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Structure and function of μ -conotoxins, peptide-based sodium channel blockers with analgesic activity

μ -Conotoxins block voltage-gated sodium channels (VGSCs) and compete with tetrodotoxin for binding to the sodium conductance pore. Early efforts identified μ -conotoxins that preferentially blocked the skeletal muscle subtype ($\text{Na}_v1.4$). However, the last decade witnessed a significant increase in the number of μ -conotoxins and the range of VGSC subtypes inhibited ($\text{Na}_v1.2$, $\text{Na}_v1.3$ or $\text{Na}_v1.7$). Twenty μ -conotoxin sequences have been identified to date and structure–activity relationship studies of several of these identified key residues responsible for interactions with VGSC subtypes. Efforts to engineer-in subtype specificity are driven by *in vivo* analgesic and neuromuscular blocking activities. This review summarizes structural and pharmacological studies of μ -conotoxins, which show promise for development of selective blockers of $\text{Na}_v1.2$, and perhaps also $\text{Na}_v1.1,1.3$ or 1.7 .

Currently, there are approximately 128 peptide-derived drugs in various stages of clinical development [1]. In 2012 alone, six peptide drugs received FDA approval, making this class of compounds second only to small-molecule drugs in approvals granted during a given year [1]. Biologics such as peptides are rapidly gaining acceptance as viable therapeutic entities and, as the search to identify new drug leads continues [2], one source of bioactive peptides that has shown particular promise is the complex venom mixtures of predatory organisms. Venoms have evolved over millions of years as efficient mediators of defense, predation and competition. They are of interest to the pharmaceutical industry for their potential therapeutic benefits, resulting largely from the fact that the individual constituents are often highly potent ligands for specific subsets of key therapeutic targets (e.g., cell-surface receptors, ion channels and transporters). Out of the six FDA-approved drugs derived from venoms, four are of peptide origin: eptifibatide [3], bivalirudin [4], ziconotide [5] and exenatide [6]. Furthermore, approximately 20 additional venom-derived peptides are currently at various stages of clinical/preclinical development [7].

The venoms of marine snails of the genus *Conus* constitute an abundant source of neuroactive peptides [8–10]. Cone snails hunt by injection of a venom cocktail containing at least 100–200 bioactive peptides designed to rapidly immobilize prey or defend against predators [11,12]. The complexity of these venoms, combined with the large number of identified *Conus* species so far (500–700 species) [13], highlights the tremendous potential of these venoms as a source of pharmacological tools for the study or even treatment of numerous neurological disorders [14].

Conotoxins are broadly classified into 16 gene ‘superfamilies’ based on the endoplasmic reticulum (ER) signal peptide sequence [15]. Each superfamily is further subdivided according to disulfide bridging framework and/or pharmacological target [15]. The M-superfamily encompasses 10 distinct cysteine frameworks and at least four distinct molecular targets. Within this superfamily are two classes of peptides that inhibit **voltage-gated sodium channels** (VGSCs): the μ -conotoxins, which block Na^+ conductance by direct occlusion of the VGSC pore [16], and the μO -conotoxins, which act as gating modifiers by binding

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to sites on the voltage-sensing domain on the extracellular surface of the VGSC [17,18]. This review will focus on the μ -conotoxins, thus far identified only in the venoms of piscivorous members of *Conus*. They are characterized by a Type III cysteine framework (i.e., CysI-CysIV, CysII-CysV, CysIII-CysVI) [16,19].

The **sodium channel α -subunit (NaV1)** is a large pore-forming protein complex comprised of four homologous domains arranged about a central pore region (D1–D4) [20]. Each domain comprises six membrane-spanning segments (S1–S6) with an extended extracellular looped region (P-loop) connecting segments 5 and 6 (Figure 1) [21]. To date, nine distinct isoforms of the α -subunit (Na_v1.1 – Na_v1.9) have been described in various excitable tissues of the CNS or PNS, where they have been shown to modulate signal transduction in neuronal, cardiac or skeletal muscle cell types (Table 1) [22]. The flux of sodium ions

through VGSCs is responsible for both the initiation of action potentials and propagation of these signals along the length of the axon. Abnormal sodium conductance, through illness or injury, can lead to spontaneous firing of neurons and hyperexcitability, resulting in channelopathies associated with neuropathic pain [23] or other neurological disorders [24]. Channelopathies can also have the opposite effect and result in the inability to perceive painful stimuli in humans [25]. A 2006 *Nature* paper first described how a congenital loss-of-function mutation (i.e., nonsense-codon mutation) in the SCN9A gene that codes for the Na_v1.7 VGSC subtype resulted in an individual's inability to perceive pain [25].

Three VGSC subtypes have demonstrated roles in the transmission of pain signals: Na_v1.3, Na_v1.7 and Na_v1.8 (Table 1) [28,29]. Na_v1.3 expression is significantly increased in various neuropathic pain

Figure 1. Voltage-gated sodium channels structure. (A) Crystal structure of the bacterial sodium channel Na_vAb (PDB code 4EKW). Structure illustrates the four homologous domains of the channel (DI–DIV) arranged around the highly selective pore region through which Na⁺ permeates. (B) Individual domain comprising six membrane-spanning subunits (S1–S6) with the site of action (P-loop site 1) for μ -conotoxins discussed throughout this review [21]. (C) Cartoon of the VGSC α - and β -subunits. Selectivity filter is formed by the looped regions between S5 and S6 (i.e., P-loop). Approximate locations of neurotoxin-binding Sites 1–5 are shown on the α -subunit. Site 1, the location of μ -conotoxin binding, is emphasized. β -subunit crystal structure from Gilchrist et al. (PDB code 4MZZ) [26]. VGSC: Voltage-gated sodium channels.

Table 1. Sodium channel subtypes and their distribution.

Na _v 1-subtype [†]	Distribution	TTX sensitivity
Na _v 1.1	DRG, CNS, Heart	×
Na _v 1.2	DRG, CNS	×
Na _v 1.3 [‡]	DRG, CNS, PNS	×
Na _v 1.4	Muscle	×
Na _v 1.5	Heart	
Na _v 1.6 [‡]	DRG, CNS	×
Na _v 1.7	DRG, SCG	×
Na _v 1.8	DRG	
Na _v 1.9	DRG	

[†]To date, nine Na_v1-subtypes have been identified in the central/peripheral nervous systems or in muscle tissues (skeletal or cardiac). Many subtypes have been observed in the DRG with six of these subtypes being sensitive to block by the alkaloid neurotoxin TTX [20,27].

[‡]In addition to Na_v1.1 and 1.5 (as indicated above, Na_v1.3 and 1.6 have also been observed in cardiac tissue).

DRG: Dorsal root ganglia; PNS: Peripheral nervous system; SCG: Superior cervical ganglion neurons; TTX: Tetrodotoxin.

states, including nerve injury, spinal nerve ligation, postherpetic neuralgia and diabetic neuropathy [30–33]. Na_v1.7 is an essential and nonredundant requirement for nociception in humans [25]. Selective Na_v1.8 mRNA axonal transport and local upregulation may contribute to the hyperexcitability of peripheral nerves in some neuropathic pain states [34]. The Na_v1.9 subtype has also been suggested as a potential target for pain. However, this subtype is localized to the PNS and Na_v1.9 inhibitors may be more useful as treatments of inflammatory rather than neuropathic pain [28,35–36]. Taken together, these results provide support for the use of VGSC inhibitors that modulate neuronal excitability and could prove useful in the development of new analgesic compounds [22,37–40].

The alkaloid neurotoxins tetrodotoxin (TTX) and saxitoxin (STX) are potent inhibitors of VGSCs and were integral in defining neurotoxin binding site 1, a binding site deep inside the VGSC pore within the re-entrant P-loop region between S5 and S6 (Figure 1) [20,41]. However, TTX and STX have shown limited therapeutic potential due to their lack of selectivity among Na_v1 subtypes (TTX-sensitive subtypes include Na_v1.1–1.4, 1.6 and 1.7) [42–44]. Subtypes that are less sensitive to the effects of TTX are called ‘TTX resistant’ (Na_v1.5, 1.8 and 1.9). To mitigate potential adverse side effects arising from the lack of subtype specificity of TTX (e.g., cognitive effects, paralysis, ataxia), clinical investigations of TTX have been limited to focal (intramuscular) administration [37,45]. Several small molecule inhibitors of Na_v1-subtypes have also been described, but often lack target specificity leading to undesirable off-target effects (Figure 2). A potential advantage of peptide-based therapeutics is their intrinsic specificity for certain classes of molecular targets [27,46,47]. This specificity continues to be a

key driver of studies of *Conus* venoms that have led to characterization of the μ -conotoxins.1.

Numerous venom-derived neurotoxins elicit their biological effects through interaction at discrete sites within the α -subunit of the VGSC [48] (Figure 1C). Venom peptides have been shown to act at Site 1 (μ -conotoxins and nonpeptidic guanidinium toxins), Site 3 (scorpion α -toxins and anemone toxins), Site 4 (scorpion β -toxins, spider β -toxins and μ O-conotoxins) and Site 6 (δ -conotoxins), while Sites 2 and 5 are targeted predominantly by small organic neurotoxins such as the batrachotoxins and brevenoxins [49]. Peptide components of non-*Conus* origin have also been shown to block Na_v1, though the site of action of many of these toxins has yet to be fully defined. Among these are Tx1, hainantoxin-I and ProTx-II (which is of interest because of its subtype selective block of Na_v1.7) [39–40,50].

The μ -conotoxins are employed as paralytic tools by fish-hunting marine gastropods from the genus *Conus*. Over the nearly three decades since the first reports of μ -conotoxins isolated from the venom of

Key terms

Conotoxins: Neuroactive peptides isolated from the venoms of marine snails of the genus *Conus*, usually 10–30 amino acids in length and often cross-linked by one or more disulfide bridges.

Voltage-gated sodium channel: Membrane-spanning ion channel responsible for initiating and propagating action potentials in excitable tissues.

Sodium channel α -subunit (Na_v1): Na_v1-subtypes have four transmembrane spanning domains arranged around a central pore through which Na⁺ ions can permeate. The α -subunit is the minimum structure required for a functional voltage-gated sodium channel.

Conus geographus (μ -GIIIA/B/C), which preferentially blocked muscle subtypes, 17 distinct μ -conotoxins have been identified (Figure 3). More recently, μ -conotoxins with preference for neuronal subtypes were reported [51]. These peptides modulate the activity

of Na_v 1-subtypes by binding at the outer vestibule at Site 1 of the sodium channel pore, in many cases with submicromolar affinities [52,53]. This review summarizes the efforts to define the structural features of μ -conotoxins responsible for their potency and selectivity for Na_v 1-subtypes as a basis for the development of both novel pharmacological tools and potential therapeutics.

Compound	Molecular Target(s)
 4,9-anhydro-tetrodotoxin	Na_v 1.2–1.8 Others: N/A
 1-benzazepin-2-one	Na_v 1.5, 1.7 & 1.8 Others: Ca_v 1-channels
 Imidizopyridine	Na_v 1.7 & 1.8 Others: GABA receptors
 Mexiletine [†]	Na_v 1.4 Others: Ca_v 1-channels
 Lamotrigene [†]	Na_v 1.5, 1.7 & 1.8 Others: NaChRs
 Carbamazepine [†]	Na_v 1.1–1.8 Others: GABA receptors, hepatic enzymes, etc.

Figure 2. Examples of small molecule inhibitors of voltage-gated sodium channels.

[†]Indicates clinically used voltage-gated sodium channels.

Data taken from [27].

Muscle-subtype preferring μ -conotoxins

μ -Conotoxins from *Conus geographus*

The first μ -conotoxins to be isolated and characterized were μ -GIIIA and its congeners μ -GIIIB and μ -GIIIC, from the venom of *C. geographus* (Figure 4) [54]. Early work showed, for the first time, that μ -conotoxin GIIIA could discriminate between sodium channels isolated from muscle ($K_d = 25$ nM) and nerve ($K_d \sim 11,000$ nM) preparations [54,55]. This information was among the first to suggest that discrete VGSC isoforms existed within different excitatory tissue types. Recent work by Wilson *et al.* [53] established the selectivity profile for μ -GIIIA in *Xenopus* oocytes expressing rat or mouse Na_v 1-subtypes (Figure 5). Consistent with previous results, μ -GIIIA showed the highest potency for the skeletal muscle subtype Na_v 1.4 (0.019 μM), followed by the neuronal subtypes Na_v 1.1 (0.26 μM), Na_v 1.6 (0.68 μM) and Na_v 1.2 (17.8 μM) [53]. μ -GIIIA is a highly basic, 22 amino acid residue peptide that is stabilized by three disulfide bridges (Cys3–Cys15; Cys4–Cys20; Cys10–Cys21). Ala-replacement studies showed that the most critical residues for VGSC blockade are localized in the C-terminal half of the peptide (Arg13, Arg19, Hyp17 and Lys16) (Figure 6) [56]. The solution structure of the μ -GIIIA[R13A] mutant showed that these residues were located on the same face of the molecule, suggesting that this region of the peptide may interact directly with the channel [57]. The structure of μ -GIIIA also revealed the close proximity of the hydroxyl group of Hyp17 to the guanidino group of Arg13, which led to the hypothesis the μ -GIIIA interacted with Na_v 1 subtypes in a similar manner to the guanidinium toxins at Site 1 [57]. Yanagawa *et al.* [58], showed that μ -GIIIA inhibited the VGSC binding of ^3H -Lys-TTX, and conversely, that ^3H -Pr-GIIIA prevented the binding of both TTX and STX, providing strong evidence that μ -GIIIA and the guanidinium toxins compete for the same binding site on muscle Na_v 1 [55,58].

μ -Conotoxins from *Conus purpurascens*

More than a decade after the first report of μ -conotoxins from *C. geographus*, another 22-residue peptide sharing an identical Cys framework, μ -PIIIA, was identified from the venom of *C. purpurascens* (Figure 4) [60]. As

Figure 3. The μ -conotoxins as an emerging class of sodium channel blocking peptides. Timeline illustrates the relative discoveries and/or characterization of the members of this family. Data taken from [53].

with μ -GIIIA, this toxin showed a strong preference for muscle Na_v1 subtypes ($\text{IC}_{50} = 44 \text{ nM}$) over those from brain ($\text{IC}_{50} = 640 \text{ nM}$). In contrast to μ -GIIIA, μ -PIIIA exhibited nearly irreversible block of Na_v1 -subtypes [60]. The solution structure of μ -PIIIA confirmed the close proximity of the guanidino group of Arg14 to the hydroxyl group of Hyp18, providing further support for the proposed interactions between μ -GIIIA/PIIIA and Site 1 of the muscle VGSC subtype [61]. Like μ -GIIIA, μ -PIIIA exhibited the highest potency against $\text{Na}_v1.4$ ($0.036 \text{ }\mu\text{M}$), although affinities for other VGSC subtypes in the central (CNS) and peripheral (PNS) nervous systems were on average higher than those for μ -GIIIA (Figure 5) [53]. Because of the strong preference of μ -conotoxins μ -GIIIA/B/C and μ -PIIIA for muscle over neuronal subtypes, these peptides have also served as valuable tools for studying the VGSC isoform selectivity and function of skeletal muscle VGSCs [62].

μ -Conotoxins from *Conus stercusmuscarum*

μ -SmIIIA was predicted from the cDNA library from the venom of *C. stercusmuscarum* in 2002 [63]. μ -SmIIIA was shown to block Na^+ current in sympathetic neurons (TTX-resistant Na_v1 channels) with near irreversibility (for $0.5 \text{ }\mu\text{M}$ μ -SmIIIA, $k_{\text{obs}} = 0.24 \text{ min}^{-1}$) [63]. Notable differences between μ -SmIIIA and previously described μ -conotoxins included only modest

noncysteine sequence homology and the presence of Trp15 in the second intercysteine loop (Figure 4). The solution structure of μ -SmIIIA showed distinct differences from the μ -conotoxins of *C. geographus* both in its topology and the positioning of certain amino acid residues; it was hypothesized that these structural differences contributed to the block of TTX-resistant Na_v1 -subtypes by μ -SmIIIA [64]. This was supported by SmIIIA-PIIIA chimeras, which retained activity against amphibian TTX-resistant channels [64]. Block of TTX-resistant channels in amphibian preparations did not, however, translate to similar activities in mammalian channels [63]. Despite low nanomolar potency for $\text{Na}_v1.4$ (0.22 nM), μ -SmIIIA exhibited relatively poor selectivity among Na_v1 -subtypes in the CNS and PNS ($\text{Na}_v1.1$, 3.8 nM , $\text{Na}_v1.2$, 1.3 nM , $\text{Na}_v1.3$, 35 nM) (Figure 5). Moreover, μ -SmIIIA exhibited low micromolar block of the cardiac subtype $\text{Na}_v1.5$ [53], ruling it out as a viable candidate for therapeutic development.

μ -Conotoxins from *Conus striolatus*

The μ -conotoxins SxIIIA and SxIIIB, reported by Walewska *et al.* [65], also inhibited the skeletal muscle subtype preferentially ($\text{Na}_v1.4 = 0.007 \text{ }\mu\text{M}$; $\text{Na}_v1.2 = 1 \text{ }\mu\text{M}$) [53]. Among the conotoxins that preferentially target this subtype, there appears to be a pattern of hydroxyproline residues in the first and

	ER Signal sequence	Propeptide sequence	Mature toxin	Toxin name
m4 branch	MSKLGVLITICLLLFPLTA	LPMDGDEPANRPVERMQDNISSEQYPLFEKR	RD <u>CC</u> TOOKK-CKDRQCKOQR-CCA RD <u>CC</u> TOORK-CKDRRCOMK-CCA RD <u>CC</u> TOOKK-CKDRRCOLK-CCA RHG <u>CC</u> GOKG-CSSRECROQH-CC ZRL <u>CC</u> GFOKS-CRSRQCKOHR-CC RCCTGKKGSCSGRACKNLK-CCA ZKCCTGKKGSCSGRACKNLK-CCA	μ -GIIIA μ -GIIB μ -GIIC μ -TIIIA μ -PIIIA μ -SxIIIA μ -SxIIIB
m5 branch	MSKLGVLITICLLLFPLTA	LPMDQDPADQLEDQMDDISSEQYPSFVRR	ZNCC-NG--GCSKWC RDHARCC ZNCC-NG---GCSKWCKGHARCC CC-N-----CCKWC RDHSRCC NGCC-N-----CCKWC RDHSRCC ZRCN-NGRRGCSSRWCRDHSRCC VTDRCCKNGKRGC G-RWC RDHSRCC VGERCCKNGKRGC G-RWC RDHSRCC GRCC-EGPNGCSSRWCKDHARCC GRCC-DVPNACSGRWCRDHAQCC ZGCC-GEPNLCFT RWCRNNARCCRQQ ZGCC-NGPKG CCKWC RDHARCC ZGCC-NVPNGCSGRWC RDHAQCC	μ -SIIIA μ -SIIIB μ -KIIIA μ -KIIIB μ -SmIIIA μ -BullIIA μ -BullIIB μ -CIIIA μ -CnIIIA μ -CnIIB μ -CnIIC μ -MIIIA

Figure 4. Summary of identified μ -conotoxins from m4 and m5 branches of the M-superfamily. ER signal and propeptide sequences for μ -GIIIA and μ -SIIIA are illustrated as examples. 'O' denotes hydroxyproline; 'Z' denotes pyroglutamic acid. Arg13 in μ -GIIIA, or the residue in the equivalent position, is underlined to illustrate the sequence differences among conotoxins that preferentially block muscle versus neuronal subtypes.
ER: Endoplasmic reticulum
Adapted with permission from [16,59].

third intercysteine loops that is absent in μ -SxIIIA/B (Figure 4). Similarly, among conotoxins that block neuronal subtypes there is a high degree of sequence homology within the third intercysteine loop. These peptides also possess a Trp residue at an equivalent position, which is absent in μ -SxIIIA/B (Figure 4). Intraperitoneal or subcutaneous injection of μ -SxIIIA resulted in paralysis in mice [65]. Furthermore, SxIIIA was found to block $\text{Na}_v1.4$, expressed in *Xenopus* oocytes, with low nanomolar potency ($\text{IC}_{50} = 7 \text{ nM}$) [65]. The selectivity profile for μ -SxIIIA was later established as: $\text{Na}_v1.4 \gg 1.1 > 1.6 > 1.2 \gg$ all other subtypes (Figure 5) [53]. Notably, μ -SxIIIA was used as a model peptide for NMR-based mapping of disulfide connectivity [65]. Cysteines I–III (with respect to the disulfide framework) of μ -SxIIIA were labeled with 100% $^{15}\text{N}/^{13}\text{C}$, while the remaining Cys-residues were labeled with a mixture of 70% ($^{14}\text{N}/^{12}\text{C}$):30% ($^{15}\text{N}/^{13}\text{C}$). This allowed for 3D triple-resonance NMR experiments to determine disulfide connectivity from Cys $\text{H}^\alpha/\text{H}^{\beta 2}/\text{H}^{\beta 3}$ NOESY cross peaks.

μ -Conotoxins from *Conus tulipa*
 μ -TIIIA from *C. tulipa* potently inhibited both $\text{rNa}_v1.2$ and rNav1.4 expressed in *Xenopus* oocytes ($K_d = 45$ and 5 nM , respectively) [53,66] (Figure 5), but

showed little effect on isolated neurons from rat dorsal root ganglion (DRG). Alanine-replacement studies on μ -TIIIA showed that mutation of His2, Glu15 or Gln19 increased block of Na_v1 subtypes, whereas replacement of Lys6, Lys9, Arg17 and His20 reduced block (Figure 6). The μ -TIIIA[E15A] mutant not only improved block but also switched subtype preference in favor of the neuronal subtype [66]. It was suggested that the negative charge at this position created an unfavorable interaction with the binding site within the sodium channel [66]. More recent work has shown that addition of either a neutral residue (μ -TIIIA[E15A, 23A]) or basic residue (μ -TIIIA[E15A, 23K]) to the C-terminus decreased overall potency for all subtypes, but increased preference for $\text{Na}_v1.2$ [67]. Based on these findings, further positional scanning of Glu15 would be of interest to explore whether the selectivity profiles of μ -TIIIA could be shifted in favor of pain-relevant Na_v1 -subtypes.

Neuronal-subtype preferring μ -conotoxins
 μ -Conotoxins from *Conus kinoshitai*
 μ -KIIIA and μ -KIIIB are the shortest members of the μ -conotoxin family reported to date (Figure 4). In contrast to previously described μ -conotoxins, μ -KIIIA/B, preferentially blocked neuronal Na_v1 subtypes [51].

Figure 5. Selectivity profiles of μ -conotoxins against Na_v1 -subtypes. Data were obtained from reported IC_{50} values; *represents data obtained from pK_d values [53]. Pain-relevant subtypes are highlighted in red. Broken bars represent values greater than 100 μM . For color images please see online <http://www.future-science.com/doi/full/10.4155/FMC.14.107>

μ -KIIIA also showed significant differences in amino acid composition and intercyysteine loop sizes compared with muscle subtype preferring μ -conotoxins (Figure 4). Another notable difference was the substitution of the typically conserved Arg residue in loop 2 with Lys. These toxins also have a Trp residue within the second intercyysteine loop and more closely resembled μ -SmIIIA in the third loop. cDNA sequence data also identified a peptide with an extended N-terminus, μ -KIIIB [68]. The major folding isoforms for the short (μ -KIIIA) and extended (μ -KIIIB) peptides exhibited comparable activities when tested in *Xenopus* oocytes expressing $\text{Na}_v1.2$ [68,69]. As all available structure–activity relationship (SAR) data were obtained from the N-terminally truncated peptide, this review will focus on analogs of μ -KIIIA rather than μ -KIIIB. μ -KIIIA exhibited nearly 17-fold preference for neuronal over skeletal muscle subtypes (K_d $\text{rNa}_v1.2$ = 0.003 μM and K_d $\text{rNa}_v1.4$ = 0.05 μM) [69], and Ala-walk studies showed that four residues within loops 2 and 3 contributed to its $\text{Na}_v1.2$ preference, namely, Trp8, Arg10, His12 and Arg14 [69]. Furthermore, block of the

muscle subtype was decreased by Ala replacement of Lys7, Trp8, Arg10, Asp11, His12 and Arg14 (Figure 6).

Arg13 in μ -GIIIA and Arg14 in μ -PIIIA were shown to be crucial for the inhibition of skeletal muscle Na_v1 -subtypes, with the conservative analog μ -GIIIA[R13K] showing 10-fold lower potency for skeletal muscle subtypes [70]. Intriguingly, the neuronal-preferring conotoxins such as μ -SIIIA or μ -KIIIA possess a lysine at the equivalent position [51], and SAR studies of μ -KIIIA showed that mutations at this position (Lys7) could influence $\text{Na}_v1.2$ subtype preference [51]. μ -KIIIA[K7A] retained similar activity to μ -KIIIA against $\text{rNa}_v1.2$, but showed reduced activity against $\text{rNa}_v1.4$, thereby increasing the selectivity window [69]. This was also shown in the block of $\text{Na}_v1.2$ by the μ -KIIIA[K7Nle] analog, which more closely mimicked the steric bulk of a lysine residue at position 7 (block = 83 vs 33%) [69]. McArthur *et al.* recently showed that Ala substitution of either Lys7 or Arg10 led to lower K_d values and decreased maximal block, but retained the wild-type selectivity profile ($\text{Na}_v1.2 > 1.4 > 1.7$), while replacement of either His12 or Arg14 decreased

potency and altered the Na_v1 selectivity profiles in favor of pain-relevant subtypes ($\mu\text{-KIIIA}[\text{H12A}] - \text{Na}_v1.2 > 1.7 > 1.4$ and $\mu\text{-KIIIA}[\text{R14A}] - \text{Na}_v1.7 > 1.2 > 1.4$) [71]. Taken together, these results demonstrated that Lys7 was important for both potency and efficacy of block by $\mu\text{-KIIIA}$.

Analogues of $\mu\text{-KIIIA}$ were also constructed that incorporated nonnatural *N*-substituted Gly monomers (e.g., *N*-methylglycine, *N*-butylglycine and *N*-octoylglycine) at position 7 to explore the effects on Na_v1 affinity (K_d or IC_{50}) and efficacy (% block) [72]. These analogues retained the ability to block $\text{Na}_v1.2$, although both the kinetics of block and affinities were affected. The *N*-methylglycine substituted peptide was the most potent of these analogues tested. Importantly, this peptide also exhibited the largest residual Na^+ current of the series demonstrating a correlation between the size of the R-group of the substituted monomer and efficacy [72].

Mutations at Trp8 led to increased discrimination between neuronal and muscle subtypes. Replacement by Ala or Leu decreased the maximal block of both $\text{Na}_v1.2$ and $\text{Na}_v1.4$ (50 and 19% decrease, respectively). These substitutions also affected the kinetics of block, where k_{obs} for $\text{Na}_v1.2$ was significantly increased and the rate of block of $\text{Na}_v1.4$ was essentially unchanged [69]. Van Der Haegen *et al.* replaced Trp8 with Arg, Gln or Glu to explore the electronic effects of varying charge at this position on Na_v1 inhibition [73]; in all instances, Na_v1 potency was decreased. More importantly, mutation of Trp8 resulted in increased selectivity for neuronal sodium channel subtypes through greater reduction in $\text{rNa}_v1.4$ potency. This work also showed that substitution of Trp8 with less hydrophobic amino acid residues, regardless of charge, increased the reversibility of blockade [73].

Figure 6. Structure–activity characterization of μ -conotoxins. Potencies of characterized μ -conotoxins against skeletal ($\text{Na}_v1.4$) and neuronal ($\text{Na}_v1.2$) subtypes. Solution structures showing the folded peptide structures with disulfide connectivity. PDB ID# 1TCK ($\mu\text{-GIIIA}$), 1R9I ($\mu\text{-PIIIA}$), 1Q2J ($\mu\text{-SmIIIA}$), 2LXG ($\mu\text{-KIIIA}$) and 2LO9 ($\mu\text{-BulIIIB}$). BMRB Entry# 20024 ($\mu\text{-TIIIA}$) and 20023 ($\mu\text{-SIIIA}$). Results of individual amino acid replacements on Na_v1 -subtype blockade. ($\uparrow$) indicates mutations that improve potency against Na_v1 -subtypes, ($\downarrow$) denotes decreased potency and ($\circ$) represents no change in potency. (desZ1) indicates deletion of pyroglutamic acid in position 1 of $\mu\text{-SIIIA}$.

Figure 6. Structure–activity characterization of μ -conotoxins (cont.). Potencies of characterized μ -conotoxins against skeletal ($\text{Na}_v1.4$) and neuronal ($\text{Na}_v1.2$) subtypes. Solution structures showing the folded peptide structures with disulfide connectivity. PDB ID# 1TCK (μ -GIIIA), 1R9I (μ -PIIIA), 1Q2J (μ -SmIIIA), 2LXG (μ -KIIIA) and 2LO9 (μ -BullIB). BMRB Entry# 20024 (μ -TIIIA) and 20023 (μ -SIIIA). Results of individual amino acid replacements on Na_v1 -subtype blockade. (↑) indicates mutations that improve potency against Na_v1 -subtypes, (↓) denotes decreased potency and (°) represents no change in potency. (desZ1) indicates deletion of pyroglutamic acid in position 1 of μ -SIIIA.

Ala-substitution of basic residues (Lys7, Arg10, His12 and Arg14) influenced the affinity of and/or block by μ -KIIIA (Figure 6) [71]. Ala-substitution of His12 further improved the selectivity profile in favor of $\text{Na}_v1.2$, followed by $\text{Na}_v1.7$, and both were significantly preferred over $\text{Na}_v1.4$. A similar effect was observed in μ -KIIIA[R14A], with a complete shift in the selectivity profile ($\text{Na}_v1.7 > \text{Na}_v1.2 > \text{Na}_v1.4$). Alignment of the sequences of $\text{Na}_v1.2$, $\text{Na}_v1.4$ and $\text{Na}_v1.7$ subtypes showed that binding specificity was dependent upon interactions between Arg14 and Asp1241 of domain III of the outer ring of the P-loop. By mutating Arg14 to Ala, tight interactions with $\text{Na}_v1.2$ and $\text{Na}_v1.4$ were disrupted; it was hypothesized that the slow off-rates of μ -KIIIA ($k_{\text{off}} = 0.002 \text{ min}^{-1}$) were a consequence of these interactions [71]. Inspection of the h $\text{Na}_v1.7$ sequence revealed that the equivalent position within the P-loop of the channel contained Ile (Ile1410) rather than the Asp observed

in the neuronal and skeletal muscle subtypes. Using this information, future analogs may offer better discrimination between Na_v1 subtypes.

Disulfide-deficient KIIIA analogs were constructed based on the ‘canonical’ μ -conotoxin framework CysI–CysIV, CysII–CysV, CysIII–CysVI (Figure 4) [74]. Electrophysiology experiments revealed that the CysII–CysV and CysIII–CysVI disulfide pairs were most critical for Na_v1 -blockade [74]. The solution structures of these analogs showed that removal of the CysI–CysIV bridge resulted in greater N-terminal flexibility, but, did not disrupt the α -helical C-terminal region known to be important for biological activity [75]. Based on these findings, the [desC1]KIIIA[S3/4Aopn, C9A] analog was constructed, which lacked the CysI–CysIV bridge and replaced less-critical amino acid residues with an Aopn (5-amino-3-oxapentanoic acid) backbone spacer unit. This analog not only blocked $\text{Na}_v1.2$ with low nanomolar potency, but also exhibited

analgesic activity in a mouse model of inflammatory pain [74]. Recently, it has been shown that the disulfide connectivity for synthetic μ -KIIIA is different from that of previously described μ -conotoxins [68,76]. These results are discussed in greater detail below.

μ -Conotoxins from *Conus striatus*

μ -SIIIA and μ -SIIIB from the venom of *C. striatus* [51,77] differ from one another by only two amino acid residues within the third intercysteine loop (Arg14 and Asp15 in μ -SIIIA vs Lys14 and Gly15 in μ -SIIIB) (Figure 4). In *Xenopus* oocytes expressing Na_v1 -subtypes, μ -SIIIA displayed a threefold preference for $\text{Na}_v1.2$ over $\text{Na}_v1.4$ (0.05 vs 0.13 μM) [53]. Greater subtype discrimination was achieved through removal of the N-terminal pyroglutamate residue. For μ -SIIIA(2-20), affinity for $\text{Na}_v1.2$ was increased while that for $\text{Na}_v1.4$ was decreased, while the opposite was true for μ -SIIIB [77]. The importance of individual residues was assessed in μ -SIIIA(2-20) using an ^{125}I -TIIIA displacement assay against $\text{Na}_v1.2$ and $\text{Na}_v1.4$. Similar to μ -KIIIA, residues in the C-terminal helix were found to be most important for VGSC blockade. Alanine replacement of Lys11, Trp12, Arg14, His16 or Arg18 leads to the most pronounced effects on VGSC binding, with the majority of these mutations retaining preference for the neuronal subtype (Figure 6). Importantly, μ -SIIIA[H16A] exhibited a significant decrease in binding affinity for both neuronal and muscle subtypes, suggesting that this residue contributes to general Na_v1 binding.

Yao *et al.* undertook detailed structural analyses of μ -conotoxins that blocked neuronal VGSC subtypes (μ -SmIIIA, μ -SIIIA and μ -KIIIA) [78]. These peptides shared near sequence identity at the C-terminus but differed significantly from the C-termini of skeletal muscle preferring μ -conotoxins. In μ -SIIIA, residues 11–16 formed a well-defined helix, while residues 3–5 associated to form a 3_{10} -helix [78]. Dynamics measurements of μ -SIIIA made using ^{13}C NMR relaxation experiments showed that the N-terminus and Ser9 had larger magnitude motions on the subnanosecond timescale, while the C-terminus was more rigid. These data were interesting in light of functional data which suggested that the N-terminus was important for subtype selectivity, while the C-terminus contributed mainly to neuronal subtype interactions [78]. Schroeder *et al.* explored the structural and functional consequences of N-terminal modification of μ -SIIIA/B [67]. Replacement of the N-terminal pyroglutamate (Pyr1 or single letter code Z) with charged residues (i.e., μ -SIIIA[Z1R] and μ -SIIIB[Z1E]) either decreased (μ -SIIIA) or increased (μ -SIIIB) preference for $\text{Na}_v1.2$. Greater selectivity could also be obtained through extension of the N-terminus. The role of negatively-charged

residues at the N-terminus for $\text{Na}_v1.2$ subtype selectivity was further demonstrated by the μ -SIIIB analogs μ -SIIIB[E0, Z1R] and μ -SIIIB(22–0)[N2E], respectively. Extension of the C-terminus of μ -SIIIA[D15A], using charged amino acids, afforded greater selectivity for the neuronal subtype [67]; specifically, the μ -SIIIA analogs μ -SIIIA(2-21)[D15A, 21K] and μ -SIIIA(2-21)[D15A, 21D] showed improved discrimination between $\text{Na}_v1.2$ and $\text{Na}_v1.4$ [67].

The C-terminal regions of neuronal subtype preferring μ -conotoxins are highly homologous. However, the N-termini of these peptides have a decreasing number of residues in the first intercysteine loop across the series μ -SmIIIA > μ -SIIIA > μ -KIIIA. We previously investigated this region as a potential site for modification by flexible backbone spacer units such as 6-aminohexanoic acid (Ahx) and 3-oxapentanoic acid (PEG) with the goal of creating polymer-peptide hybrid analogs of μ -SIIIA with analgesic activity (e.g., polytides) [79]. At 25 μM , backbone spacer containing analogs of μ -SIIIA demonstrated approximately 80% block of TTX-sensitive sodium currents from mouse DRG neurons, compared with 65% block by wild-type μ -SIIIA. The μ -SIIIA polytide analogs produced not only increased block but also faster onset of action compared with the native peptide. These results provided further evidence that the μ -conotoxin scaffold could accommodate significant structural modifications and lent support to the concept of peptide engineering to enhance pharmacological properties. This work also demonstrated that μ -SIIIA analogs were potent analgesics in an *in vivo* pain model, with the most promising of the analogs (PEG-SIIIA) having higher potency of 0.05 mg/kg (μ -SIIIA = 0.9 mg/kg) following intraperitoneal administration [79].

μ -Conotoxins from *Conus consors*, *Conus catus* & *Conus magus*

In addition to a high degree of homology among the C-termini of μ -CnIIIA, μ -CnIIIB, μ -CIIIA and μ -MIIIA, these peptides also possess a characteristic Pro-Asn dipeptide in the first intercysteine loop (Figure 4) [80]. These peptides blocked sodium conductance of TTX-resistant Na_v1 in frog DRG neurons with varying potency and selectivity, with μ -CnIIIA (87% block – neuronal subtype selective) and μ -CIIIA (96% block – nonselective) being the most potent of this group. However, when tested in a mammalian system (mouse), μ -CnIIIA and μ -CIIIA blocked only weakly and lacked significant subtype selectivity [80]. μ -CnIIIA was nearly equipotent against neuronal and skeletal muscle subtypes (0.25 vs 0.27 μM). Likewise, μ -MIIIA showed poor discrimination between these two subtypes (0.45 vs 0.33 μM) (Figure 5) [53].

Recently, another μ -conotoxin from *C. consors* was reported by Favreau *et al.* [81]. This peptide was interesting in that the N-terminus of μ -CnIIIC was homologous to μ -TIIIA (loop 1), whereas the C-terminus resembled neuronal subtype-preferring conotoxins such as μ -KIIIA and μ -SIIIA in that μ -CnIIIC possesses a lysine in the equivalent position to Arg14 of μ -GIIIA (Figure 4). This peptide blocked $\text{Na}_v1.2$ ($\text{IC}_{50} = 1.3 \text{ nM}$) and $\text{Na}_v1.4$ (100% block at $1 \text{ }\mu\text{M}$) with little or no effect on cardiac subtypes in HEK 293 cells. μ -CnIIIC also blocked the seemingly disparate nicotinic acetylcholine receptors (nAChR) $\alpha3\beta2$ subtype and inhibited global nerve action potentials from sciatic and olfactory nerves with slow reversibility [81]. The authors suggested that the effects of μ -conotoxins on nAChR targets responsible for the modulation of pain signals may be more widespread than originally thought, perhaps offering a potential new molecular target for these potent analgesic compounds [81].

μ -Conotoxins from *Conus bullatus*

Three μ -conotoxins (μ -BuIIIA, μ -BuIIIB and μ -BuIIIC) from the venom of *C. bullatus* blocked $\text{Na}_v1.4$ with near irreversibility [59]. These peptides had an extended N-terminus and a shorter second intercysteine loop compared with previously described μ -conotoxins (Figure 4). Wilson *et al.* determined the subtype-selectivity profiles for μ -BuIIIA and μ -BuIIIB and confirmed that $\text{Na}_v1.4$ was the preferred target, followed by $\text{Na}_v1.2$ and $\text{Na}_v1.3$ (Figure 5) [53].

Thus far, the μ -conotoxins in this review have been compared for their abilities to block either neuronal ($\text{Na}_v1.2$) or skeletal muscle ($\text{Na}_v1.4$) subtypes. However, μ -BuIIIB is also a potent inhibitor of $\text{Na}_v1.3$ (Figure 5) [53]. The exact pharmacological role of this subtype remains to be confirmed, but studies by Haines *et al.* demonstrated upregulation of $\text{Na}_v1.3$ following peripheral nerve axotomy, implicating this subtype as a potential target for the treatment of neuropathic pain [82,83]. Moreover, there are currently no selective probes of this channel subtype, making μ -BuIIIB an attractive lead compound to study $\text{Na}_v1.3$. Recent studies have focused on determination of interactions between μ -BuIIIB and $\text{Na}_v1.3$ [84,85]. The solution structure was determined by Kuang *et al.* [84] and it was hypothesized that the signature characteristics (an N-terminal extension and differences in intercysteine loop sizes) might contribute to the block of $\text{Na}_v1.3$ [84].

Ala-replacement of the N-terminal residues of μ -BuIIIB influenced its potency but not its selectivity (Figure 6). Substitution of either Gly2 or Glu3 resulted in significant improvements to $\text{Na}_v1.3$ potency. Importantly, substitution of Gly2 with D-Ala resulted

in a μ -BuIIIB analog with greater than 40-fold higher potency for $\text{Na}_v1.3$, presumably through stabilization of a type II β -turn at the N-terminus [84]. Interestingly, complete removal of the N-terminal extension still resulted in a fourfold improvement in the block of $\text{Na}_v1.3$ over wild-type μ -BuIIIB [84]. Despite the gains in potency, selectivity for $\text{Na}_v1.3$ was not generated. These observations suggested, however, that further SAR studies of μ -BuIIIB would be valuable in order to identify the key structural components involved in $\text{Na}_v1.3$ blockade.

Detailed SAR of μ -BuIIIB has been largely inhibited by synthetic inaccessibility owing to its propensity to form numerous folding isoforms during oxidative folding. A strategy was therefore devised that directed folding toward a single bridging pattern through incorporation of a diselenide bridge and disulfide bridge removal (Figure 7). The rationale for this strategy lies in two previous reports on μ -conotoxin KIIIA [72,74]. A disulfide-deficient, diselenide-containing scaffold was constructed in which Cys5 and Cys17 were replaced with **selenocysteine** and the Cys6–Cys23 pairing was removed by alanine substitution (i.e., ddSecBuIIIB) [85]. These studies provided valuable insight into which amino acids contributed to block of the $\text{Na}_v1.3$ subtype. Similar to the other μ -conotoxins, residues near the C-terminus of μ -BuIIIB were most important for Na_v1 block (Figure 6). In ddSecBuIIIB, the aromatic residues Trp16 and His20 were shown crucial for $\text{Na}_v1.3$ inhibition, with alanine-substituted analogs having K_d values of $>30 \text{ }\mu\text{M}$ each, compared with $0.2 \text{ }\mu\text{M}$ for wild-type μ -BuIIIB [85].

The highly basic character of μ -conotoxins is thought to contribute to their activity in the negatively charged pore of VGSCs. Early reports highlighted the critical role of a single basic residue near the C-terminus of μ -conotoxins (e.g., [μ -GIIIA[Arg13]] for VGSC blockade [56,57]. Ala substitution of Arg15, Arg18 or Arg22 in ddSecBuIIIB resulted in K_d values of 1.84, 15.7 and $3.81 \text{ }\mu\text{M}$, respectively [85]. Interestingly, the most ‘critical’ of these basic residues was not located at the position equivalent to Arg13 in μ -GIIIA. Instead, multiple basic residues were shown to be important for activity (Figure 6), consistent with models previously constructed by McArthur *et al.* using μ -PIIIA [71].

Key term

Selenocysteine: Often referred to as the 21st proteinogenic amino acid, selenocysteine is an isosteric replacement for cysteine. In selenocysteine, the sulfur-containing thiol of cysteine is replaced by a selenium-containing selenol.

Figure 7. Description of the ddSec strategy used to facilitate structure–activity relationship studies of μ -BullIIB.

(A) Cartoon depiction of μ -BullIIB showing disulfide connectivity between cysteine thiols. (B) Cartoon of ddSecBullIIB scaffold with disulfide depletion by removal of the Cys6–Cys23 bridge and diselenide bridge formation by selenocysteine replacement of Cys5 and Cys17. (Panels C and D) Upper and lower chromatograms show linear and folded elution patterns of μ -BullIIB (C) and ddSecBullIIB (D), respectively. *Indicates the properly folded isoform. Positional scanning of all noncysteine residues was subsequently performed using the ddSecBullIIB scaffold. Reproduced with permission from [85] © 2014 FEBS.

The ddSecBullIIB analog also served to identify residues for which Ala-substitution did not greatly affect biological activity (e.g., Val1, Gly2, Asn8, Gly14 and Ser21), suggesting these positions as possible sites for chemical modification to achieve enhanced pharmacological/physicochemical properties [85]. The most striking outcome of these studies was that the substitution of Glu3 increased $\text{Na}_v1.3$ potency ($K_d = 0.07 \mu\text{M}$). These findings were in agreement with the report by Kuang *et al.*, which suggested that the negative charge of Glu3 may result in unfavorable interactions with $\text{Na}_v1.3$ [84]. Current efforts are directed at modifying the above-mentioned positions, particularly position 3, with the goal of developing potent and selective inhibitors of $\text{Na}_v1.3$.

Disulfide connectivity in μ -conotoxins

Disulfide bridges serve to stabilize the global structure of conotoxins [86]. With three disulfide pairs, μ -conotoxins have the potential to form up to 15

distinct structural isomers. Some of these conformations will be naturally selected against because they are energetically disfavored, but ‘noncanonical’ isoforms do occur and may be observed in some abundance even upon reaching folding equilibrium. Following oxidation of the fully reduced peptide, the species found in highest abundance within the folding mixture has traditionally been assumed to be the native fold, although this has not been confirmed in most cases.

Until recently, the disulfide connectivity of all Na_v1 -targeting μ -conotoxins within the m4 and m5 branches of the M-superfamily (Figure 4) was assumed to be the same (CysI–CysIV; CysII–CysV; CysIII–CysVI). A recent report by Tietze *et al.* showed that three major folding isoforms, with differing disulfide connectivities, resulted from the direct oxidation of synthetic μ -PIIIA, namely, μ -PIIIA-1 (CysI–CysV; CysII–CysVI; CysIII–CysIV), μ -PIIIA-2

(CysI–CysIV; CysII–CysV; CysIII–CysVI) and μ -PIIIA-3 (CysI–CysII; CysIII–CysIV; CysV–CysVI) [87]. All three isoforms were shown to block $\text{Na}_v1.4$, albeit with different potencies: $\text{IC}_{50}\mu$ -PIIIA-1 = 46.7 nM, $\text{IC}_{50}\mu$ -PIIIA-2 = 103.2 nM and $\text{IC}_{50}\mu$ -PIIIA-3 = 203.7 nM [87]. These results showed that the ‘canonical’ disulfide bridging pattern for μ -PIIIA did not result in the most potent folding isoform [87]. Molecular dynamics simulations of each isoform in this study showed that the side-chain of Arg14 in μ -PIIIA-1 penetrated deeper into the binding pore, presumably contributing to the increased potency of this isomer [87].

Another study re-examined the ‘canonical’ μ -framework of μ -KIIIA [76]. Poppe *et al.* noted that, because through-bond scalar couplings between the two sulfur atoms are undetectable by NMR, interactions are typically inferred based on interproton distance constraints. To circumvent this problem, they developed a method known as the pattern of disulfides from local constraints (PADLOC) and suggested an alternative connectivity for the native fold of μ -KIIIA (CysI–CysV; CysII–CysIV; CysIII–CysVI) [76]. This was recently confirmed by Khoo *et al.*, who isolated the major folding oxidation products of synthetic μ -KIIIA and studied connectivity by collision-induced dissociation MS [68,88]. This confirmed the connectivity of the major folding isoform and determined the connectivity of the isomer in the next highest abundance as CysI–CysVI; CysII–CysIV; CysIII–CysV, neither of which matched the assumed connectivity for μ -conotoxins reported previously. Whether these connectivities are a consequence of the unique structural features of this peptide (μ -KIIIA has only a single residue between CysII and CysIII), or an indication of greater toxin diversity within the μ -conotoxin family remains to be determined. An intriguing possibility is that *Conus* produces multiple active folding isoforms to increase its toxin repertoire.

A recent study by Safavi-Hemami *et al.* [89] provided evidence for the presence of both the globular (CysI–CysIII; CysII–CysIV) and ribbon (CysI–CysIV; CysII–CysIII) forms of α -conotoxin ImI in the venoms of *Conus imperialis* [89]. Pharmacological studies have traditionally focused on the activities of the globular form, assuming this to be the native disulfide connectivity. However, the authors point out that in numerous cases (e.g., α -AuIB), it is actually the ribbon form that elicits the greatest biological effect [89,90]. Although these studies emphasized the presence of multiple folding isoforms of α -conotoxins, it is conceivable that multiple ‘noncanonical’ isomers of μ -conotoxins are also present in venom. In light of this, an in-depth re-examination

of μ -conotoxin connectivities would be beneficial, particularly for the remaining members of the m4 branch and for toxins belonging to the more recently described m5 branch.

Influence of $\text{Na}_v\beta$ subunit expression on μ -conotoxin activities

In vivo, VGSCs comprise both a pore-forming α -subunit (260 kDa) and one or more accessory **sodium channel β -subunits** (32–36 kDa) (Figure 1) [91]. To date, four β -subunit subtypes have been identified ($\text{Na}_v\beta1$ – $\text{Na}_v\beta4$). The odd-numbered subtypes ($\beta1$ and $\beta3$) associate with the α -subunit via noncovalent interactions whereas $\beta2$ and $\beta4$ subtypes are covalently linked to the α -subunit via disulfide bridges [92]. Despite these interactions, the α -subunit, which possesses the minimal essential features of a fully functioning VGSC, has traditionally been used to measure the effects of sodium channel agonists or antagonists in electrophysiology assays. However, co-expression of the β -subunit with the α -subunits influenced the steady-state activation of $\text{Na}_v1.8$ -subtypes expressed in *Xenopus* oocytes, suggesting a more significant role of the β -subunit in channel activation [91]. Furthermore, the β -subunits are of interest because expression levels are altered following spinal cord injury in multiple animal models [91].

Wilson *et al.* showed that co-expression of $\text{Na}_v1.8$ with any of the four β -subunits resulted in higher affinity block by $\mu\text{O-MrVIB}$, resulting from an increased k_{on} and modest decreases in k_{off} [93]. Most recently, Zhang *et al.* reported the importance of these accessory proteins with respect to the activities of a number of μ -conotoxins [94]. The binding kinetics of μ -conotoxins PIIIA, TIIIA, KIIIA and SmIIIA were examined by **two-electrode voltage-clamp electrophysiology** in *Xenopus* oocytes co-expressing Na_v1 with one of the four β -subunits. The key findings of this work were that co-expression of the $\beta1$ or $\beta3$ subunits in the presence of the Na_v1 α -subunit increased the on-rates (k_{on}) of μ -conotoxins, whereas k_{on} was dramatically

Key terms

Sodium channel β -subunit ($\text{Na}_v\beta$): Accessory proteins associated with the α -subunit by either noncovalent ($\text{Na}_v\beta1$ or $\beta3$) or covalent ($\text{Na}_v\beta2$ or $\beta4$) interaction. These subunits modulate channel gating, regulate VGSC trafficking/ expression and promote cell adhesion/migration; $\text{Na}_v\beta$ -subtypes can also modulate biological activities of venom-derived toxins.

Two-electrode voltage-clamp electrophysiology:

A method that assesses the effects of ligands on heterologously expressed VGSCs, typically in *Xenopus* oocytes. Kinetics of block are measured by changes in ionic current resulting from Na^+ flow-through VGSCs.

decreased in the presence of either $\beta 2$ or $\beta 4$ with $\text{Na}_v 1$. Conversely, little or no effect was observed upon treatment of the same system with either of the alkaloid toxins TTX or STX [94]. These results highlight the importance of β -subunits in obtaining a complete and physiologically relevant assessment of engineered subtype-selective blockers based on μ -conotoxins.

Analgesic activities of μ -conotoxins

Discovery of so-called 'neuronal-preferring' μ -conotoxins encouraged exploration of these compounds in pain models. Three reports have documented the abilities of the neuronal subtype-preferring conotoxins μ -KIIIA and μ -SIIIA to suppress nociception in animal models [69,74,79]. In all instances, compounds were tested in the formalin-induced pain assay in mice following intraperitoneal administration. In this assay, μ -KIIIA was effective in suppressing paw-licking times during the Phase II nociceptive response (inflammatory pain) with a calculated ED_{50} of 0.1 mg/kg [69]. Similarly, μ -SIIIA and its analogs were shown to be analgesic in this model with an ED_{50} of 0.9 mg/kg. The analog in which noncritical residues were replaced with a PEG backbone spacer, possessed an ED_{50} of 0.05 mg/kg [79]. These results exemplified the relative ease by which peptidic scaffolds can be modified to engineer enhanced pharmacological and physicochemical properties. Analgesic effects were also observed with the minimized peptide analog des[C1] KIIIA[S3/4Aopn, C9A], where intraperitoneal administration of 10 nmol resulted in a 54% decrease in the inflammatory pain response compared with the saline control [74]. Despite these encouraging results, further work is necessary to transform neuronal-subtype preferring μ -conotoxins into analgesic drug leads.

Future perspective

Accelerating the pace of μ -conotoxin discovery & development

A very small fraction (< 1%) of the total complement of neuroactive peptides in *Conus* venoms has been identified to date, let alone characterized [95]. The discovery of new conotoxins has traditionally relied on bioassay-directed fractionation of whole venoms, followed by sequencing of individual peptide components [96], and this approach has been responsible for the discovery of nearly all μ -conotoxins described to date. However, assay-driven identification of large numbers of unique toxin sequences is impractical because it is often labour intensive, time consuming and costly. New methodologies such as transcriptomics [12,96,97] and proteomics/peptidomics [98,99] are already accelerating the pace at which novel peptide sequences are discovered and characterized. Proteomic/peptidomic approaches can

be effective when coupled to other methods such as *de novo* sequencing. However, transcriptomics alone is not a viable option for the identification of new μ -conotoxins because of a higher propensity for base call errors (compared with traditional sequencing methods) and an inability to identify post-translationally modified residues, which are common in *Conus* toxins [100]. The most effective means for identifying new toxins from *Conus* venoms lies in the combination of the above-mentioned technologies with high-resolution MS [101,102]. In a recent article, Prashanth *et al.* suggested that the combination of transcriptomic and peptidomic methods, in conjunction with bioinformatics, could lead to the construction of large, searchable repositories for important sequence data [100]. Recently, such an approach was employed to identify new toxins and toxin families in the venoms of *C. consors* [11], *C. flavidus* [103] and *C. marmoreus* [102].

Future research on μ -conotoxin research will increasingly involve a combination of transcriptomic/proteomic approaches and high-throughput screening methods for the correlation of biological activities with new sequence information. Traditionally, determination of the biological activity of a newly identified conotoxin relied on patch-clamp or voltage-clamp electrophysiology assays, the major drawback of which is the inability to screen large numbers of peptide against multiple molecular targets. Calcium imaging studies employing Ca^{2+} -sensitive dyes to study the effects of conotoxins against their molecular targets [104,105] have proven to be highly amenable to implementation in high-throughput screening assays, as discussed in [100].

Another issue faced in the study and development of conotoxin-based drugs is one of production. Ideally, bacterial or yeast expression systems would help to solve this problem, but the abundance of post-translational modifications in these peptides has made these approaches particularly challenging [86,106]. Furthermore, the reducing environment of the bacterial cytoplasm commonly results in misfolded forms of the peptide or sequestration to inclusion bodies [107]. Peptides can be recovered from the insoluble fraction, but misfolded peptides must undergo reduction and *in vitro* oxidative refolding to obtain the final product [107]. Recent advances in recombinant methods have shown promise in addressing issues of misfolded conotoxin isoforms and have proven useful for sequences that draw from the naturally occurring 20 amino acid repertoire [107,108]. However, a major impediment to large-scale expression of conotoxins still remains, that being the inability of recombinant expression systems to 'decorate' the primary sequence with PTMs commonly found in conotoxins. Several enzymes responsible for such modifications have been identified in *Conus* and, in the future it may be fea-

Figure 8. Conotoxins at various stages of preclinical and clinical development.

n.a.: Not available

Data taken from [9,132].

sible to modify recombinantly expressed peptide with such enzymes [109–111]. In the meantime, synthetic approaches such as solid-phase peptide synthesis remain the more practical approach to synthesis of conotoxins for structural or functional characterization.

Synthetic approaches for producing conotoxins have been largely dependent on solid-phase peptide synthesis using Fmoc or Boc chemistries. As new chemistries are developed, the pace at which new conotoxin sequences can be produced is expected to increase. A major issue in synthesizing conotoxins is ensuring proper cyclization patterns. Early efforts focused on stepwise oxidation of Cys thiols using trityl (Trt), acetamidomethyl (Acm) or other orthogonal protecting groups to direct folding to a major isoform [112]. The major drawback to this approach is the requirement for multiple purification steps, resulting in decreased yields of the fully folded product. Recently, proxies for disulfide bridges such as dicarba- [113,114], diselenide- [115–117] or thioether bridges [118] have been developed. A significant advantage of these methods is the independent formation of macrocycles either on-resin or following cleavage from solid support without additional purification steps.

An incomplete understanding of the interactions between μ -conotoxins and their molecular targets (i.e., VGSCs) has made the rational design of peptide analogs with improved pharmacological action challenging. Early studies of interactions between μ -conotoxins and Na_v1 -subtypes were almost entirely reliant on mutational studies [71,119].

However, computational studies and the emergence of bacterial sodium channel models are anticipated to increase the rate at which potent and selective blockers of VGSCs can be developed. Such studies can provide useful information about subtle interactions between the toxin and the channel that may lead to improved pharmacology. A recent example of how computational studies can aid in the design of potent peptide analogs can be found in a publication describing mutants of the *Stichodactyla helianthus* potassium channel-blocking toxin ShK, which were designed on the basis of modeling and docking studies and exhibited improved selectivity for $\text{K}_v1.3$ [120]. Unfortunately, in the absence of accurate models of human VGSCs, similar studies with μ -conotoxins have not been possible to date. The earliest modeling studies docked μ -GIIIA to a skeletal muscle VGSC model based upon the potassium channel and available mutational data [119]. These studies were valuable in defining the binding orientation of μ -GIIIA in the pore region of Domain II and they highlighted the importance of interactions between positively-charged μ -conotoxins and the negatively charged outer ring of the pore vestibule [119].

Recent crystal structures of bacterial VGSCs have provided another opportunity to discover important interactions between VGSCs and their ligands. In 2005, Pavlov *et al.* confirmed the critical role of the pore region for channel blockade using NaChBac, a prokaryotic VGSC [121]. The NaChBac model was useful from

the perspective of being a simplified VGSC structure that possessed multiple common features to eukaryotic VGSCs, but showed greater sequence similarity to calcium channels than sodium channels [121]. Recently, the crystal structures of a number of prokaryotic sodium channel orthologs have provided valuable insights into the structure and function of VGSCs [21,122]. These structures may eventually yield structural information to help in the design of μ -conotoxin analogs with improved VGSC potency and/or selectivity. Payandeh *et al.* reported the crystal structure of a bacterial sodium channel (Na_vAb) in its closed conformation in 2011 [21,123]. This structure helped explain the intrinsic selectivity of VGSCs for Na^+ ions and how these ions are translocated across the membrane. It also aided in the understanding and interpretation of previous SAR data since the pore regions of Na_vAb and the corresponding regions in vertebrate VGSCs share a significant degree of sequence homology [21]. Shortly after the first studies of Na_vAb , McCusker *et al.* reported the crystal structure of the pore domain of another prokaryotic VGSC, Na_vMs [122], which differed from Na_vAb in that it was in an open conformation. The open state of Na_vMs resembled the closed conformation of Na_vAb in that the selectivity filters appeared similar, but a major difference between the two structures was observed in the fenestrations within the transmembrane region. Na_vMs exhibits a much wider opening, suggesting that it may be able to accommodate large, hydrophobic pore-blocking drugs in the open state [122]. These models also differed in their putative mechanisms of

sodium conductance across the membrane. Although significant differences exist between prokaryotic and eukaryotic VGSC subtypes (i.e., lack of tetrameric symmetry, differences in amino acid composition and selectivity filter loop size), elucidation of bacterial sodium channel structures has served as a valuable starting point from which interactions of VGSC subtypes and their ligands (i.e., μ -conotoxins) can be understood [124]. Computational studies using these structures [124,125] are expected to result in more accurate depictions of channel–ligand interactions than those based on other ion channel families.

μ -conotoxins as pharmacological tools & drug leads for the treatment of pain

The μ -conotoxins are interesting not only from the perspective of identifying potent therapeutic leads, but also as scaffolds upon which novel functionalities may be presented. SAR studies on disulfide-rich peptides have identified single amino acids or short di- or tri-peptide segments between Cys-residues that can be removed or replaced without diminishing biological activity. Short peptide sequences have been ‘grafted’ into such regions of the ‘knottin’ peptides to generate powerful tumor imaging agents [126] or peptide analogs with additional therapeutic potential [127]. The μ -conotoxins are extremely hypervariable in these loop regions and, as such, are well suited for such studies. This hypervariability also makes these molecules ideal candidates for directed evolution experiments using scaffold-constrained random libraries in a manner described

Executive summary

Background

- Voltage-gated sodium channels (VGSCs) represent an attractive target for the treatment of neuropathic pain, epilepsy and a number of neurological disorders.
- More than 20 μ -conotoxins have been described to date, many with distinct selectivity profiles for VGSC subtypes in the CNS or PNS.

μ -Conotoxin inhibitors of VGSCs

- Early work identified μ -conotoxins that were selective for skeletal muscle subtypes and have been valuable in determining the structure and function of VGSCs.
- Neuronal subtype preferring μ -conotoxins have been important for discrimination of VGSC subtypes and for exploration as potential analgesics.
- Structure–activity relationship studies have identified critical residues in various μ -conotoxins as a basis for enhancing potency and/or selectivity.

Applications of μ -conotoxin inhibitors of VGSCs

- Muscle-preferring μ -conotoxins have been important for the study of VGSCs; these peptides may also be useful as myorelaxant drugs when administered focally.
- Neuronal subtype-preferring μ -conotoxins have shown analgesic activity and may be useful as treatments for pain, epilepsy or other neurological disorders.

Future perspectives

- The complexity of the more than 50,000 *Conus* venom peptides highlights the potential for discovery of numerous therapeutic peptides, including new μ -conotoxins.
- Differences in subtype-selectivity, in addition to the amenability of the μ -conotoxin scaffold to chemical modification, are expected to yield useful pharmacological probes in addition to potential drug leads.

in [128]. Another promising application of μ -conotoxins is bioconjugation with small molecule VGSC inhibitors for increased analgesic effect. Sata *et al.* have shown that neurosteroids such as allopregnanolone sulfate and endocannabinoids such as anandamide can inhibit pain-relevant Na_v1 -subtypes expressed in *Xenopus* oocytes [129,130]. Coupling of these molecules to μ -conotoxin scaffolds could conceivably generate peptide analogs with enhanced analgesic activity. Moreover, such peptides may display the target specificity that is often lacking in small molecule inhibitors.

The μ -conotoxins are clearly valuable pharmacological tools for identifying different Na_v1 -subtypes in neuronal membrane preparations [53]. The differences in selectivity profiles among this family have most recently allowed for examination of specific TTX-sensitive Na_v1 -subtypes in small and large DRG and superior cervical ganglion neurons [131]. It is anticipated that μ -conotoxins will continue to be used in these types of studies and that they will have prominent roles in probing the structure and function of VGSC subtypes in the future.

Several conotoxin-derived therapeutics are currently in various stages of preclinical or clinical evaluation (Figure 8). The development of μ -conotoxins has focused largely on identifying peptides that act as potent analgesics through inhibition of pain-relevant Na_v1 -subtypes in the periphery [71,85]. However, blockade of the muscle subtype $\text{Na}_v1.4$, long viewed as an undesirable characteristic of many μ -conotoxins, may in fact have clinical applications, as suggested by a recent report on μ -CnIIIC highlighting such inhibitors as potential myorelaxant drugs [81].

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