs discovered during the last decade have good prospects to be transformed into novel drugs. For example, mambalgins are perfect candidates for the design of powerful analgesics, and on the basis of CTX-I possessing insulinotropic activity, new therapeutics for the treatment of diabetes may be created. The data presented in this minireview (Table 1) show that TFTs continue to be important and promising for both basic science and medicine.

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