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*Review*

## **Conotoxins: Therapeutic Potential and Application**

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**Abstract:** The pharmacological variety of conotoxins, diverse peptides found in the venoms of marine cone snails, is well recognized. Venoms from each of the estimated 500 species of cone snails contain 50 to 200 distinct biologically active peptides. Most conotoxins characterized to date target receptors and ion channels of excitable tissues, such as ligand-gated nicotinic acetylcholine, N-methyl-D-aspartate, and type 3 serotonin receptors, as well as voltage-gated calcium, sodium, and potassium channels, and G-protein-coupled receptors including  $\alpha$ -adrenergic, neurotensin, and vasopressin receptors, and the norepinephrine transporter. Several conotoxins have shown promise in preclinical models of pain, convulsive disorders, stroke, neuromuscular block, and cardioprotection. The pharmacological selectivity of the conotoxins, coupled with the safety and efficacy demonstrated in preclinical models, has led to their investigation as human therapeutic agents. In the following review, we will survey the pharmacology and therapeutic rationale of those conotoxins with potential clinical application, and discuss the unique challenges that each will face in the course of their transition from venom component to human therapeutic.

**Keywords:** conotoxin, conopeptide, cone snail, venom, analgesia

**Abbreviations:** 5-HT<sub>3</sub>R, type 3 serotonin receptor; AChR, nicotinic acetylcholine receptor; CaV, voltage-gated calcium channel; CNS, central nervous system; KV, voltage-gated potassium channel; NaV, voltage-gated sodium channel; NMDAR, N-methyl-D-aspartate receptor; TCA, tricyclic antidepressant; TTX, tetrodotoxin.

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## 1. Introduction

Since the Cambrian era, predators have developed numerous strategies for finding, incapacitating, and killing other organisms for nutritional purposes. Of interest to the pharmacologist and physician are those predators that have developed a strategy of envenomation to overcome their prey. Typical animal venoms contain complex mixtures of proteins, enzymes, peptides, and low molecular weight molecules, and represent a rich source of structurally unique, bioactive compounds [1-3]. One group of venomous animals with a particular talent for developing peptide toxins is the marine mollusks of the genus *Conus*, comprising approximately 500 species. Cone snails hunt a diverse array of prey animals, and specific cone snail species may hunt fish, polychaete worms or other snails. To accomplish this task, cone snails produce a complex venom composed of peptide toxins, known as conopeptides or conotoxins, with high selectivity for many of the receptors and ion channels found in their prey [4, 5]. This pharmacological profile, coupled with small size and structural stability, make the conotoxins promising candidates for development as therapeutic compounds [1, 2, 6-8].

Cone snails produce conotoxins in a venom duct and inject them into prey through a long, distensible proboscis and finally through a barbed hollow tooth that serves as both harpoon and hypodermic needle [4, 5]. Each of the 500 different *Conus* species produces a venom containing 50-200 different biologically active peptides. Peptides found in one species of cone snail are distinct from peptides found in other species. Typical conotoxins are small (12-30 amino acids) and exhibit a highly constrained structure stabilized by intramolecular disulfide bridges and posttranslational modifications. Conotoxins are typically grouped into families based on sequence homology, cysteine bond structure, and function [4, 5, 9]. Most families of conopeptides described to date target receptors and ion channels associated with nervous and muscle tissue. For example,  $\alpha$ -conopeptides target specific subtypes of nicotinic receptors [10],  $\delta$ -conopeptides delay inactivation [11] whereas  $\mu$ - and  $\mu$ O-conopeptides block voltage-gated sodium channels ( $\text{Na}_V$ ) [12, 13],  $\sigma$ -conopeptides block type 3 serotonin receptors (5-HT<sub>3</sub>Rs) [14],  $\omega$ -conopeptides block subtypes of voltage-gated calcium channels ( $\text{Ca}_V$ ) [15, 16],  $\kappa$ - and  $\kappa$ A-conopeptides block voltage-gated "Shaker" potassium channels ( $\text{K}_V$ ) [17, 18], conantokins inhibit N-methyl-D-aspartate receptors (NMDARs) [19-22],  $\chi$ -conopeptides inhibit the norepinephrine transporter [23], conopressins are agonists at vasopressin receptors [24],  $\rho$ -conopeptides inhibit  $\alpha$ -adrenergic receptors [25], and contulakin-G is an agonist at neurotensin receptors [26].

The therapeutic potential of peptide drugs is well recognized, however, endogenous peptides like the enkephalins are often poor drug candidates due to rapid degradation and poor biodistribution. While these properties of endogenous peptides are consistent with and essential for their biological roles as molecular signals, they are problematic properties for therapeutic drugs. In important ways, the problems faced by physicians intending to use peptides as human therapeutics are similar to the problems faced by cone snails using peptides to capture their prey. Both groups of users seek to alter the behavior of excitable tissues in the recipient, and both groups of users encounter obstacles to this goal. The peptides in each case must enter the body of the recipient, avoid premature metabolism and excretion, find their way into the physiological space wherein resides the target protein, and produce a selective action at that target of sufficient duration to accomplish the intended task (producing a beneficial response in a patient or incapacitating a prey species until digested). In contrast to

physicians, cone snails have had the benefit of 50 million years of natural selection to find solutions to these problems. Thus, relative to endogenous neurotransmitter peptides, many conotoxins are endowed with superior pharmaceutical properties.

There are, however, important distinctions between a physician's patient and a cone snail's prey. Target proteins in prey species may be similar to target proteins in humans, but small differences may alter the potency, selectivity, or efficacy of the conotoxin. In addition, the target protein may subserve functions in a prey species that are distinct from those in a patient, and may be found in protected physiological spaces of patients, like the central nervous system (CNS). Thus, while conotoxins have intrinsic properties that may be advantageous with respect to drug development, some clinical indications will be more amenable to therapy with conotoxins than others. We will review the pharmacology of those conotoxin families with potential clinical application, and discuss the unique challenges that each will face in the course of their transition from venom component to human therapeutic.

## 2. Therapeutic Applications

### 2.1. Pain

Great progress has been made in the last 30 years toward understanding the neural substrates of pain and identifying novel molecular targets for analgesic drug development. Fortuitously, since many of these targets also figure prominently in the envenomation strategies of cone snails, a number of conotoxins with analgesic properties have been identified.

Pain is a form of somatic sensation that occurs in response to actual or potential tissue damage (for review see [27]). Conotoxins act at various locations in the sensory systems that mediate pain, including the periphery, the spinal levels, and higher CNS centers. The sensation of pain is initiated by noxious chemicals or extremes of temperature or pressure that activate a variety of proteins in the terminals of specific peripheral nerve fibers, called nociceptors, resulting in depolarization and the generation of action potentials. Peripheral nociceptors (thinly myelinated A $\delta$  fibers and unmyelinated C fibers) have cell bodies in the dorsal root ganglia and trigeminal ganglia and terminate largely in the various layers comprising the dorsal horn of the spinal cord. Neurotransmitters released from peripheral nociceptors activate receptors on spinal dorsal horn neurons which project through several ascending pathways to the brainstem and thalamus and ultimately to the cerebral cortex. In addition, pain information from peripheral nociceptors is modulated by input from other sensory fibers, local interneurons, and projections descending from the brain stem.

Pain states fall into two broad categories, acute and chronic. While unpleasant, acute pain is important for survival. It alerts an organism to important, potentially life threatening problems that need attention. Acute pain is also transient, and resolves upon either removal of tissue from the noxious stimulus that is causing the pain, or healing of tissue damaged by the noxious stimulus. Acute pain, such as pain following surgery, is generally well controlled by existing drugs like nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids. Chronic pain, in contrast, does not serve an obvious survival function. It is best considered as a disorder resulting from a dysfunctional reorganization of the pain system, and persists for long periods of time, often long after an initial injury has healed.

Chronic pain syndromes include spinal cord injury pain, low back pain, cancer pain, complex regional pain syndrome, post herpetic neuralgia, diabetic neuropathy, carpal tunnel syndrome, HIV/AIDS neuropathy, phantom limb pain, and trigeminal neuralgia. Unfortunately, current treatment strategies for chronic pain syndromes often lack efficacy or are associated with undesirable side effects. There remains a great need for effective treatments for chronic pain states. Ideally, new treatments for chronic pain states will have high anatomic or pharmacologic specificity to avoid the side effects that limit the use of drugs like opioids. Since some conotoxins have high selectivity for pain targets, and can be coupled with appropriate administration strategies such as intraspinal infusion or subcutaneous injection, they are well suited for use as analgesics for chronic pain. One conotoxin has been approved for use as an analgesic, several other conotoxins are in development, and still more are being explored for this indication (Table 1). These conotoxins are reviewed below.

**Table 1.** Amino Acid Sequences of Analgesic Conopeptides.

| Name                            | Sequence <sup>1</sup>           | <i>Conus</i> Species |
|---------------------------------|---------------------------------|----------------------|
| MVIIA<br>(Prialt <sup>®</sup> ) | CKGKGAKCSRLMYDCCTGSCRSGKC*      | <i>C. magus</i>      |
| CVID<br>(AM336)                 | CKSKGAKCSKLMYDCCSGSCSGTVGRC*    | <i>C. catus</i>      |
| Contulakin-G<br>(CGX-1160)      | ZSEEGGSNAT <sub>g</sub> KKPYIL* | <i>C. geographus</i> |
| MrIA<br>(Xen-2174) <sup>a</sup> | NGVCCGYKLCHOC*                  | <i>C. marmoreus</i>  |
| Conantokin-G<br>(CGX-1007)      | GEXXLQXNQXLIRXKSN*              | <i>C. geographus</i> |
| Vc1.1<br>(ACV-1)                | GCCSDPRCNYDHPEIC*               | <i>C. victoriae</i>  |
| MrVIB<br>(CGX-1002)             | ACSKKWEYCIVPILGFVYCCPGLICGPFVCV | <i>C. marmoreus</i>  |

O is 4-trans-hydroxyproline; \* indicates amidated C-terminus; Z is pyroglutamate; T<sub>g</sub> is threonine with the disaccharide Gal(β1→3)GalNAc(α1→) attached; <sup>a</sup> Xen2174 is a more chemically stable analogue of MrIA.

### 2.1.1. Conotoxins acting on Spinal Pain Targets

The dorsal horn of the spinal cord is an important site for processing pain information, and is rich in analgesic targets for conotoxin drugs. Since the spinal cord, like the rest of the CNS, lies within the privileged domain protected by the blood-brain-barrier, delivery of conotoxins to the CNS is a challenge requiring special technology.

An available technology for delivery of conotoxins to the CNS is intrathecal drug infusion. Intrathecal drug infusion systems utilize fully implantable pumps that continuously deliver drugs

through catheters placed into the intrathecal space. Intrathecal drug infusion has been used for several years and has proven to be safe and effective means for delivering drugs for pain, spasticity and dystonia, chemotherapeutics, and contrast media for visualizing pathology in patients with spinal disorders. Since intrathecal infusion requires the surgical implantation of a pump and catheter, this means of delivery is only used for chronic pain that is unresponsive to orally or intravenously administered agents. However, intrathecal delivery has several advantages for analgesic drugs. First, intrathecal delivery concentrates the analgesic drug within the spinal cord where analgesic actions take place, minimizing concentrations at distant sites in the brain and periphery where side effects are often mediated [28]. Second, drugs are continuously infused, allowing constant delivery of the minimally effective drug concentration, and avoiding the fluctuations in drug concentrations typical of intermittent oral dosing. Third, constant infusion through an implanted pump results in greater patient compliance.

Despite the advantages of intrathecal infusion, existing intrathecal therapies such as opioids are not effective in all patients and still have significant side effect profiles. Conotoxins may therefore represent safe and effective non-opioid intrathecal therapies for the treatment of chronic intractable pain.

#### 2.1.2. MVIIA (Prialt®)

Nervous systems exist largely to enable organisms possessing them to respond quickly and appropriately (i.e. in ways that promote their survival) to changes in their environments. Neurons, the cells that comprise nervous systems, rely on rapid changes in transmembrane electrical potential to communicate detection of environmental changes and instructions for appropriate response. Transmembrane electrical potential is controlled by large protein assemblies, called ion channels, which allow passage of distinct sets of ions into and out of the cell. Critical to this process is the movement of calcium ions through voltage-gated calcium channels. Calcium channels respond to decreases in membrane potential (depolarization) by allowing calcium ions to flow into the cell. Calcium influx is critical for many cellular processes, including the initiation of neurotransmitter release. A variety of  $Ca_v$  channels exists (including L-, P-, Q-, R-, and N-type channels), each with distinct protein subunits, physiological and pharmacological properties. N-type  $Ca_v$  channels are densely localized in the dorsal horn of the spinal cord [29, 30], and are well positioned to modulate the release of neurotransmitters from primary nociceptive neurons.

MVIIA (at various times also called SNX-111, ziconotide, and most recently, Prialt®) was first isolated from the venom of *C. magus* [31] and is a member of the  $\omega$ -conotoxin family that inhibits presynaptic neurotransmitter release [32, 33] through blockade of N-type  $Ca_v$  channels [34, 35]. MVIIA has been demonstrated to attenuate nociception in a variety of animal models (for review see [36]), including models of persistent pain (i.e. the formalin test) [37-39], post-operative pain [40], chronic inflammatory pain [41], and neuropathic pain [37, 42, 43]. MVIIA was effective in morphine tolerant rats [44], and prolonged (7 days) intrathecal infusion of MVIIA did not produce tolerance to its analgesic effects [39, 44].

Based on this preclinical profile, MVIIA has been studied extensively in the clinic. Prior to its approval by the FDA in December of 2004 for the management of severe chronic pain in patients who

are refractory to other analgesic treatments, MVIIA was administered to over 1,200 patients in three double-blind, placebo controlled multi-center studies and four open label long-term studies [45]. In the largest reported study, a double-blind, randomized, placebo-controlled trial in 111 cancer or AIDS patients with treatment refractory chronic pain, intrathecal MVIIA provided clinically and statistically significant analgesia. Specifically, to be eligible for this study, patients needed to have VASPI (Visual Analog Scale of Pain Intensity) scores of 50 mm or greater. The VASPI is a pain assessment scale in which patients rate their pain on a scale of 0 mm (no pain) to 100 mm (worst pain imaginable). Of the evaluable population, mean VASPI scores improved 53.1% in the MVIIA group and 18.1% in the placebo group. Five patients receiving MVIIA achieved complete pain relief, and 50.0% of patients receiving MVIIA responded to therapy compared with 17.5% of those receiving placebo [46]. Clinical experience with MVIIA has revealed a significant side effect profile for MVIIA, which includes abnormal gait, dizziness, nystagmus, confusion, somnolence, fever, postural hypotension, urinary retention, nausea, and vomiting [46]. However, these side effects can be minimized by starting with a low beginning dose of intrathecal MVIIA followed by a slow titration [45].

### 2.1.3. CVID (AM336)

While MVIIA is effective, its side effect profile will likely limit its clinical use. CVID, first isolated from *C. catus*, represents another  $\omega$ -conotoxin selective for N-type  $Ca_v$  channels [47]. Consistent with a role as an antinociceptive drug, CVID was shown to inhibit potassium ion-evoked release of the nociceptive neurotransmitter, substance P from rat spinal cord slices [48]. Based on its preclinical profile to date, CVID, also called AM336, may have a superior side effect profile relative to MVIIA, and is in clinical development for chronic pain management.

CVID was effective in the spinal nerve ligation model of neuropathic pain in the rat, and showed a higher ratio of efficacy to behavioral toxicity than GVIA and MVIIA [49]. In addition, intrathecal administration of CVID was effective in a model of chronic inflammatory pain and doses producing side-effects were 10-fold larger than the doses required to produce antinociception [48]. It is possible that the greater therapeutic ratio of CVID relative to MVIIA is due to greater selectivity for an N-type  $Ca_v$  channel variant expressed in preganglionic nerve terminals [50].

The preclinical efficacy of MVIIA and CVID, coupled with the clinical success and FDA approval of MVIIA, has demonstrated that  $\omega$ -conotoxins (and conotoxins in general) can be developed as human therapeutics, and has validated the N-type  $Ca_v$  channel as an important target for future analgesic development.

### 2.1.4. Contulakin-G (CGX-1160)

Neurotensin is an endogenous, 13 amino acid neuropeptide that functions as a neurotransmitter in the CNS and hormone in the periphery. Neurotensin produces a number of effects upon central administration including hypothermia, modulation of dopamine transmission, and analgesia [51]. Three neurotensin receptor subtypes have been identified, including the G-protein coupled receptors NTS1 and NTS2, and NTS3, a single transmembrane spanning protein identical to gp95/sortilin [51]. Neurotensin and its receptors are localized in several regions of the CNS including those important for

spinal processing of pain information such as the superficial layers of the dorsal horn [52-54] and the dorsal root ganglia [55]. Intrathecal administration of neurotensin is effective in models of acute pain [56-58].

CGX-1160 (Contulakin-G) is a synthetic 16 amino acid O-linked glycopeptide originally isolated from the venom of *C. geographus* [26]. CGX-1160 appears to be an agonist at neurotensin receptors since the last six amino acids in the C-terminal portion of CGX-1160 are homologous to those in the C-terminal portion of neurotensin, CGX-1160 inhibits the binding of [<sup>3</sup>H]neurotensin to its receptors, and upon central injection CGX-1160 produces a behavioral syndrome in mice that is similar to that produced by neurotensin [26].

Interestingly, while neurotensin was a far more potent inhibitor of [<sup>3</sup>H]neurotensin binding than CGX-1160 at rat neurotensin receptors rNTR1 and rNTR2, and the mouse neurotensin receptor mNTR3, CGX-1160 was 1 to 2 orders of magnitude more potent in an *in vivo* assay (a visually rated assessment of locomotor activity) following intracerebroventricular administration to mice [26]. This finding is supported by results in the mouse formalin test, a preclinical model of analgesic efficacy, indicating that CGX-1160 is more potent than neurotensin by greater than 100-fold [59]. CGX-1160 has subsequently been shown in mice to potently produce full efficacy in models of chronic inflammatory and neuropathic pain with a wide margin of safety [60]. In rats, intrathecal administration of CGX-1160 through a chronic lumbar intrathecal catheter was effective in the formalin test with no changes in motor function or corneal reflexes seen at doses tested [61]. In the thermal (62.5°C) skin twitch test in dogs, CGX-1160 produced a dose-dependent increase in the skin twitch latency lasting for approximately 6 h. In this study, no changes in motor function, body temperature, heart rate, or blood pressure were seen at any dose tested [61]. These results indicate that intrathecal CGX-1160 has analgesic efficacy in mice, rats, and dogs at doses that do not elicit motor or cardiovascular side effects.

CGX-1160 has been evaluated in two Phase I clinical trials. The first clinical trial was a randomized, double-blind, placebo-controlled study to investigate the safety and pharmacokinetics of a single intravenous infusion of CGX-1160 in healthy volunteers. No serious adverse events occurred and no subjects discontinued the study due to adverse events. This study demonstrated that CGX-1160 was safe and well tolerated in this normal healthy population. The second clinical study was an open label, dose-escalation study designed to investigate the safety, cerebrospinal fluid pharmacokinetics, and analgesic activity of intrathecally administered CGX-1160 in subjects with central pain following spinal cord injury. CGX-1160 was well tolerated in this study, and patients reported pain relief, a reduction in pain intensity, and a decrease or absence of allodynia. While these promising results are consistent with the results from nonclinical studies, analgesic efficacy remains to be confirmed in randomized, double-blind, placebo-controlled studies.

#### 2.1.5. MrIA (and Xen-2174)

Descending noradrenergic projections from brain stem nuclei form part of an endogenous pain control system. These descending neurons release the neurotransmitter norepinephrine and suppress the activity of nociceptive neurons in the dorsal horn. Inhibition of norepinephrine reuptake has long been recognized as a strategy for the treatment of neuropathic pain, and tricyclic antidepressants (TCAs) have been used for this purpose for many years. Unfortunately, many patients are

unresponsive to these drugs, or are unable to tolerate their side effects (typically orthostatic hypotension, dry mouth, and sedation).

Recently,  $\chi$ -conopeptide MrIA ( $\chi$ -MrIA), a 13-residue peptide originally isolated from the venom of *C. marmoreus* [62], was shown to inhibit the activity of norepinephrine transporter [63]. This inhibition was selective, since  $\chi$ -MrIA had no effect on the activity of the dopamine or serotonin transporters [23]. The binding site  $\chi$ -MrIA appears to overlap with that for TCAs, since the conopeptide competitively inhibited the binding of [<sup>3</sup>H]nisoxetine and [<sup>3</sup>H]mazindol to the expressed rat and human norepinephrine transporter [23]. However, a comparison of the interactions of  $\chi$ -MrIA, desipramine, and cocaine with human norepinephrine transporter mutants revealed that while the binding sites overlap, they are not identical [64]. The solution structure of  $\chi$ -MrIA has also been determined using NMR spectroscopy [65].

Consistent with its mechanism of action,  $\chi$ -MrIA has been shown to produce analgesia in several preclinical pain models. The conopeptide produced potent and dose-dependent analgesia in the hot plate test following intrathecal administration, at doses that did not produce motor impairment as measured by the rotarod test [62]. Intrathecal  $\chi$ -MrIA also reduced mechanical allodynia in rats with a chronic constriction injury of the sciatic nerve, a model of neuropathic pain [66].

Although the sequence has not been made public, Xen2174 is a more chemically stable analogue of  $\chi$ -MrIA that also inhibits the norepinephrine transporter. Intrathecal Xen2174 reversed the mechanical allodynia induced in rats in two models of neuropathic pain, the chronic constriction injury of the sciatic nerve and the L5/L6 spinal-nerve injury. At doses that reversed the allodynia, side-effects were mild for both  $\chi$ -MrIA and Xen2174 [66]. Intrathecal administration of Xen2174 also reduced the tactile hypersensitivity that develops following paw incisional surgery in rats, a model of post-operative pain. Finally, these data suggest that intrathecal administration of Xen2174 at the time of spinal anesthesia might produce postoperative analgesia in humans [67]. These results suggest that  $\chi$ -conopeptides may represent a new treatment for post-operative pain, and, due to their selectivity, may provide relief to some chronic pain patients with fewer side effects than conventional TCAs like nortriptyline and desipramine. These results also demonstrate that chemical stability limitations of some natural conotoxins (like  $\chi$ -MrIA) can be circumvented by the synthesis of more stable analogs (like Xen2174).

#### 2.1.6. Conantokins (i.e. CGX-1007)

NMDA receptors comprise a large family of heterooligomeric ligand-gated ion channels that contain multiple, allosterically coupled recognition sites for the neurotransmitter glutamate, the co-neurotransmitter glycine, as well as for polyamines, ions and use-dependent channel blockers (for review see [68]). Activation of NMDARs expressed on neurons results initially in calcium influx, which subsequently can influence gene activation, neuronal survival, axon outgrowth, and synaptic strength. Activation of NMDARs also plays a major role in central sensitization, a phenomenon initiated by excessive activity of peripheral nociceptors (such as occurs following nerve or tissue injury) and a major component of chronic pain states [69]. Native NMDARs are composed of subunits from three families, NR1 (a single subunit with eight known splice variants), NR2 (four different subunits: NR2A, NR2B, NR2C and NR2D), and NR3, with different subunit compositions resulting in

different functional and pharmacological characteristics [70-72] (for more details see the introductory minireview by Arias in this special issue). In addition, distinct NMDARs comprised of different subunits display more restricted tissue distributions [73-76]. The development of selective inhibitors for distinct subtypes of NMDARs represents a promising strategy for the development of NMDAR antagonists with improved efficacy and side effect profiles.

NMDAR antagonists are effective in animal models of peripheral tissue or nerve injury, and have demonstrated efficacy in man, however, unacceptable side effects have limited their use. Recently, due to a more favorable side effect profile, antagonists with selectivity for NMDARs containing NR2B subunits have emerged as potential treatments for persistent pain states [77]. One reason for the improved therapeutic index of NR2B selective antagonists may be the more restricted localization of the NR2B subunit to nociceptive primary afferents (sensory neurons carrying pain information) and the neurons of dorsal horn [78, 79].

The conantokins are a novel family of post-translationally modified, small peptide (17-27 amino acids long) antagonists of the NMDAR that are found in a variety of cone snail venoms and exhibit a range of potencies and selectivities at distinct NMDARs [80]. Conantokin-G (also referred to as CGX-1007), the most extensively characterized conantokin, selectively inhibits NMDARs containing the NR2B subunit [81, 82].

Conantokins-G and -T were effective in several mouse models of persistent pain (formalin test), chronic inflammatory (complete Freund's adjuvant induced allodynia), and neuropathic pain (partial sciatic nerve ligation allodynia) [83]. In the formalin test, for both conantokin-G and conantokin-T the effective dose (ED<sub>50</sub>) was well below the toxic dose (TD<sub>50</sub>), and efficacy was observed for four hours after administration [83]. These data indicate that the conantokins represent a class of NMDAR inhibitors with potential utility in the control of chronic pain states with fewer side effects relative to non-subtype selective NMDAR antagonists.

Anticonvulsant drugs are often used in the treatment of chronic pain. Furthermore, in addition to their major role in initiating and sustaining pain states, the release of glutamate and subsequent activation of NMDARs also play critical roles in the initiation and propagation of seizure activity. Accordingly, the conantokins have also been shown to be effective anticonvulsants in a variety of preclinical models of seizure disorders. In Frings audiogenic seizure susceptible (AGS) mice, intracerebroventricular administration of either conantokin-G or -R potently suppressed audiogenic seizures at doses that did not impair rotorod performance [84, 85]. In CF#1 mice, intracerebroventricular administration of either conantokin-G or -R was effective in suppressing seizures induced by threshold tonic extension, maximal electroshock, and subcutaneous pentylenetetrazol. Anticonvulsant effects occurred at doses well below those that produce rotorod impairment [84, 85]. Conantokin-G was also effective in suppressing seizures induced by subcutaneous picrotoxin and subcutaneous bicuculline [85]. Furthermore, chronic infusion of conantokin-G in Frings AGS mice for 28 days *via* an osmotic pump system produced no cumulative toxicity or tolerance to the anticonvulsant effect [85]. In rats, conantokin-G blocked maximal electroshock seizures and fully expressed corneal-kindled seizures, but failed to prevent the acquisition of corneal-kindled seizures [85]. In amygdala kindled rats, conantokin-G dose dependently blocked the expression of secondarily generalized seizures, while another NR2B selective NMDAR antagonist, CI-1041, was ineffective [86]. Similar to the results in corneal-kindled rats, neither conantokin-G nor CI-

1041 were able to prevent or delay acquisition of amygdala kindling [86]. Overall, these studies indicate that conantokins display a broad efficacy profile in experimental models of both pain and seizure disorders with a larger separation between effective and toxic doses than other NMDAR antagonist drugs.

#### 2.1.7. Conotoxins Acting on Systemic Pain Targets

Not all analgesic conotoxins will require intrathecal delivery. A variety of pain targets exist in the periphery, and conotoxin families that interact with these targets have been identified. Since these targets are accessible following parenteral routes of administration such as intravenous, subcutaneous, and intramuscular administration. Appropriate coupling with sustained release formulations or chronic parenteral delivery devices would make sustained delivery of conotoxins medically feasible.

#### 2.1.8. Vc1.1 (ACV-1)

Neuronal nicotinic receptors (AChRs), ligand-gated ion channels composed of pentameric assemblies of  $\alpha$  ( $\alpha 2$  through  $\alpha 10$ ) and  $\beta$  ( $\beta 2$  through  $\beta 4$ ) subunits, are expressed in many regions of the central and peripheral nervous systems, and are known to play an important role in modulating pain pathways. Nicotinic agonists such as epibatidine (originally isolated from the skin of the Ecuadorian tree frog, *Epipedobates tricolor*), and its analogs like ABT-594 produce antinociception in animal models [87, 88], and mice lacking  $\alpha 4$  and  $\beta 2$  subunits demonstrate reduced antinociception in response to administration of nicotinic agonists [89].

Many species of cone snails utilize antagonists of neuronal AChRs in their venoms [90]. The largest family of conopeptides targeting the neuronal AChR is the  $\alpha$ -conopeptides [91]. Interestingly, an  $\alpha$ -conopeptide from *C. victoriae*, Vc1.1 (ACV-1), shows potent analgesia in a number of animal models. Like other  $\alpha$ -conopeptides, Vc1.1 inhibits the binding of [ $^3$ H]epibatidine to neuronal AChRs and inhibits nicotine-induced catecholamine release from bovine adrenal chromaffin cells [92]. Local perfusion of Vc1.1 into the footpads of rats was also able to inhibit the vascular inflammatory response activated by antidromic electrical stimulation of C-fibers, suggesting an ability to inhibit C-fiber function [92]. It is possible that Vc1.1 is inhibiting C-fiber function through an action on the neuronal AChRs that are found on C-fibers, including human C-fibers [93].

In *in vivo* studies, intramuscular injection of Vc1.1 suppressed pain behaviors (mechanical hyperalgesia) in two rat models of neuropathic pain, the chronic constriction injury model and the partial sciatic nerve ligation model. Seven days of treatment with Vc1.1 (after the development of hyperalgesia) resulted in a reduction of mechanical hyperalgesia in both models for up to a week following cessation of treatment. Vc1.1 also accelerated functional recovery of injured neurons as measured by the ability of the terminals to mount an inflammatory vascular response upon perfusion of the blister base (raised over the footpad innervated by the injured nerve) with substance P [94].

#### 2.1.9. Conotoxin Inhibitors of Voltage-Gated Sodium Channels

$\text{Na}_V$  channels control membrane excitability in neurons by allowing sodium ions to enter the cell, resulting in depolarization and propagation of action potentials. The family of  $\text{Na}_V$  channels comprises

a variety of related proteins with distinct physiological and pharmacological properties. Nav channel subtypes also differ in their tissue expression patterns, with specific subtypes localized to brain regions, skeletal muscle, peripheral neurons, and heart. Local anesthetic drugs work by blocking these channels and interrupting the flow of action potentials in neurons carrying nociceptive (and other sensory) information from the periphery to the CNS. Local anesthetics also produce toxic side effects by interacting with sodium channels in non-nervous tissue such as cardiovascular muscle.

Certain Nav channel subtypes are expressed selectively in nociceptors, and represent attractive targets for drug development. These subtypes include the tetrodotoxin (TTX)-resistant subtypes Nav1.8 and Nav1.9, the TTX-sensitive subtype Nav1.7, as well as Nav1.3, a TTX-sensitive channel that up-regulates in damaged peripheral nerves (for review see [95]). The development of compounds with improved selectivity at these specific Nav channels is proceeding rapidly (see reviews in this special issue) and holds great promise toward the treatment of a variety of pain states.

Several conotoxins have been identified that interact with these Nav channel subtypes that are expressed selectively in nociceptors. The  $\mu$ O-conotoxins MrVIA and MrVIB, from *C. marmoratus*, inhibit transient TTX-resistant sodium current in rat dorsal root ganglion neurons, as well as TTX-sensitive sodium currents [96]. The  $\mu$ -conotoxins SIIIA (from *C. striatus*), KIIIA (from *C. kenoshitai*), and SmIIIA (from *C. stercusmuscarum*) inhibit TTX-resistant sodium currents in neurons from frog sympathetic and dorsal root ganglia but only poorly block action potentials in frog skeletal muscle that are mediated by TTX-sensitive sodium channels [97, 98]. Finally, crude venom from *C. ventricosus* is able to produce a selective block of TTX-resistant Nav1.8 currents in rat dorsal root ganglion neurons [95]. These studies indicate that sodium channel-inhibiting conotoxins may represent systemically active analgesic candidates.

## 2.2. Stroke

Stroke is a leading cause of death and adult disability, including cognitive and functional impairment, in the United States and around the world. Despite intensive research, the development of safe and effective drugs for the treatment of stroke has yet to meet with much clinical success. The cognitive and functional impairment that follows ischemic stroke results from occlusion of blood vessels in the brain, often a consequence of atherosclerotic plaque formation or blood clots originated in the heart. Blood vessel occlusion results in loss of oxygen and glucose supply, depletion of brain ATP, and the consequent loss of ability to maintain and restore ionic gradients in neurons. As neurons depolarize, intracellular calcium concentrations rise to toxic levels, glutamate is released and extracellular glutamate increases, and the cycle of depolarization is exacerbated. Neurons in the core of the infarcted region swell and burst, resulting in the initiation of inflammatory processes. The death of cells in the core of the infarct initiates a progressive series of degenerative events, including secondary apoptotic processes, in the neuronal tissue surrounding the core (the penumbra). The neuronal damage and cell death originally confined to the core expands over time to the much larger surrounding penumbral region.

Since calcium influx through both Cav channels and NMDARs has been implicated in neuronal loss following ischemic stroke, compounds that inhibit these targets remain promising candidates for stroke treatment.  $\omega$ -Conotoxins and conantokins inhibit Cav channels and NMDARs respectively, and have

shown efficacy in animal models of stroke. Importantly, these peptides can be administered *via* acute bolus injections or systemic infusions, both feasible approaches to drug delivery upon diagnosis of acute stroke. These studies are reviewed below.

### 2.2.1. MVIIA (Prialt®)

The synthetic  $\omega$ -conotoxin MVIIA is effective in a variety of preclinical models of stroke (reviewed in [36]). Intravenous administration of MVIIA was neuroprotective in a model of transient forebrain ischemia, the rat model of four-vessel occlusion, even when administered 24 h after ischemia [99, 100]. Similarly, intravenous administration of MVIIA was neuroprotective in the rat middle cerebral artery occlusion (MCAo) model of focal cerebral ischemia [99, 101], and in a rabbit model of focal cerebral ischemia [102].

In rats with permanent occlusions of the right middle cerebral and right common carotid arteries and transient occlusion of the left common carotid artery, intravenous infusion of MVIIA reduced the cortical volume of infarction as well as the amount of extracellular glutamate [103]. Based on these results, a phase I safety study of intravenous MVIIA in healthy volunteers was performed [104]. The major side effect observed in this study was orthostatic hypotension. Phase II neuroprotective efficacy trials of MVIIA were suspended [36].

### 2.2.2. Conantokins (i.e. CGX-1007)

Activation of NMDARs is also a critical contributor to the dangerous rise in intracellular calcium concentrations during ischemia. While NMDAR antagonists have been successful in animal models of stroke (focal ischemia), they have demonstrated a lack of efficacy or unacceptable levels of side effects in the clinic [105, 106]. Despite these results, studies in animal models of stroke suggest that conantokins may still be effective in the clinic.

The most extensively studied conantokin, conantokin-G (also called CGX-1007), produced a dose-dependent and complete neuroprotection of primary cultures of rat cerebellar neurons against neuronal injury produced by hypoxia/hypoglycemia, NMDA, glutamate, or veratridine [107]. Subsequently, intracerebroventricular administration of conantokin-G provided significant neuroprotection in the rat MCAo model of focal cerebral ischemia, along with significant improvement in neurological score at 24 h post-occlusion [107]. Intrathecal delivery represents a more feasible route of administration in an emergency room situation following a stroke. In the rat MCAo model of focal cerebral ischemia, intrathecal administration of conantokin-G reduced infarct volume assessed at 24 h post-occlusion. Moreover, intrathecal administration of conantokin-G beginning at 8 h post-occlusion also significantly reduced core infarct size and improved the neurological score [108].

One possible explanation for the clinical failure of some NMDAR antagonists in stroke trials is an inability to reduce secondary apoptotic processes that lead to delayed cell death. The protein kinase inhibitor staurosporine induces apoptotic cell death in primary cultures of rat forebrain neurons, defined morphologically by cell shrinkage, chromatin condensation, and appearance of apoptotic bodies. In primary cultures of rat forebrain neurons, conantokin-G inhibited staurosporine-induced cell damage [109]. Changes in gene expression may also contribute to delayed cell death following

ischemic brain injury. Intracerebroventricular administration of conantokin-G reduced the expression of *c-fos* mRNA (a gene associated with delayed cell death), increased Bcl-2 immunoreactivity (an anti-apoptotic gene), and reduced DNA fragmentation (a hallmark of apoptosis), following occlusion of the middle cerebral artery in rats [110].

The ability of conantokin-G to reduce core infarct size in rats when given up to 4 to 8 h post-occlusion [107, 108], coupled with the unique ability (relative to non-selective NMDAR antagonists like MK-801) to reduce staurosporine-induced apoptotic cell death [109], and the ability to up-regulate the expression of the anti-apoptotic protein Bcl-2 [110], indicates that conantokins may represent an important therapeutic tool in the treatment of ischemic stroke, and should theoretically be effective in hemorrhagic stroke where current “clot-busting” drugs are contraindicated.

Since conantokin-G is effective in animal models of stroke, reduces staurosporine-induced apoptotic cell death, up-regulates the expression of the anti-apoptotic protein Bcl-2, and exhibits a favorable preclinical safety profile, conantokins may represent promising compounds for use in the acute treatment of stroke.

### 2.3. Neuromuscular Block

Neuromuscular blocking drugs inhibit the actions of acetylcholine on AChRs of the neuromuscular junction through either depolarization block (i.e., succinylcholine) or competitive antagonism (i.e., d-tubocurarine). They are used to provide muscle relaxation during surgery, to facilitate positive pressure ventilation during and after anesthesia in the intensive care unit, and to facilitate tracheal intubation [111]. Neuromuscular blocking drugs facilitate tracheal intubation by relaxing the muscles of the larynx. Many situations, including trauma, emergency cesarean section, crises in intensive care, and unconscious patients at risk for vomiting and aspiration, require rapid protection of the airway by tracheal intubation to insure adequate ventilation. Due to its fast onset and short duration of action, succinylcholine is the drug of choice for facilitating tracheal intubation. Unfortunately, succinylcholine also elicits many undesirable side effects including fasciculations (rippling, uncoordinated muscle contraction), postoperative muscle pain, hyperkalemia (which can elicit severe cardiovascular consequences), vagally mediated bradycardia, histamine-induced hypotension and anaphylaxis, and a life threatening malignant hyperthermia. There is therefore a major need for alternatives to succinylcholine.

The primary paralytic toxins in fish hunting cone snails of the Indo-Pacific region comprise a subclass of the  $\alpha$ -conopeptides. Known as  $\alpha$ 3/5 conotoxins, they are characterized structurally by two conserved disulphide bridges which form two loops of three and five amino acids (Table 2). They produce immobilization of prey through highly selective competitive inhibition of AChRs of the neuromuscular junction [90]. The  $\alpha$ 3/5 conotoxins display distinct binding site selectivity despite a relatively high degree of homology. For example, conopeptides MI and GI are ~10,000 times more potent for the  $\alpha/\delta$  interface than for the  $\alpha/\gamma$  interface mammalian neuromuscular AChRs. Conopeptide SI, on the other hand, is less potent and does not distinguish between these two agonist/competitive antagonist binding sites [112-115]. At nicotinic receptors from *Torpedo* electric organ, the selectivity of  $\alpha$ -conopeptide MI is reversed, with much higher affinity for the  $\alpha/\gamma$  interface [113, 114]. Due to their selectivity and mechanism of competitive blockade, the  $\alpha$ 3/5 conotoxins may represent new

neuromuscular blocking drugs that lack the side-effects of depolarizing neuromuscular blockers like succinylcholine.

**Table 2.** Amino Acid Sequences of some  $\alpha 3/5$ -Conotoxins.

| Name | Sequence <sup>1</sup> | Conus Species        |
|------|-----------------------|----------------------|
| GI   | ECCNPACGRHYSC*        | <i>C. geographus</i> |
| MI   | GRCCHPACGKNYSC*       | <i>C. magus</i>      |
| SI   | ICCNPACGPKYSC*        | <i>C. striatus</i>   |

<sup>1</sup>The disulphide connectivity is C1-C3, C2-C4. \* indicates amidated C terminus.

In anaesthetized cats,  $\alpha$ -conopeptides GI and MI have previously been shown to produce neuromuscular blockade at doses of 20-80  $\mu\text{g}/\text{kg}$ . Both were rapidly reversed by the acetylcholinesterase inhibitor neostigmine. At doses that produced neuromuscular blockade, neither MI or GI had effects on arterial blood pressure, heart rate, or responses to vagal and preganglionic stimulation [116]. In addition, GI competitively blocked AChRs at neuromuscular junctions in the rat sciatic nerve-gastrocnemius muscle and frog abdominal muscle preparations. In this study, GI did not produce ganglionic blockade in the parasympathetic ganglion, or an anti-muscarinic effect [117]. These results further indicate the specificity of the  $\alpha 3/5$  conopeptides for nicotinic receptors of the neuromuscular junction.

While hundreds of neuromuscular blocking drugs have been synthesized since the first clinical use of d-tubocurarine in 1942 [118], none are ideal, particularly for facilitating intubation. The properties of  $\alpha 3/5$  conopeptides, however, have led to speculation that their analogs might represent rapidly biodegradable drugs which permit the use of high doses in order to gain rapid onset of action [119]. Importantly, the  $\alpha 3/5$  conopeptides are effective following acute intravenous injection, which is how the current generation of neuromuscular blocking drugs are administered. Since the therapeutic goal is a quick acting agent of short duration, potential susceptibility to peptidase degradation and rapid systemic clearance are advantages rather than liabilities.

#### 2.4. Cardioprotection

Acute myocardial infarction occurs when the blood supply to a part of the heart muscle (myocardium) is stopped. Often this is a result of a coronary thrombosis. Coronary thrombosis occurs when atherosclerotic plaques tear and trigger the formation of blood clots that block the coronary artery. The major treatment goal is to limit the size of the infarction by restoring the blood supply to the myocardium (reperfusion). This is currently accomplished pharmacologically with thrombolytic drugs or surgically through percutaneous coronary intervention in the catheterization laboratory. Despite these treatments, acute myocardial infarction resulting from coronary heart disease is a leading cause of death in the world. There is therefore a major need for drugs which can complement thrombolytic therapy or catheterization and further reduce the size of an infarction. Many drugs have

been demonstrated in preclinical models to limit infarct size. Unfortunately, these drugs either do not reduce infarct size when given after initiation of ischemia, or are associated with hypotension, and are therefore of limited usefulness in the clinic.

$\kappa$ -PVIIA, also called CGX-1051, is a 27 amino acid conopeptide originally isolated from the venom of *C. purpurascens* [17] that blocks  $K_V$  “Shaker” channels with high affinity [17, 120, 121]. In rabbits, rats, and dogs, acute intravenous administration of  $\kappa$ -PVIIA substantially reduces infarct size in models of acute myocardial infarction. Specifically, in rabbits, intravenous administration of  $\kappa$ -PVIIA 5 min before a 3 h reperfusion reduced the size of a myocardial infarct following a 30 min occlusion of a branch of the left coronary artery. The cardioprotection produced by  $\kappa$ -PVIIA was sustained even when reperfusion was permitted to last for 72 h, indicating that the peptide prevents, rather than delays, the loss of myocardium [122]. In rats, intravenous administration of  $\kappa$ -PVIIA 5 min before a 3 h reperfusion reduced the size of a myocardial infarct following a 25 min occlusion of the left coronary artery [123]. Finally, in dogs, intravenous administration of  $\kappa$ -PVIIA 5 min before a 3 h reperfusion reduced the size of a myocardial infarct following a 60 min occlusion of a branch of the left anterior descending coronary artery [123]. Administration of  $\kappa$ -PVIIA caused no adverse alterations in cardiovascular hemodynamics at any dose tested in rabbits, rats, or dogs [122, 123].

While the mechanism underlying the cardioprotective efficacy of  $\kappa$ -PVIIA is unclear, it is unlikely to depend on circulating leukocytes since the peptide reduced infarct size in crystalloid-perfused, isolated rabbit hearts [122]. In addition, prior administration of either the mitochondrial  $K_{ATP}$  inhibitors glibenclamide or 5-hydroxydecanoate reversed the ability of  $\kappa$ -PVIIA to reduce infarct size in rabbits. Likewise, prior administration of the MEK1/2 inhibitor PD 98059 also reversed the effect of  $\kappa$ -PVIIA indicating that functionally active mitochondrial  $K_{ATP}$  channels and extracellular receptor kinase (ERK) are necessary for protection [122].

Based on these preclinical results,  $\kappa$ -PVIIA may represent a valuable adjunct to coronary artery thrombolytic therapy and percutaneous transluminal coronary angioplasty in the management of acute myocardial infarction. Importantly,  $\kappa$ -PVIIA is effective following acute intravenous injection just before the time of reperfusion. It remains to be seen if continuous intravenous infusion is more effective, but either mode of administration is feasible upon diagnosis of myocardial infarction and initiation of steps to restore cardiac perfusion.

### 3. Conclusions

The pharmacological variety of the conotoxins found in the venoms of marine cone snails, and their synthetic analogs, is well recognized. The ability of many conotoxins to distinguish between closely related subtypes of target proteins has led to their widespread use as pharmacological tools. Most conotoxins characterized to date target receptors and ion channels of excitable tissues, including such diverse targets as ligand-gated AChRs, NMDARs, and 5-HT<sub>3</sub>Rs, as well as  $Ca_V$ ,  $Na_V$ , and  $K_V$  channels, G-protein-coupled receptors including  $\alpha$ -adrenergic, neurotensin, and vasopressin receptors, and the norepinephrine transporter. Several conotoxins have shown promise in preclinical models of pain, convulsive disorders, stroke, neuromuscular block, and cardioprotection. Despite their promise in these models, conotoxins possess some characteristics, like size, charge, and susceptibility to peptidase degradation that can limit their clinical usefulness. However, when combined with appropriate

delivery technologies, such as intrathecal infusion or acute intravenous injection, conotoxins emerge as very promising strategies for the treatment of a variety of human pathologies. Moreover, conotoxin selectivity is useful in the validation of clinical targets, like the N-type Ca<sub>v</sub> channel for neuropathic pain. The unique pharmacological profiles of the conotoxins, adapted over millions of years as synergistic components of a sophisticated envenomation strategy, coupled with their safety and efficacy as demonstrated in preclinical models, has led to their investigation as human therapeutic agents. The recent approval by the FDA of MVIIA (Prialt®) for chronic pain indicates that the pharmaceutical challenges posed by conotoxins are surmountable, and holds out the hope that other safe and effective treatments for human pathologies remain to be found in the venom ducts of marine snails.

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