Back to basics with receptors and ligands – molecules, mechanisms and medicine

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1. General introduction

Drug discovery is a complex, substantially long, technology-driven, labour-intensive and inordinately expensive process involving target discovery and validation, lead identification by high-throughput screening, and lead optimization by medicinal chemistry, preclinical evaluation in animal models, pharmacological screening (ADME screen: administration, distribution, metabolism, elimination) and studies of toxicology, specificity, and drug interactions[1,2]. While comprehensive drug discovery workflows are predominantly undertaken in the big pharma domain, drug discovery in academia must not be discounted. Research relevant to drug discovery in academia is generally at the fundamental level, with a focus on drug mechanisms and the identification and validation of potential therapeutic targets, often in commercially unattractive, but relevant therapeutic areas particularly for the developing world, including rare disorders, parasitic diseases, and in research into natural products[3,4]. Investigating natural sources for drug leads include compounds from venomous animals; with illustrious examples of success such as captopril (angiotensin converting enzyme inhibitor for the treatment of hypertension), exenatide (a glucagon-like peptide-1 receptor agonist for the treatment of type 2 diabetes) and ziconotide (an N-type calcium channel blocker for intractable pain)[5,6].

Drug discovery in academia is usually associated with identification of drug targets and mechanisms, often with the overarching goal of progressing to proof-of-concept studies and translational research with commercial viability – the so-called 'bench to bedside' vision. Nevertheless, outcomes such as development of probe molecules that can serve as research or diagnostics tools to dissect molecular mechanisms or pathophysiological pathways, or contribution to the structural and functional characterization of receptors, ion channels, enzymes and other molecular targets are also critical for drug discovery[2,7].

This oration paper outlines an overview of two decades of research in the academia, focusing on the neurotransmitter-gated family of ion channel receptors, specifically the nicotinic acetylcholine receptor (nAChR); investigating both, novel ligands and drugs for the receptor, as well as characterizing their binding sites within the receptor. Collectively, these studies have made substantial contributions of clinical, pharmacological and neurobiological significance.

2. Methods

2.1 Experimental procedures for identification of drug binding sites within nicotinic acetylcholine receptors

Membranes rich in nAChRs were isolated from *Torpedo californica* (Pacific electric ray) electric organs as described previously[8]. Photo-reactive derivatives of the general anaesthetic drug etomidate were chemically synthesized[9-11] and their effects on the equilibrium binding of tritium ($[^3H]$)labelled acetylcholine or non-competitive nAChR antagonists, $[^3H]$tetracaine, or $[^3H]$phencyclidine, to nAChR-rich membranes were studied using radio-ligand binding assays[12-14]. Two-electrode voltage clamp (TEVC) electrophysiology was used to study the effects of etomidate derivatives on *Torpedo* $\alpha_2\beta\gamma\delta$ nAChRs expressed in *Xenopus* oocytes[10,12]. Photolabelling of nAChRs

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with photo-reactive drugs was carried out as described[10,11,14]. In brief, nAChR-rich Torpedo membranes were incubated with [^{3}H]-labelled photo-reactive etomidate, irradiated for 30 min at 360 nm and the photolabelled membranes were solubilized and separated by SDS-polyacrylamide gel electrophoresis into nAChR α, β, γ, and δ subunits. The incorporation of ^{3}H into individual nAChR subunits was quantified by liquid scintillation counting of excised gel slices containing the polypeptide bands of the nAChR subunits. The gel bands containing the α, β, γ, and δ subunits were passively eluted and the proteins digested with proteolytic enzymes. The digests of the nAChR α, β, γ, and δ subunit proteins were fractionated and retrieved by reverse-phase HPLC. Amino acid sequencing was performed on each peptide fragment and the product of each amino acid cycle was collected for scintillation counting to determine the specific amino acid(s) into which there was incorporation of ^{3}H, representing photolabelling by the drug. Based on the data generated, a homology model of the *Torpedo californica* nAChR was constructed using the Accelerys Discovery Studio software to visualise the binding of the drug within the nAChR.

2.2 Experimental procedures for the isolation, purification, characterisation and structure-function analyses of novel scorpion toxins

Studies on novel toxins from the Malaysian black scorpion *(Heterometrus spinifer)* utilized venom extracted from scorpions housed at the National University of Singapore. Pooled scorpion venom was subjected to ultrafiltration, and the ultrafiltrate of *Mr* < 5000 subjected to a single-step, reverse-phase HPLC to isolate and purify novel toxins to homogeneity, as verified by capillary electrophoresis as well as analytical HPLC[15,16]. The molecular masses of the purified scorpion toxins were determined by electrospray ionization mass-spectrometry (ESI-MS) or matrix-assisted laser desorption ionization-time of flight mass-spectrometry (MALDI-TOF-MS). Amino terminal sequencing of novel scorpion toxins was done by automated Edman degradation. Given the very low yield of the native toxin in venom, synthetic analogues of the scorpion toxins were synthesized using solid phase methodology with Fmoc chemistry[16,17]. Molecular modelling was carried out using the program MODELER in the Accelrys Discovery Studio software to determine the predicted structure of toxins[16,17]. TEVC electrophysiological studies determined the pharmacological actions of scorpion toxins on voltage-gated ion channels[16,18,19].

2.3 Experimental procedures for the isolation, purification, characterisation and structure-function analyses of novel snake toxins

The isolation and purification of peptide toxins from snake venoms is a well-established, widely utilized and streamlined protocol in our laboratory[20-23]. Briefly, commercially purchased lyophilised snake venom was subjected to multi-stage high performance liquid chromatography (HPLC) – firstly, separation of venom based on the molecular weight of the components, followed by separation of selected protein fractions based on the hydrophobicity of proteins using reverse-phase HPLC. The molecular mass and homogeneity of snake toxins were determined as described above. The structural characterization of novel snake toxins, from primary to quaternary protein structure, was carried out as previously described[22-25], with the amino acid sequence determined by the Edman degradation and the three-dimensional structures determined by x-ray crystallography[22,23] or nuclear magnetic resonance (NMR)[26,27]. The pharmacological characterisation of novel snake toxins was carried out *in vitro*, on isolated tissues in organ bath studies as well as *in vivo*, in anaesthetised rodents employing widely utilised pharmacological techniques in our laboratory[20,21,28,29] and nerve-skeletal muscle preparations that are representative models of the avian and mammalian neuromuscular junctions[30,31]. The pharmacological effects of snake toxins on the contractile responses of the isolated muscle were then investigated to provide a quantitative measure of neurotoxicity and recovery of the muscle from complete neuromuscular blockade was also evaluated[29]. TEVC electrophysiological studies were utilised to identify the specific molecular target(s) of the novel snake toxins[20,22,23,29].

2.4 Ethical considerations

All animal studies were in compliance with the National University of Singapore's and Harvard Medical School's Institutional Animal Care and Use Committee Regulations and the Griffith University Animal Ethics Guidelines (Australia), which conform to the World Health Organization's International Guiding Principles for Animal Research[32].

3.   Results and discussion

3.1 Drug discovery in academia: identification and characterization of drug binding sites within the nicotinic acetylcholine receptor

The excitatory nAChR is a member of the superfamily of neurotransmitter-gated ion channels that also includes the inhibitory GABA$_A$ receptors (GABA$_A$Rs). GABA$_A$Rs and nAChRs are primary molecular targets of drugs widely used in general anaesthesia, psychiatry, neurodegeneration and neurology as well as substances of abuse[33-35]; which elicit their actions by acting as agonists, competitive antagonists, non-competitive antagonists and /or positive allosteric modulators[33,35-37]. The nAChRs and GABA$_A$Rs are composed of five homologous subunits, each with a large amino-terminal extracellular domain where neurotransmitter binding sites are located at subunit interfaces, and a transmembrane domain (TMD) consisting of four α-helices (M1-M4) which associate around a central, selective, ion channel [38,39]. Within the nAChR TMD, amino acids from each M2-helix contribute to the lumen of the channel, which constitutes the binding site of many nAChR non-competitive inhibitors[10], and there are also pockets within each subunit helix bundle and at subunit interfaces that are potential binding sites for allosteric modulators[40].

Positive allosteric modulators of agonist binding represent an important class of therapeutic agents, which enhance the efficacy of endogenous neuro-transmitter signalling while avoiding the prolonged, non-physiological pattern of receptor activation produced by agonists[41-44]. Without reliable structural data pertaining to the receptor, in particular the specific sites for drug binding and interaction, the mechanistic basis for pharmacological modulation of nAChRs and GABA$_A$Rs remains largely unknown[36].

3.1.1 Identification of the binding site for the general anaesthetic drug, etomidate, within the nicotinic acetylcholine receptor

To identify the binding sites in a ligand-gated ion channel for etomidate, an intravenous general anaesthetic, nAChR-rich membranes were photolabelled with a photoactivatable analogue, [3H]azietomidate[10]. In radio-ligand binding assays, both etomidate and azietomidate acted as a non-competitive inhibitor of the nAChR since they inhibited the binding of the non-competitive antagonist [3H]phencyclidine, which is known to bind inside the ion channel. Furthermore, azietomidate and etomidate bind with 10-fold higher affinity to nAChRs in the desensitized state (IC_{50}=70μM) than in the closed channel state. nAChR-rich membranes were photolabelled with [3H]azietomidate, which showed preferential photo-incorporation of [3H]azietomidate into the α and δ subunits of the nAChR. Specifically, the labelled amino acids were identified by Edman degradation to be αGlu-262 and δGln-276 at the extracellular end and δSer-258 and δSer-262 toward the cytoplasmic end of the nAChR ion channel (see Figure 1). These studies revealed the primary binding site for azietomidate, and hence, likely for etomidate, to be within the ion channel of the nAChR[10].
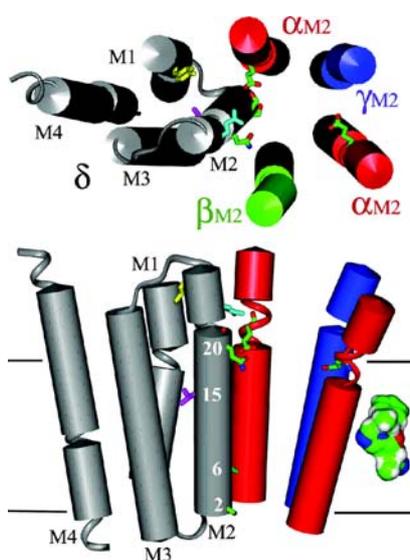

Figure 1. **Binding site in the nicotinic acetylcholine receptor for azi-etomidate, a photoreactive analogue of the general anaesthetic etomidate.** The location of amino acids in the nAChR ion channel photolabelled by [3H]azietomidate is shown in this tube and ribbon representation of the nAChR δ subunit transmembrane domain and the M2 α-helices of the other subunits that form the lumen of the ion channel are shown, as viewed from the extracellular domain (top panel) and in cross-section (bottom panel, extracellular surface up). Shown in green are αGlu-262 (αM2-20) and δSer-258 (δM2-2), δSer-262 (δM2-6), and δGln-276 (αM2-20), which are labelled by azietomidate. Shown in magenta is αM2-15, a position in the GABA$_A$R δ subunit identified as an etomidate affinity determinant. Shown in yellow is δTyr-228, in δM1, which is labelled by [14C]halothane. Azietomidate, which measures 13 Å in an extended conformation, is shown in a space-filling representation for comparison of dimensions (to the right in the bottom panel).

3.1.2 Identification of binding sites for positive allosteric modulators within the nicotinic acetylcholine receptor

TDBzl-etomidate (4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzyl-1-(1-phenylethyl)-1H-imidazole-5-carboxylate), an analogue of the general anaesthetic, etomidate, was chemically synthesized[9]. Interestingly, in electrophysiological studies, TDBzl-etomidate acted as a positive allosteric nAChR modulator, rather than an inhibitor like etomidate[12]. TDBzl-etomidate (>10 μM) increased the submaximal response of nAChRs to acetylcholine in a concentration-dependent manner, with a 2.5-fold increase seen at 60 μM. At higher concentrations, TDBzl-etomidate inhibited the binding of non-competitive nAChR antagonists [³H]tetracaine and [³H]phencyclidine (IC$_{50}$ = 0.8 mM).

nAChR-rich membranes were photolabelled with [³H]TDBzl-etomidate, and the labelled amino acids identified by Edman degradation[11] to be in the ion channel at positions M2-9 (δLeu-265), M2-13 (αVal-255, αVal-269), and M2-10 (αSer-252). In addition, there was labelling in δM3 (γMet-299), but not in M3, and labelling at the lipid interface (αCys-412 and αMet-415). These data reveal that TDBzl-etomidate binds at two distinct sites – within the lumen of the ion channel (labelling of M2-9 and 13), an inhibitory site for nAChR; and at the interface between the   and   subunits (labelling of αM2-10 and Met-299), likely to be a site for positive allosteric modulation of nAChRs (see Figure 2). This was the first report of the detailed characterization of a positive allosteric site within nAChRs, with implications for drug design for diseases underpinned by impaired cholinergic function (e.g. Alzheimer's disease).
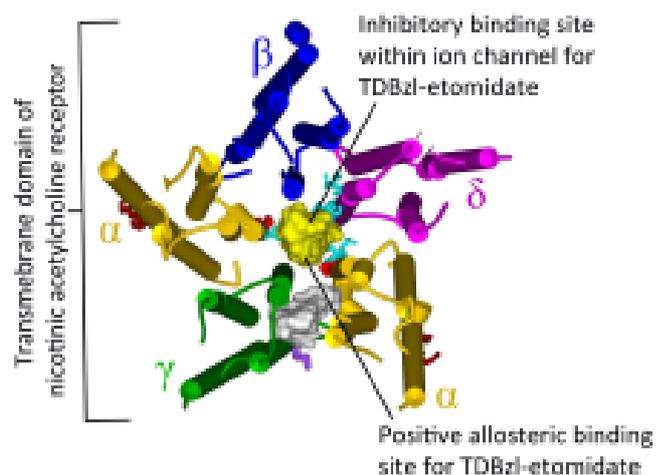

Figure 2. **Binding sites in the nicotinic acetylcholine receptor for the positive allosteric modulator (TDBzl-etomidate)**. Three-dimensional structural model of the of the transmembrane domain of the *Torpedo californica* nicotinic acetylcholine receptor (subunits α, gold; β, blue; γ, green; δ, magenta) from perspective of looking down into the ion channel from the extracellular side. Amino acid residues photo-labelled by [³H]TDBzl-etomidate are shown in stick format, color-coded for αM2-10 (red), M2 channel lumen (cyan), γM3 (purple), and αM4 (brown). The binding site for TDBzl-etomidate within the ion channel lumen (inhibitory site; occupied at high concentrations) is shown in yellow; or in a novel binding pocket between the γ and α subunits (positive allosteric site; occupied at low concentrations) are shown in Connolly surface representations.

3.1.3 Identification of binding sites for partial agonists in the nicotinic acetylcholine receptor

APFBz-choline (4-azido-2,3,5,6-tetrafluorobenzoylcholine), a photoreactive analogue of benzoylcholine, a partial agonist of nAChRs, was synthesised and its pharmacology and binding site within the nAChR deciphered using electrophysiology and photo-labelling[11]. In TEVC experiments, APFBz-choline acted as a low-efficacy partial agonist, eliciting maximal responses that were 0.3 - 0.1% of that of acetylcholine for nAChRs. Equilibrium binding studies of [³H]APFBz-choline with nAChRs revealed that it bound to the acetylcholine-binding site (affinity of Keq = 12 μM). Photolabelling experiments further supported the binding of APFBz-choline to the acetylcholine-binding site of the receptor, since incorporation of [3H]APFBz-choline into the nAChR was inhibited in the presence of an agonist. Photolabelled amino acids in the agonist binding sites were identified to be γLeu-109/δLeu-111, γTyr-111, and γTyr-117 in binding site segment E as well as αTyr-198 in α subunit binding site segment C of the acetylcholine-binding site (see Figure 3)[11]. Insight into partial agonist binding within nAChRs in the context of its pharmacology is useful for new therapeutic interventions, such as for nicotine addiction and cognitive decline[45,46].
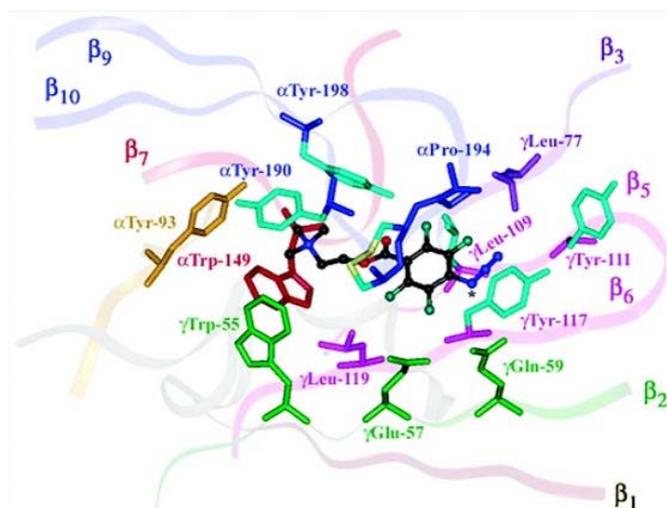
Figure 3. **Binding site for the partial agonist APFBz-choline in the nicotinic acetylcholine receptor.** Three-dimensional structural model of the extracellular region of the *Torpedo californica* nicotinic acetylcholine receptor showing the binding site for APFBz-choline, located within the acetylcholine (agonist) binding site located at the α-γ subunit interface. APFBz-choline is shown as a ball-and-stick structure with atom types coloured as follows: black for carbon, blue for nitrogen, red for oxygen, and teal for fluorine. The amino acid residues of the receptor contributing to the binding of APFBz-choline are shown in stick representation with their colours corresponding to the different agonist site segments (ribbons): segment A in gold, segment B in red, segment C in blue, segment D in green, and segment E in magenta.

3.2 Drug discovery in academia: discovery of novel lead molecules from scorpion venoms

Voltage-gated potassium (K⁺) ion channels are present in a wide variety of cells and play a key role in electrical excitability, cell proliferation, apoptosis, and volume regulation[47,48]. Of clinical interest is the demonstration that Kv1.3 channels are widely expressed by the autoreactive memory T lymphocytes, believed to be associated with autoimmunity; suggesting that Kv1.3 channels maybe valid targets for diagnoses and treatment of autoimmune disorders[48,49]. Furthermore, Kv1.3 channels have also been considered as a potential molecular target for the diagnostics and therapy of some cancers[50]. A multitude of scorpion toxins targeting K⁺ channels, with diverse pharmacological selectivity for various K⁺ channels have been characterized[51,52]. These toxins typically consist of 23-43 amino acid residues and are classified into four subfamilies, of which α-KTx is the largest subfamily that shares a common cysteine-stabilized α/β motif[53]. Given the high specificity and affinity of scorpion toxins for K⁺ and other ion channels, their potential as lead candidates for drug discovery for ion channelopathies has been widely recognized[5,52,54].

3.2.1 Refining the structural determinants of peptide toxins for interacting with voltage-gated potassium channels

A new family of K⁺ channel toxins (designated κ-KTx) with a novel "xbi-helical" protein scaffold was discovered from the Indian black scorpion *Heterometrus fulvipes* venom in our laboratory[55,56]. κ-Hefutoxin-1 (κ-KTx1.1), the first example of this family, had a protein structure that included amino acids Y5 and K19, considered to be the minimum functional dyad required for peptide toxins to bind and block K⁺ channels[17,57]. Accordingly, κ-hefutoxin-1 was investigated and found to block Kv1.2 (IC$_{50}$ ~ 40 µM) and Kv1.3 (IC$_{50}$ ~ 150 µM) channels[55].

Subsequently, κ-KTx1.3, a scorpion toxin that shares ~60% identity with κ-hefutoxin 1, was isolated from the Malaysian black scorpion *H. spinifer venom*[15,16]. Interestingly, despite the presence of the functional dyad (Y5 and K19), κ-KTx1.3 failed to reproduce the K⁺ channel blocking activity of κ-hefutoxin-1. Since the dyad lysine in κ-KTx1.3 was flanked by another lysine (K20) (see Figure 4), it was hypothesized that this additional positive charge could hinder the critical electrostatic interactions known to occur between the dyad lysine (K19) and the K⁺ channel selectivity filter. Hence, mutants of κ-KTx1.3, substituting K20 with a neutral (K20A) or a negatively charged (K20E) amino acid were synthesized. κ-KTx1.3 K20E, in congruence with κ-hefutoxin 1, produced blockade of Kv1.2 (IC50 = 37 µM) and Kv1.3 (IC$_{50}$ = 54 µM) but not Kv1.1 channels. κ-KTx1.3 K20A produced blockade of both Kv1.2 (IC$_{50}$ = 37 µM) and Kv1.3 (IC$_{50}$ = 116 µM) and in addition, acquired affinity for Kv1.1 channels (IC$_{50}$ = 111 µM). These data suggest that the presence of an additional charged residue in a position adjacent to the dyad lysine impedes the functional block of Kv1 channels produced by κ-KTx1.3, and may determine selectivity for peptide toxins for K+ channel subtypes[16]. This work exemplifies the rationale and strategy for assigning a distinct pharmacological action to an otherwise inert toxin, highlighting the importance of the amino acids surrounding the putative functional site of a peptide.
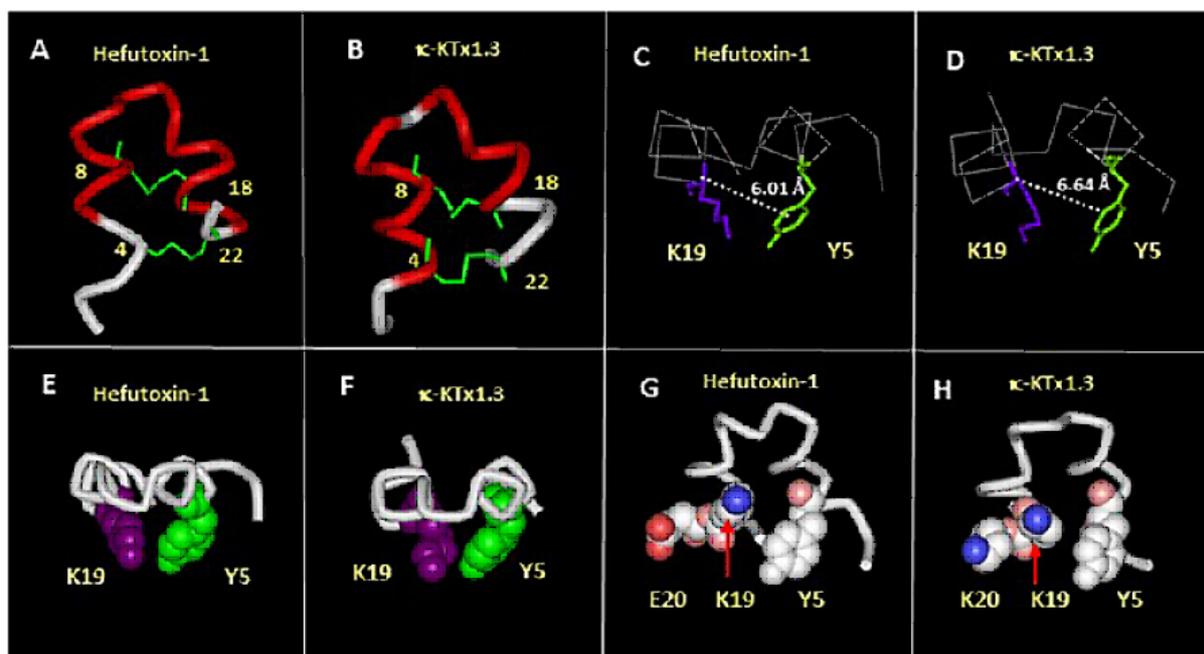
Figure 4. **Comparison of the tertiary structures of κ-KTx 1.1 (κ-hefutoxin) and pharmacology inert homologue κ-KTx 1.3. (Panels A-B)** Comparison of the tertiary structures of κ-KTx 1.3 and κ-hefutoxin 1 showing a compact structure consisting of two parallel α-helices that are held together by the two disulfide bridges (C4-C22 and C8-C18) (shown in green). **(Panels C-D)** The potassium channel blocking activity of κ-hefutoxin 1 was deduced based on the presence of the functional dyad, Y5 and K19, positioned at a distance of 6.01 Å between the α-carbon of the lysine and the center of the aromatic face of the tyrosine (C). κ-KTX1.3 also showed the presence of this dyad (Y5, K19) positioned at a distance of 6.64 Å (D). The α-carbon backbone structure is presented. **(Panels E-F)** In both, κ-hefutoxin 1 (E) and κ-KTX1.3 (F), the dyad residues (shown in Corey-Pauling-Koltun presentation) K19 (violet) and Y5 (green) protrude out of a flat surface formed by the edges of the two parallel α-helices, in conformity with the general architecture of other pore-blocking potassium channel toxins. **(Panels G-H)** The amino acid residue at position 20, Glu20 in κ-hefutoxin 1 (G) and Lys20 in κ-KTX1.3 (H) is also shown in relation to the functional dyad (K19, Y5) in the respective toxins. In (G) and (H), both toxins are shown in similar orientations along the long-axis of the side chain of K19 with the selected residues in Corey-Pauling-Koltun representation. The amino acid residues are coloured according to their charge, with blue and red depicting positive and negative charge, respectively.

3.2.2 Delineating structure-function relationships of a novel scorpion toxin

Spinoxin, a 34-amino acid neurotoxin, was isolated from the Malaysian black scorpion *Heterometrus spinifer* venom, sequenced, and chemically synthesized[58]. Spinoxin (α-KTx6.13), was a potent inhibitor of voltage-gated Kv1.2 (IC_{50} = 2.5 nM) and Kv1.3 (IC_{50} = 63 nM) K^+ channels[18,19]. Spinoxin has four conventionally paired disulfide bridges, like other α-KTx6 subfamily members that share 50-65% sequence similarity with it. Molecular modelling of spinoxin revealed a compact molecule with a cysteine-stabilized α/β-fold, typical of scorpion toxins that target K^+ channels[18].

Utilising the "alanine scanning" approach, 25 analogues of spinoxin were chemically synthesised and the role of each amino acid in the toxin analysed for delineating the active site of the toxin[18]. Alanine replacements at Lys23, Asn26, and Lys30 resulted in loss of activity against Kv1.3 channels, with Lys23 appearing to be the key residue that underpins Kv1.3 channel inhibition. To investigate the role of the individual disulfide bonds in the structure-activity relationship of spinoxin, four analogues in which each pair of cysteine residues was replaced by

alanine residues were synthesised. These studies concluded that three of the disulfide bridges (Cys1-Cys5, Cys2-Cys6, and Cys3-Cys7) were critical for structural stability and function, but interestingly, the analogue lacking Cys4-Cys8 retained both native secondary structure and inhibitory activity on Kv1.3 channels[19].

3.3 Drug discovery in academia: discovery of novel lead molecules from snake venoms

The identification and characterization of the first neurotransmitter protein, the nAChR, was a significant milestone in molecular pharmacology; which was made possible only with the concurrent discovery of α-bungarotoxin, the prototypical snake neurotoxin antagonist of the receptor[59]. Toxins from venomous animals including snakes have evolved to target various nAChR subtypes with cruise missile-like precision. Three-finger α-neurotoxins (α-3FNTxs), which have a characteristic protein fold consisting of three finger-like β-stranded loops converging at a disulfide-rich hydrophobic core, constitute one of the largest families of snake toxins that produce neurotoxicity during envenomation[60,61].

α-3FNTxs are classified into short-chain (60-62 amino acid residues with four disulfide bonds) and long-chain (66-75 amino acid residues with five disulfide bonds) subfamilies. Both bind with high affinity (Kd ~ 10^{-10}-10^{-11} M) to muscle $\alpha 1\beta 1\gamma\delta$ nAChRs, whereas long-chain, but not short-chain α-3FNTxs, are capable of binding with high affinity (K_d = 10^{-8} to 10^{-9} M) to neuronal $\alpha 7$, $\alpha 9$ and $\alpha 9/\alpha 10$ nAChRs[60-62]. Although the interactions of most α-3FNTxs with nAChRs are effectively irreversible, novel α-3FNTxs that produced rapid and complete reversible blockade of muscle nAChRs have been described, including candoxin from the Malayan krait *Bungarus candidus*, which belongs to a new 'non-conventional' class of α-3FNTxs[29,63]. The primary challenge to developing nicotinic acetylcholine receptor-targeted therapeutics is the lack of selective ligands as investigative tools or drug leads, and in this context, the discovery of new snake toxins with novel pharmacology is paramount[34,46,64].

3.3.1 Dimerization of snake neurotoxins as a strategy to broaden the scope of selectivity for nicotinic acetylcholine receptor targets

Although most α-3FNTxs are monomers, a small number exist as dimers[60,61]. κ-Bungarotoxin is a homodimer composed of two non-covalently bound identical long-chain α-3FNTxs, which binds with high affinity to $\alpha 3\beta 2$ (IC_{50} = 3 nM), but not to muscle ($\alpha 1\beta 1\gamma\delta$) nAChRs[65]. We described haditoxin isolated from King cobra *Ophiophagus hannah* venom that was a non-covalent homodimer composed of short-chain α-3FNTxs, which inhibited muscle ($\alpha 1\beta 1\gamma\delta$) ($IC_{50}$ = 0.5 μM) as well as neuronal $\alpha 7$, $\alpha 3\beta 2$ and $\alpha 4\beta 2$ nAChRs (IC_{50} = 0.5 - 2.5 μM)[23]. We have also characterized the first heterodimeric α-3FNTx, irditoxin (from brown tree snake *Boiga irregularis* venom), in which the subunits are covalently-linked by a single intermolecular disulfide bridge, that exhibits potent (IC_{50} = 10 nM) taxa-specific neurotoxicity for avian $\alpha 1\beta 1\gamma\delta$ nAChRs[22].

Recently, we detailed the pharmacological and structural characterization fulditoxin, from American coral snake *(Micrurus fulvius fulvius)* venom[66]. Fulditoxin's crystal structure revealed a non-covalent homodimer of two short-chain α-3FNTxs, which, interestingly, produced potent neuromuscular blockade in avian skeletal muscle (IC_{50} = 20 nM), which was completely reversible, unlike that produced by typical snake α-3FNTxs. Furthermore, fulditoxin produced blockade of human muscle $\alpha 1\beta 1\gamma\delta$ (IC_{50} = 2.5 μM) as well as neuronal $\alpha 7$ (IC_{50} = 7 μM), h$\alpha 4\beta 2$ (IC_{50} = 1.8 μM) and h$\alpha 3\beta 2$ (IC_{50} = 12 μM) receptors, revealing a breadth of nAChR selectivity, which is unusual for short-chain α-3FNTxs that target only muscle nAChRs[67].

Therefore, dimeric α-3FNTxs represent a structurally heterogeneous group of unique snake toxins (see Figure 5) exhibiting different receptor specificities from that expected for their monomeric components. Thus, dimerization presents novel structural conformations for α-3FNTxs to enable interactions with new targets, diversifying the biological activity of snake venom. This opens significant opportunities for identifying new drug targets and the design of research and diagnostic tools as well as therapeutic agents.

Figure 5. **Dimeric three-finger neurotoxins from snake venoms.** Non-covalently linked homodimer – haditoxin (*Opiophagus hannah*; Elapidae) and fulditoxin (*Micrurus fulvius*; Elapidae); Covalently linked heterodimer irditoxin (*Boiga irregularis*; Colubridae) (2H7Z). The figures were generated using the respective PDB structures (accession number indicated in parenthesis) of each toxin. The species and family of the source are indicated. Disulfide bonds are shown in yellow.

4. Conclusions, and clinical and scientific implications

4.1 Aiding drug discovery by delineating the binding sites for positive allosteric modulators, non-competitive antagonists and partial agonists in the nicotinic acetylcholine receptor

Drug discovery for Alzheimer's disease, schizophrenia and pain has turned to positive allosteric modulators of nAChR and GABA$_A$Rs, as means to enhance endogenous neurotransmitter signalling while avoiding the prolonged, non-physiological pattern of receptor activation produced by agonists[41-44,68,69]. In this context, detailed structural data pertaining to the receptor of interest and its binding pockets for pharmacological modulation by positive allosteric modulators is critical for lead identification and optimisation. The identification of a novel lead (TDBzl-etomidate)[9] and the detailed characterization of the positive allosteric modulator binding site in the nAChR[12] has been regarded as a discovery of significance for therapies for neurodegenerative diseases *(Forman S, Massachusetts General Hospital, Boston; Faculty of 1000 in Medicine, Post-Publication Expert Review).* Likewise, detailed information about the binding sites for non-competitive antagonists and partial agonists within the nAChR, and by extrapolation GABA$_A$R, provide valuable information for rational drug design.

4.2 Scorpion toxins as therapeutic leads and molecular probes for potassium channel associated pathologies

Given the role of voltage-gated K$^+$ channels in the pathophysiology of autoimmune disorders and cancer, and their potential as therapeutic targets[48-50], knowledge of the structural and functional determinants by which scorpion toxins interact with K$^+$ channels will help develop therapies targeting K$^+$ channel dysfunction[51,70]. Our studies on Asian scorpion toxins (*Heterometrus* species)[16-19] provide valuable information about the structural determinants of toxins for K$^+$ channel selectivity; and demonstrates that a cyclic mini-peptide, a fifth of the size of the native, toxin is able to replicate the pharmacological selectivity of the parent molecule for K+ channels.

4.3 Understanding molecular mechanisms underpinning snake envenomation syndromes

The WHO has designated snakebite as a neglected tropical disease due to the significant morbidity and mortality following envenomation in Asia, Africa and Latin America[71,72]. Sri Lanka in particular, has one of the highest global incidence rates of venomous snakebites[73-75]. Globally, the venom composition and molecular actions of snake toxins of a majority of clinically relevant species have remained elusive[76,77]. Greater insight into the pharmacology of snake neurotoxins and their interactions with nAChRs provided by the discovery and characterization of new clades of snake toxins including dimeric neurotoxins will enhance our understanding of the molecular basis for their clinical envenoming syndromes, with potential for refining current management practices.

4.4 A novel snake neurotoxin as a molecular probe for labelling neuronal α4β2 nicotinic acetylcholine receptors implicated in neurodegenerative diseases

To the best of our knowledge, fulditoxin is the only reported snake neurotoxin to produce potent (IC_{50} ~1.8 µM) inhibition of neuronal α4β2 nAChRs implicated in the pathophysiology of pain, Alzheimer's and Parkinson's disease. Further elucidation of fulditoxin's structure-function relationships will offer a unique pharmacological probe to localise α4β2 nAChRs in histopathological analysis as well as a research tool for neurobiology and medicine.

4.5 Application of a snake toxin-derived peptide in targeted drug delivery for brain tumours

The blood-brain barrier poses a significant challenge in treating diseases of the central nervous system. In this context, the therapeutic efficacy of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and paclitaxel (PTX) for the treatment of intracranial glioblastoma is suboptimal due to limitations in the penetration of the blood-brain barrier and blood-tumour barrier by drugs[78]. A 16-amino acid long peptide (CDX) was designed and developed from candoxin, a snake neurotoxin with high affinity (IC_{50} = 50 nM) for binding human neuronal α7 receptors[20,29]. CDX was conjugated with TRAIL and paclitaxel loaded micelles, and utilised as a brain-targeted drug delivery system capable of permeating the blood-brain barrier with great efficacy[79, 80]. The anti-glioblastoma effects and *in vivo* bio-distribution observed with this targeted drug-delivery underscores the significant potential of CDX as brain-targeted drug delivery system.

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