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Pain Therapeutics from Cone Snail Venoms: From Ziconotide to Novel Non-Opioid Pathways

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Abstract

There have been numerous attempts to develop non-opioid drugs for severe pain, but the vast majority of these efforts have failed. A notable exception is Ziconotide (Prialt®), approved by the FDA in 2004. In this review, we summarize the present status of Ziconotide as a therapeutic drug and introduce a wider framework: the potential of venom peptides from cone snails as a resource providing a continuous pipeline for the discovery of non-opioid pain therapeutics. An auxiliary theme that we hope to develop is that these venoms, already a validated starting point for non-opioid drug leads, should also provide an opportunity for identifying novel molecular targets for future pain drugs.

This review comprises several sections: the first focuses on Ziconotide as a therapeutic (including a historical retrospective and a clinical perspective); followed by sections on other promising *Conus* venom peptides that are either in clinical or pre-clinical development. We conclude with a discussion on why the outlook for discovery appears exceptionally promising. The combination of new technologies in diverse fields, including the development of novel high-content assays and revolutionary advancements in transcriptomics and proteomics, puts us at the cusp of providing a continuous pipeline of non-opioid drug innovations for pain.

Keywords

ziconotide; pain; cone snail venoms; non-opioid therapeutics; non-opioid pain pathways

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[1] Nomenclature. Nomenclature used for cone snails has not been applied uniformly. In the older literature, a single genus, *Conus*, regarded as the only living genus in the family Conidae, encompassed all living cone snail species. Molecular phylogeny has revealed that there are three cone snail lineages that are divergent from the main group, and thus, in one modern scheme, cone snails comprise four living genera, *Conus* (including the vast majority of species), *Conasprella* (with ca. 80–100 species) and two smaller groups, *Californiconus* and *Profundiconus*. Most statements in this review primarily apply to the genus *Conus*, *sensu stricto.*

Introductory overview

Conus venom peptides ("conotoxins", "conopeptides"). A well-validated resource for the study of non-opioid pain pathways and analgesics are the venoms of the ca. 800 species of predatory marine snails in the family Conidae ("cone snails")*. Each species has evolved its own distinctive venom. The genes encoding the bioactive venom components have been characterized for many species; the gene products these encode are relatively small peptides (mostly 10–35 amino acids in length), the majority of which are structurally constrained by multiple disulfide bonds. Many conopeptides carry additional post-translational modifications [1]. Cone snail venom peptides are encoded by gene superfamilies, and a single species has a repertoire of ~100–400 venom peptides [2–4]. A remarkable feature of the genes encoding bioactive venom components is their accelerated evolution; conopeptides are probably among the most rapidly evolving gene products known [5, 6]. The consequence is that each cone snail species has its own distinctive complement of venom peptides.

Each individual venom peptide presumably has a specific, physiologically-relevant molecular target in the envenomated animal (i.e., prey, predator or competitor). Cone snail venoms have proven to be a rich source of highly selective ligands for ion channels, receptors and transporters in the nervous system. This is consistent with the overall goal of envenomation: the alteration of the normal physiological behavior of the targeted animal to benefit the venomous predator. Almost all venom characterization studies that have been carried out have examined species in the genus *Conus sensu stricto* (i.e. the genus in the strict sense) with a strong bias for the species that hunt fish. Relatively few studies have been done with the minor genera in the family Conidae (*Conasprella*, *Californiconus* and *Profundiconus*) (Fig. 1, left).

Cone snails are not the only venomous marine snails; in terms of species numbers, they comprise only a minor component of venomous snail biodiversity. All taxa that use venom including *Conus*, are assigned to the Superfamily Conoidea [7]. Classically, in addition to the cone snails, two other major groups of venomous conoideans have been recognized, the auger snails (family *Terebridae*) and the turrids (classically, the family *Turridae*, which is clearly polyphyletic, and has been split into at least half a dozen additional family groups) (Fig. 1, right). It is the turrids that encompass most of the biodiversity of venomous snails — it is estimated that Conoidea contains 10,000 venomous species (and this could be a significant underestimate) [7]. Very little work has been done with venoms outside the family Conidae but initial analyses suggest that these are just as complex as cone snail venoms. However, compared to *Conus*, many of the venom components in some of the Turrid groups are significantly larger polypeptides [7]; cone snails are unusual in that a majority of their highly expressed venom components are relatively small (10–35 amino acids).

Ziconotide (Prialt®) (For additional reviews, see [8–12])

(A) Historical Retrospective

The cone snail venom peptide that ultimately became Prialt®, an approved non-opioid therapeutic for intractable pain was discovered as part of a basic research investigation of two fish-hunting cone snails, *Conus geographus* (the Geographer Cone), a species that can cause human fatality, and *Conus magus*, a smaller species with a narrower biogeographic distribution in the Western Pacific. The venoms of both cone snails have components that are capable of paralyzing fish, and these include two physiologically and genetically related peptides, ω-conotoxin GVIA (from *C. geographus*) and ω-conotoxin MVIIA (from *C. magus*) (Fig. 2). The discovery of these peptides was made possible by key contributions of two University of Utah undergraduates, Craig Clark and J. Michael McIntosh. In retrospect, it clearly was not a drug development program that led to the discovery of Ziconotide, but rather, resulted from an effort to understand how the two fish-hunting cone snail species were able to capture their fish prey (and why one of these species was also capable of killing people).

Application of what is in effect a simple high-content assay to cone snail venoms was a key innovation introduced by Craig Clark. Craig injected each venom component intra-cranially into mice; when the venoms of *Conus geographus* and *Conus magus* were examined, he discovered that the different venom components elicited different behavioral phenotypes. There were components in both species that elicited a characteristic tremor that was an easily scored symptomatology; these were initially dubbed the “shaker” activities in these venoms [13].

Using the behavioral phenotype elicited, Michael McIntosh purified and characterized the shaker component from *Conus magus*; simultaneously, the corresponding venom component from *Conus geographus* was also characterized (Fig. 2). Both peptides have three disulfide linkages, with the disulfide connectivity of the two peptides being similar. After the amino acid sequences of the peptides were established, chemical synthesis was carried out by Jean Rivier’s laboratory at the Salk Institute, USA. The chemically synthesized peptides exhibited the expected biological activities (the highly characteristic shaker phenotype in mice, as well as paralysis in fish) [14].

The biochemical characterization and successful chemical synthesis of these peptides made a systematic investigation of the physiological mechanism underlying their bioactivity feasible. The peptides caused paralysis in fish by blocking neuromuscular transmission at the pre-synaptic terminus —release of the neurotransmitter, acetylcholine was inhibited. Electrophysiological evidence suggested that the molecular target was a specific voltage-gated calcium channel [15, 16]. At the time, calcium channels had not been defined at a molecular level — no ion channels had been cloned, and it was therefore uncertain how many different voltage-gated calcium channels were present in the vertebrate nervous system. The isolation of these peptides provided key pharmacological tools to define different types of voltage-gated calcium channels. The peptides had unprecedented selectivity for a calcium channel subtype that had not previously been recognized (known as the N-type calcium channel, and later as Cav 2.2).

After the peptides were characterized, the chemically synthesized compounds were used for electrophysiological studies. These experiments revealed that while ω-conotoxin GVIA from *Conus geographus* bound almost irreversibly to the targeted calcium channel, the peptide from *Conus magus*, ω-conotoxin MVIIA, though a high-affinity, selective ligand could be washed out. This was a feature that made it desirable for electrophysiological experiments. ω-conotoxin GVIA remains a defining ligand for Cav 2.2, and has become a standard tool for assessing the role of Cav 2.2 in synaptic transmission. To date ω-conotoxins have been been used in more than 3,000 published studies.

The progression from pharmacological tools for basic neuroscience to a therapeutic candidate can be credited to one individual, George Miljanich. He was one of the academic researchers using ω-conotoxin MVIIA as a pharmacological tool to study neurotransmitter release (in this case, using synaptosomes from the electric organ of the electric ray). When he joined a nascent biotech company, Neurex Corp (Menlo Park, USA), he persuaded the company CEO to consider exploring biomedical applications of this highly specific blocker of Cav 2.2. The company initially assessed whether the compound might be neuroprotective in animal models of stroke. In the course of their initial characterization, they radiolabeled the peptide to determine binding sites in the vertebrate nervous system. It was the results of these radiolabeling studies that led to the first indication that there might be applications for pathological pain. In the spinal cord, the peptide was surprisingly specific in binding to only the layers of the dorsal horn previously established to be important for the perception of pain. This key finding led George Miljanich, Scott Bitner and J. Ramachandran to systematically assess whether ω-conotoxin MVIIA might have potential as an analgesic. The studies at Neurex quickly revealed the suitability of the peptide for this specific biomedical application ([10] and personal communications).

The early preclinical research involved standard studies to further advance the peptide as a potential non-opioid analgesic, including an extensive structure/function study. Basically, every amino acid in ω-conotoxin MVIIA was mutated to determine whether there might be an analog with significantly better pharmacokinetic properties than the native peptide, but in the end, the company did not change a single functional group. Thus, a remarkable feature of the therapeutic drug is that it is chemically identical to the 25 amino acid natural product evolved by the Magician's Cone, *Conus magus*.

Following the discovery of Cav2.2 as a molecular target for pain several other attempts were made to develop selective antagonists of this channels, including clinical developments of Leconotide by Xenome Ltd (coded as AM-336), an ω-conotoxin from the venom of *Conus catus* (Fig. 2), and small molecule inhibitors developed by Neuromed Pharmaceuticals Ltd. (Vancouver, Canada). However, to the best of our knowledge, these did not advance to human clinical trials [17].

(B). Clinical Development and Commercialization

From ω-conotoxin MVIIA to Ziconotide (Prialt®). Because Ziconotide is a peptide, it does not cross the blood brain barrier. A collaboration between Neurex and Medtronic (Medtronic Inc., Minneapolis, USA) led to the intrathecal delivery of the peptide through a pump that could be implanted. Neurex was able to move the compound to human trials, and because these were the heady days of the Biotechnology bubble, on the basis of their success in pushing ω-conotoxin MVIIA to Phase II Human Clinical Trials, the biotech company was acquired by Elan Pharmaceuticals for $760 million, with the venom peptide being the major asset.

The further clinical development of the peptide was ultimately achieved by Elan, but it was a longer and more complex process than had been anticipated; approval was finally given in 2004 by the Federal Drug and Food Administration (FDA) and marketed as Prialt® (the **Pri**mary **Alt**ernative' to Morphine). The clinical development and applications of Ziconotide have been reviewed in more detail elsewhere [18–21]. Some of the extended gestation period could be explained by the fact that this was a peptide from a venom and larger than most small molecule drug leads. Thus, the FDA was conservative, carefully monitoring all of the clinical data. In addition, the financial situation of the biotechnology industry went through a series of major upheavals, and thus, not all delays were due to problems with the clinical trials. Some years after, approval in both the U.S. and the European Union was obtained. Elan sold the commercial rights for the peptide to Jazz Pharmaceuticals in the U.S., and to Takeda in the EU, which remains as the commercial situation of Ziconotide today.

A Clinical Perspective: Ziconotide vs. Opioids

In the quest for improved analgesic compounds, it is perhaps useful to imagine the 'ideal' drug while contemplating the limitations and failures of existing therapies. This *idealized* agent would selectively attenuate nociceptive signaling while leaving other physiologic modalities such as light touch, proprioception, and motor function intact. All existing analgesics (with the exception of non-steroidal anti-inflammatories) demonstrate inhibitory properties and may result in undesirable side-effects such as sedation, constipation, psychomotor retardation and respiratory depression. This elusive ideal drug would eliminate or minimize these undesirable inhibitory physiologic characteristics while offering a robust analgesic response.

Opioids, while excellent in the management of acute nociceptive pain, fall short in the management of neuropathic pain, or nociceptive pain of longer duration [22, 23]. Numerous issues have made opioids a less than ideal therapy for long-term pain management. First, a lack of long-term efficacy has been observed, with diminishing analgesic effect due to factors such as tolerance and hyperalgesia. Second, opioid-related side effects are common and may prohibit further dose escalation or result in poor compliance. Third, selected pain conditions are resistant to mu-receptors agonism and simply fail to respond to opioid therapy even at high doses. Finally, opioids have the unfortunate pharmacologic profile of physiologic and sometimes psychological dependence after prolonged use, which can result in an unpleasant withdrawal experience or a tendency towards addiction. This latter problem has received a lot of media attention lately, and for good reason – it is estimated that the "opioid epidemic" claims over 90 lives every day in the United States [24]. The source of the offending opioids is largely from legitimately prescribed drugs for the management of chronic pain, so we now have an even stronger imperative to identify novel analgesics that lack the aforementioned problems, and therefore mitigate the individual and societal risks of opioid therapy.

Ziconotide, when approved by the FDA in 2004, was indeed a welcome addition to the armamentarium of the pain specialist as an agent with a non-opioid mechanism, no respiratory depression, and lack of a withdrawal phenomenon. Clinical trials of intrathecally administered ziconotide had demonstrated good efficacy in both chronic and cancer-related pain [25]. Ziconotide has been used as a single agent, but increasingly, has been combined with an intrathecal opioid to harness a potentially synergistic effect in the treatment of refractory chronic and cancer pain [26, 27]. Clinically, ziconotide is often used a ‘last resort’ due to its relatively high cost and the requirement of an intrathecal pump. However, recent guidelines encourage the use of ziconotide as a first-line agent in various pain conditions including neuropathic and nociceptive pain [28].

Nevertheless, clinical experience with ziconotide over the last decade has demonstrated some significant shortfalls. Most significantly, and in contrast to the good oral bioavailabilty of opioids and other analgesics, the small peptide requires direct delivery to the cerebrospinal fluid, necessitating the implantation of an intrathecal drug delivery system – a relatively costly and invasive procedure that will always be a barrier to more widespread use. Until less invasive pharmacologic strategies for delivering peptides, to the CNS are developed, the widespread use of peptides will be limited.

An additional impediment to a greater acceptance of ziconotide has been its unfortunate tendency to induce a range of psychomotor side effects ranging from mild ataxia and auditory hallucinations (typically completely reversible with a small dose reduction) to more debilitating ataxia and psychosis. While the latter phenomena are rare now due to more careful dosing protocols, the very narrow therapeutic window nevertheless presents a significant clinical challenge prohibiting optimal dosing for analgesia.

In summary, while chronic pain remains undertreated and a major healthcare issue, and the ‘opioid epidemic’ rages, the pain clinician and the patients they treat remain in desperate need of novel agents that offer a specific analgesic effect without the side effects and societal risks of our current and limited therapies.

The Dual Role of Therapeutic Venom Components

Pain therapeutics from cone snail venoms evolved for a specific physiological role. This is likely true of every natural product, but in general, we have very little insight into what the exact physiological role of a specific natural product might be. However, because of the unusually striking and well-studied biological interactions between cone snails and their prey, the therapeutic peptides derived from the venoms discussed in this review can be assigned physiological roles. We believe that understanding these specific evolutionary functions can greatly accelerate drug discovery from venoms and other natural products [29]. We discuss two examples, Ziconotide, the approved commercial drug discussed in the previous sections, and Contulakin G, which is a cone snail venom peptide that has reached human clinical trials, discussed in the next section. In this overview, a rationale for why the snails evolved these peptides is presented. We discuss how repurposing the venom peptides for use as human therapeutics that relieve pain by non-opioid mechanisms is related to their presumptive endogenous roles.

As discussed above, Ziconotide is identical to the gene product of a venom peptide evolved by *Conus magus*. This species hunts fish as its major (and perhaps exclusive) prey, and belongs to a lineage of cone snail species known as the Pionoconus clade, all of which are fish-hunting (piscivorous). All Pionoconus capture prey using a similar prey capture strategy; two videos illustrating this is included with this review (Supporting Videos 1 and 2). There are probably two-dozen different species of cone snails that belong to the Pionoconus clade [30]. Basically, when these snails sense the presence of a fish primarily using chemosensory cues, they extend their highly distensible proboscis towards the fish. Once they are within striking distance, when the tip of their proboscis contacts the skin of a fish, a hollow harpoon-like tooth is extruded with sufficient force to pierce through the surface of the fish. Because the harpoon-like tooth is barbed, it serves as both a hypodermic needle to inject venom and a harpoon that tethers the fish mechanically. As shown in the video, once the snail strikes, the tethered fish immediately jerks, and in a few seconds is completely immobilized. This initial phase of envenomation is a consequence of injected venom components that cause a massive depolarization of axons in the neighborhood of the injection site. Meanwhile, a second group of venom components are taken up by the circulatory system of the fish, and when these reach the neuromuscular junctions that control the fin musculature, they block synaptic transmission, causing an irreversible block of the fin musculature. One set of these peptides target the pre-synaptic calcium channels present at the motor nerve ending.

By blocking pre-synaptic calcium channels, even though an axonal action potential may be elicited, no neurotransmitter release from the pre-synaptic terminus occurs because calcium entry is essential for the exocytosis of neurotransmitter vesicles. Thus, when injected into fish, these peptides are paralytic. The peptide from *Conus magus*, ω-conotoxin MVIIA, is in fact, Ziconotide. Thus, the snail evolved this peptide as part of its pharmacological armamentarium for prey capture. How did a peptide evolved to cause paralysis in fish become a therapeutic drug for pain? What the discovery of these peptides revealed is that fish express a very specific calcium channel subtype, Cav 2.2, at their neuromuscular junctions. However, this is not the subtype expressed at the neuromuscular junction of mammals (instead, it is Cav 2.1). ω-conotoxin MVIIA and all homologous ω-conotoxins of this type in Pionoconus snails can discriminate between Cav 2.2 and Cav 2.1 in all vertebrate systems — they are highly selective for Cav 2.2 in every synapse tested. Thus, the peptide is not paralytic to mammals, but retains its high affinity and selectivity for mammalian Cav 2.2.

One of the sites where this Ca^+ channel (Cav 2.2) is expressed in the mammalian nervous system (the only major site in the spinal cord) is in the dorsal horn; the pre-synaptic termini of pain fibers that synapse with spinal cord neurons express Cav 2.2, required for the release of neurotransmitter at these synapses. Thus, when the peptide is present, even though pain fibers fire, the signal is not transmitted to the central nervous system because ω-conotoxin MVIIA blocks these synapses. Thus, repurposing the peptide as a therapeutic is feasible because the structure of the calcium channel targets is highly conserved (all vertebrate calcium channels can be unambiguously assigned to a particular subtype based on homology in the amino acid sequences). The key scientific basis that made the peptide feasible for development as a therapeutic is the difference in calcium channel expression at specific synapses in different vertebrate systems.

The compound that will be discussed in the next section, which has reached human clinical trials is Contulakin G, a peptide expressed in the venom of *Conus geographus*. Although like *Conus magus*, *Conus geographus* is a fish-hunting cone snail, it uses an entirely different strategy for capturing fish, and it belongs to a different clade of fish-hunting cone snails from *Conus magus* (it does not belong to the *Pionoconus* clade, but to the *Gastridium* clade, a smaller group of fish-hunting cone snail species on a different branch of the phylogenetic tree). *Conus geographus* hunts fish by using a "net strategy". This is shown in the second enclosed video (Supporting Video 3) and at https://www.youtube.com/watch?v=FYh2zeAsRXY. Instead of extending its proboscis towards the fish, *Conus geographus* opens its false mouth (rostrum) and releases a subset of venom components; one of these is Contulakin G. The net effect of the venom components released is to make the fish sedated, hypoactive and hypoglycemic. Contulakin G plays a role in this by stimulating neurotensin receptors (the exact role of neurotensin in fish physiology is not well defined).

In many ways, the use of Contulakin G may be analogous to its endogenous role; the implication is that activation of a neurotensin receptor quiets down key neuronal circuitry in fish, and the effects of injecting Contulakin G into the intrathecal space seems to be to quiet down pathologically overactive pain circuitry. Thus, in contrast to what is found for Ziconotide, the endogenous role of Contulakin G may be directly related to its therapeutic potential as a non-opioid drug for pain. The following section provides an overview Contulakin-G, from discovery to clinical development.

Contulakin-G, a potent analgesic from the venom of Conus geographus

Contulakin-G is a 16 amino acid long peptide from the venom of *Conus geographus* that was originally isolated based on its "sluggish" activity in mice [31]. Typically, mice injected intracerebroventricularly (*i.c.v*) with Contulakin-G had difficulty righting after a few minutes, became unresponsive when prodded and rested on their stomachs within less than one hour [31]. The Filipino word "tulakin" means "has to be pushed or prodded". Based on this characteristic behavioral phenotype Contulakin-G was suggested to be part of the nirvana cabal; a set of toxins that is released into the water by net-hunting cone snails to induce hypoactivity in prey. Because of their ability to lower the activity of neuronal circuitries nirvana cabal toxins have provided unique drug leads for human pathologies that are characterized by an overstimulated nervous system (e.g., epilepsy and intractable pain).

The full amino acid sequence of Contulakin-G was obtained by Edman sequencing after treatment of the native peptide with pyroglutamate aminopeptidase to remove an N-terminal pyroglutamate residue [31]. Edman sequencing provided a low signal for a threonine at position 10 suggesting that this amino acid carried a post-translational modification. PCR sequencing confirmed the presence of a threonine residue at this position. Subsequent electrospray tandem mass spectrometry (ESI-MS/MS) revealed that Contulakin-G was O-glycosylated with the major glycoform corresponding to a galactose/N-acetylgalactosamine (Gal/GalNAc) group at threonine 10 [31] (Fig. 3).

Contulakin-G shared high sequence similarity with neurotensin, a vertebrate neuropeptide that is widely expressed in the central nervous system where it modulates dopamine signaling (Fig. 3). Administration of neurotensin into the *periaqueductal gray, the brain's* primary control center for descending pain modulation, had been shown to reduce pain signaling by activating the pain inhibitory system [32]. As expected based on its sequence similarity with neurotensin, Contulakin-G was shown to activate the human G-protein coupled neurotensin receptor 1 and 2 albeit with lower activity compared to human neurotensin [31]. Despite its lower binding affinity, Contulakin-G proved to be a much more potent analgesic than neurotensin in three different animal models of pain following intrathecal delivery, namely in tail-flick (acute pain), formalin test, and complete Freund's adjuvant (CFA)-induced allodynia inflammatory pain [33, 34]. In addition to its analgesic properties, the behavioral toxicity of Contulakin-G appeared to be much lower than that of neurotensin [35]. Its weaker agonist activity at the neurotensin receptor and more potent analgesic properties were later attributed to the presence of the glycosylation and negatively charged amino acids at the N-terminus that together induce significantly less desensitization following receptor activation [36].

Because of these promising pharmacological properties Contulakin-G was licensed to Cognetix Inc. in 2000 (coded as CGX-1160) and granted an orphan drug designation by the US Food and Drug Administration (FDA) for the treatment of chronic intractable pain. Following positive outcome in animal pain studies with high doses that were well tolerated with low side-effects, Contulakin-G entered a Phase Ib clinical study in 2004 for the treatment of chronic intractable spinal cord injury pain at Brigham and Women's Hospital, Harvard. In a 6-patient study CGX-1160 showed significant reduction of pain and was generally well tolerated with wide therapeutic index when administered intrathecally at doses up to 1000 μg/h [37]. However, the FDA placed the study on partial clinical hold because the preclinical toxicity studies in dog did not adequately determine the maximum tolerated dose. In 2005 Cognetix shut down when funding discontinued and the clinical development of Contulakin-G came to a halt despite its very promising therapeutic properties. More recently, neurotensin receptor agonists have been implicated in promoting cell proliferation in certain types of cancer [38] providing new challenges for preclinical and clinical development of neurotensin-based drug leads. However, given the urgent need for non-opioid based pain drugs, the beneficial analgesic effects of Contulakin-G may outweigh its potential harmful properties, particularly for the treatment of end-of-life pain in patients that are unresponsive to opioids and Ziconotide.

Vc1.1 and RgIA revealed the role of α9α10 nicotinic acetylcholine receptors in neuropathic pain

Livett and co-workers were the first to show that α-conotoxin Vc1.1, an antagonist of nicotinic acetylcholine receptor (nAChRs), induced analgesia in several animal models of pain [39–41]. Until then it was believed that activation (by nicotine and other agonists) but not inhibition of nAChR induced analgesia [42, 43]. The discovery of Vc1.1 ultimately led to the identification of a new molecular target and mechanism for the treatment of pain, inhibition of the α9α10 nAChR subtype [44].

Vc1.1 was first identified by PCR sequencing of venom gland cDNA from *Conus victoriae,* a snail-hunting cone snail species from Western Australia [41]. The native peptide containing post-translational modifications was later identified in the venom of *C. victoriae* by LC-MS/MS [45, 46] (Fig. 4). Vc1.1 was shown to inhibit nAChRs expressed in bovine chromaffin cells and proved to be a potent analgesic in several animal models of neuropathic pain, namely the chronic constriction nerve injury (CCI) model, partial nerve ligation (PNL) model [40] and the streptozotocin- induced model of diabetic neuropathy [47]. In addition to its analgesic properties Vc1.1 altered the pathophysiology of the disease state. Administration of Vc1.1 accelerated functional recovery of injured neurons potentially by reducing the inflammatory response at the site of injury [40]. Vc1.1 was developed by Metabolic Pharmaceuticals Limited (coded as ACV1) and reached Phase 2 Clinical Studies where it failed in 2007 due to low efficacy in humans. When ACV1 entered human clinical studies the specific target subtype of nAChR was not yet known. Work by McIntosh et al. later showed that Vc1.1 was a potent antagonist of the α9α10 nAChR subtype [44] and provided a clinical rationale for why Vc1.1 failed in human trials. Vc1.1 was several orders of magnitude less potent at the rodent vs. the human α9α10 nAChR subtype [48–50]. Nevertheless, the work on Vc1.1 led to the discovery of a new, non-opioid-based target for the treatment of pain and initiated research into the discovery of additional antagonists of the α9α10 nAChR. The second cone snail venom peptide targeting the α9α10 nAChR, α-RgIA, was discovered by cDNA sequencing of the venom gland of the worm-hunting cone snail *Conus regius* [51]. Similarly to Vc1.1, native RgIA (termed Reg1E) was post-translationally modified [52] but all pharmacological studies were carried out with a synthetic analog lacking modifications (Fig. 4). The work on RgIA provided additional evidence for the analgesic and disease modifying effects of α9α10 nAChR inhibition. RgIA alleviated neuropathic pain in the CCI model of neuropathic pain and reduced edema and inflammatory infiltrate at injury site consistent with a disease-modifying effect [53]. An alternative hypothesis has been advanced that analgesic activity of a-conotoxins is related to agonist activity at $GABA_B$ receptors (for in depth reviews of this topic see [54, 55]). More recently, an analog of RgIA, RgIA4, was developed that exhibits high affinity for both, the rodent and human α9α10 nAChR, but lacks activity at $GABA_B$ receptors. This analog prevented chemotherapy-induced neuropathic pain expanding the potential clinical applications of RgIA to cancer-related neuropathies [56]. Remarkably, the analgesic effects have been shown to last for at least 3 weeks after last injection consistent with a disease modifying effect [57]. RgIA4 was licensed by the University of Utah to Kineta Inc. (coded as KCP-400) and is currently in preclinical development.

To date three additional compounds have been shown to exert analgesic effects in various animal models of pain by inhibiting the α9α10 nAChR, including another cone snail venom peptide, αO-conotoxin from *Conus generalis* [58, 59] and two small molecules that mimic α-conotoxin binding to the receptor [60, 61].

MrIA, an inhibitor of the norepinephrine transporter (NET) with analgesic properties

MrIA was originally isolated from the venom of *Conus marmoreus* based on its ability to induce a "sluggish" hypoactive phenotype following *i.c.v.* injection in mice [62]. The full sequence of the purified peptide was obtained using a combination of Edman sequencing, ESI-MS and cDNA sequencing. Intrathecal administration of synthetic MrIA (termed mr10a in the original study) was analgesic in a mouse model of pain (hot plate assay) suggesting its potential as a drug lead for pain. Two additional peptides from *C. marmoreus* venom with high sequence similarity to MrIA were simultaneously identified by another research group [63] (Fig. 5) and shown to have interesting biological activity in mice. Shortly after these discoveries the molecular target of this group of peptides was identified: MrIA and MrIB were potent, non-competitive inhibitors of the norepinephrine transporter (NET) and were classified as χ-conotoxins based on their pharmacological activity [64]. NET is a monoamine transporter that is responsible for the reuptake of extracellular norepinephr ine. This work provided a rationale for the analgesic properties of MrIA. Norepinephrine is an endogenous mediator of *analgesic* mechanisms in the descending pain pathway [65, 66]. An analog of MrIA carrying two modifications (Xen2174) proved to be a more stable inhibitor of NET and potent analgesic in the CCI model of neuropathic pain [67]. Xen2174 was licensed to Xenome Ltd and entered various stages of clinical trials for the treatment of cancer and post-surgical pain [68]. A recent publication on the pharmacodynamics and the cerebrospinal fluid pharmacokinetics of Xen2174 in healthy patients concluded that no statistically significant effect on evoked pain was observed [69]. Additionally data from previous clinical studies provided no conclusive results on the analgesic properties of Xen2174 in humans [69] suggesting that this peptide drug does not exhibit sufficient efficacy for the treatment of pain in humans.

The future of non-opioid pain drug discovery from cone snail venoms

Ziconotide, Contulakin-G, Vc1.1 and MrIA were novel drug leads for pain and simultaneously revealed new pathways of pain signaling. These peptides only represent a miniscule fraction of the potential diversity of pharmacological agents found in cone snail venoms. With the exception of Vc1.1 and RgIA these drug leads were discovered by *in vivo* activity screening of fractionated venom followed by Edman and/or mass spectrometry-based sequencing. This "activity- first" approach was traditionally limited to species for which large amounts of venom could be collected and, until recently, a comprehensive biochemical characterization of venom components for rare species or those with very small venom glands was not possible. However, advances in DNA and protein sequencing technologies combined with the development of novel high-content assays now provide a more straightforward path to efficient bioactivity-guided discovery and characterization. Briefly, newer high-content methods, such as zebrafish behavioral screens and constellation pharmacology can be used to identify a desired bioactivity for further characterization. Phenotypic compound screening in zebrafish has the advantage of requiring relatively little material for a comprehensive analysis of behavioral changes in the presence of a library of compounds [70]. Several zebrafish models of pain have recently been described [71, 72] that have the potential to provide a novel avenue for the discovery of analgesic compounds from venoms. Constellation pharmacology is a newly emerging technology that allows the simultaneous assay of a large number of molecular targets in the nervous system including those that are implicated in pain signaling [73–75].

While many prior discovery efforts from cone snail venoms were based on the dominant paradigm in the pharmaceutical industry that high-throughput screening of combinatorial libraries against a specific set of molecular targets would maximize discovery, we believe that a shift from high-throughput to high-content is essential. The compelling scientific rationale for this is that, in contrast to the average library used for high throughput screening, each component of a venom has a distinctive bioactivity and is potentially acting at it’s own specific molecular target. Instead of testing for one molecular target at a time, which is standard using the high throughput approach, high content screening allows the simultaneous identification of diverse bioactivities. Compounds of interest can then be purified in parallel and their sequences determined for subsequent synthesis and in-depth characterization. In combination with new techniques in DNA and protein sequencing, that now ease sequence analysis of a compound of interest, these new high-content activity-screening methodologies have the potential to transform drug discovery from venoms.

Next-generation transcriptomics can now generate a comprehensive overview of venom components from a particular species, even from a single venom gland [2–5, 76]. This means that venom compounds exhibiting a desired bioactivity can be subjected to MS/MS analysis and the data obtained can be rapidly matched against a transcriptome database using search algorithm software such as Mascot [77] and pFind [78]. This eliminates the need for further Edman sequencing or *de novo* MS/MS sequencing and greatly accelerates the speed by which the primary structure of the desired compound, including posttranslational modification, can be identified. Based on this information the active compound can then be synthesized or recombinantly expressed and subjected to a more detailed characterization using *in vitro* and *in vivo* pain-specific assays.

A unique advantage for drug discovery in the cone snail venom system is that the phylogeny of cone snails has been well studied, and relationships between species are relatively well understood. Thus, once bioactive components from one venom are characterized, venom transcripts from closely related *Conus* species can be examined. Because of the accelerated evolution of the genes that encode cone snail venom components, there is significant divergence expected in the amino acid sequence of homologs that may have the same molecular target, even between very closely related cone snail species (for examples see [79, 80]). Nevertheless, this is a relatively direct way to obtain not just one sequence of interest, but a whole family of related sequences. Thus, after a potential non-opioid lead from a cone snail venom is identified, a significant database of closely related sequences from other species is straightforward to obtain. Such sequence comparisons provide structure/function information by identifying amino acids that are conserved (and therefore potentially essential for bioactivity) and which amino acids not conserved. Very often, the different homologs may show significant differences in affinity for a molecular target in a particular mammalian species. (such as a rodent receptor). Thus, the identification of a non-opioid drug lead, particularly when the molecular target is defined, can quickly lead to a very considerable accompanying structure/function database that accelerates preclinical development.

The combined use of transcriptomics and proteomics provides a comprehensive overview of an entire venom repertoire, and increasingly, this requires very little material (enabling the analysis of rare or miniscule species). This has led to the identification of new gene classes of toxins, new sequences with homology to known toxins with interesting activity and of toxins that may have unique structural features. The identification of venom components from sequencing alone without any activity screening was previously used for discovery of drug leads, including Vc1.1 and RgIA [81, 82]. Recent technological advances have made this “sequence-first” approach more facile and feasible [83]. Additionally, sequences of toxins that have very low expression levels and would not be detected by activity-based analysis can be obtained. A significant disadvantage is that compounds have to be synthesized before activity can be assessed. However, recent advances in high-throughput toxin expression and purification may soon overcome this limitation [84] for those venom peptides that do not require post-translational modifications for activity or structural integrity. Using the activity- first and sequence-first approach in parallel should synergistically accelerate the generation of a pipeline of novel therapeutics for pain in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

The current opioid epidemic is the deadliest drug crisis in American history. Thus, this review on the discovery of non-opioid pain therapeutics and pathways from cone snail venoms is significant and timely.

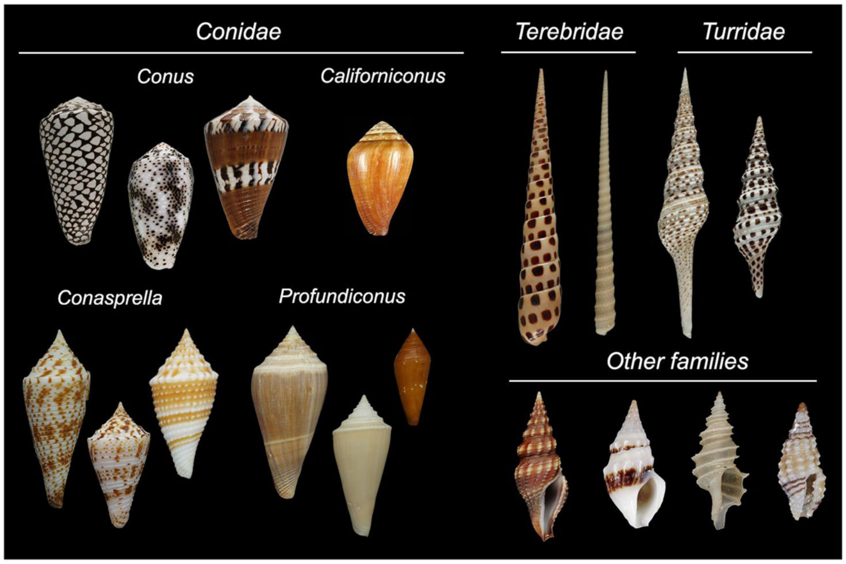

Fig. 1. The Conoidean Superfamily

Left panel: Cone snails (genus *Conus*) belong to the family Conidae that comprises three additional minor genera (*Californiconus*, *Conasprella* and *Profundiconus*). Shown are representatives from each genus. From top left to bottom right: *Conus marmoreus, Conus stercusmuscarum, Conus capitaneus, Californiconus californiconus, Consprella comatosa, Conasprella memiae, Conasprella pagodus, Profundiconus profundorum, Profundiconus termachii* and *Profundiconus lani*. Right panel: The shells of two auger snails (genus: *Terebra*), two turrids (genus *Turridae*) and members of several other families within the superfamily *Conoidea* are shown. From top left to bottom right: *Terebra subulata, Terebra triserata, Turris grandis*, *Turris babylonia, Fun asp, Clavus exasperates, Veprecula polycantha* and *Pseudodaphnella granosa.*

Fig. 2.

Sequences of MVIIA (Ziconotide, Prialt®) from *Conus magus*, GVIA from *Conus geographus* and CVID (Leconotide) from *Conus catus*. Post-translationally modified amino acids are in blue (O: hydroxyproline, *: C-terminal amidation) and the disulfide connectivity is shown. Shells are depicted next to sequences.

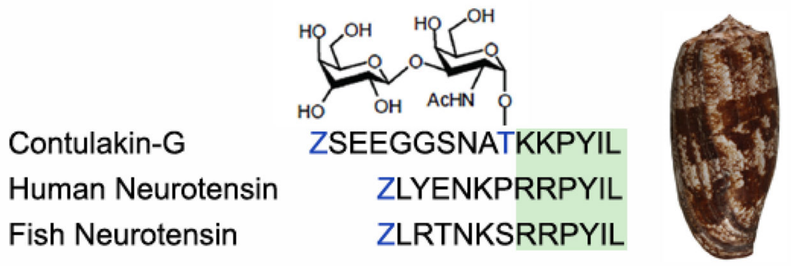

Fig. 3.

Alignment of Contulakin-G isolated from the venom of *Conus geographus* with human and zebrafish neurotensin. Post-translationally modified amino acids are shown in blue. N-terminal glutamines are modified to pyroglutamic acid (Z) and the threonine in Contulakin-G carries a galactose/N-acetylgalactosamine (Gal/GalNAc) group. C-terminal amino acids critical for neurotensin receptor binding are highlighted in green. The zebrafish hormone was predicted from a precursor sequence based on similarity to human neurotensin (Uniprot accession: A0A0A7H8E2). The shell of *Conus geographus* is depicted.

Fig. 4.

Sequences of Vc1.1 from *Conus victoriae* and RgIA from *Conus regius*. The native peptides containing post-translational modifications (Vc1a and Reg1E) are highlighted in yellow. Disulfide connectivities are provided. O: hydroxyproline, γ: γ-carboxyglutamate, *: C-terminal amidation. Shells of *C. victoriae* and *C. regius* are depicted next to sequences.

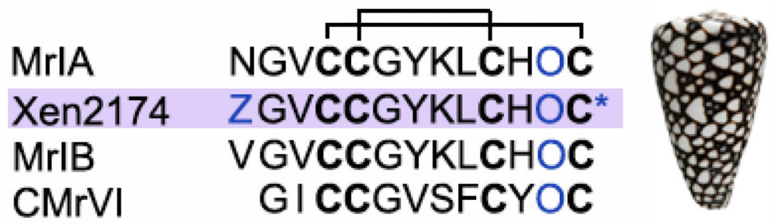

Fig. 5.

Sequences of MrIA and related peptides (MrIB and CMrVI) from *Conus marmoreus*. The peptide analog developed as a drug lead for pain (Xen2174) carries two additional posttranslational modifications and is shown in purple. Disulfide connectivities are provided. *: C-terminal amidation, O: hydroxyproline, Z: pyroglutamic acid.