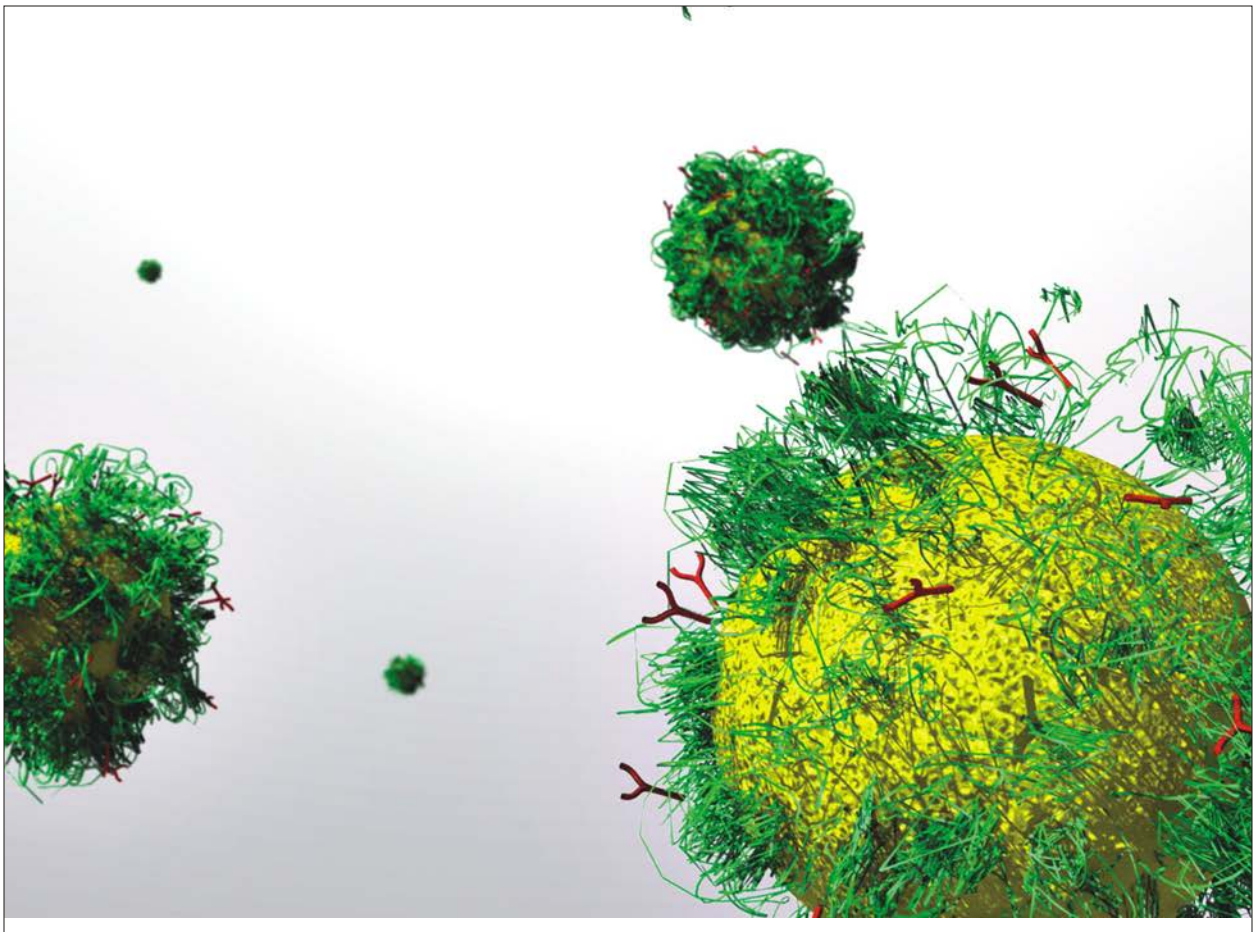

American Journal of Drug Discovery and Development

ISSN 2150-427X

Academic Journals Inc.

www.academicjournals.com

American Journal of Drug Discovery and Development 1 (1): 49-57, 2011
ISSN 2150-427x / DOI: 10.3923/ajdd.2011.49.57

Molecular and Combinatorial Array of Therapeutic Targets from Conotoxins

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ABSTRACT

Marine gastropod molluscs known as cone snails produce a complex array of over 50,000 peptides evolved for defense and prey capture. These venom peptides of predatory snails represent a rich combinatorial like library of evolutionary selected, neuro pharmacologically active. A major portion of the venom components are small, disulphide rich peptides that potently and specifically target and modulate components of the neuromuscular system, particularly ligand gated, voltage gated ion channels and transporters, making them a valuable source of new ligands for studying the role of these targets play in normal and disease physiology. Conotoxins are genetically encoded a propeptides which following expression and cleaved by specialized endoprotease to produce the mature venom peptide. A vast number of conuspeptides reduce neuropathic pain in animal models. Though several peptide drugs are in preclinical and clinical development for the treatment of severe pain often associated with diseases such as cancer, less than 1% of cono peptides are pharmacologically explored. This review focuses on the fundamental aspects and families of conotoxins in addition to some of the novel conopeptides acting at different types of Voltage gated and ligand gated ion channels which may lead to molecular and therapeutic targets.

Key words: Venome, conotoxins, herapeutic targets, cono peptides

INTRODUCTION

Cone snails are large group (>500) of recently evolved, widely distributed marine molluscs of the family Conidae. They hunt prey using a highly developed envenomation apparatus (Fig. 1) that can paralyse prey within seconds encrusting its capture and reducing exposure to predation by larger fishes. The evolution of conotoxin in the venom of predator snails may be influenced by selective pressures imposed by the nature of the prey, with peptides mixtures from piscivorous, molluscivorous and vermivorous snails exhibiting differences (Olivera, 1997). Each venom contains unique array of over 100 different peptides. A striking feature of many Conus peptides is the unusually high frequency of post translational modification observed (Menez *et al*., 1992; Mandal *et al*., 2007). Many of the modified amino acids are unusual and some unprecedented for example, bromination of tryptophan, C terminal amidation, isomerisation of L to D aminoacid, hydroxylation of proline, sulfation of tyrosine residues, glycosylation of serine and threonine residues and γ carboxylation of glutamyl residues. Conotoxins are genetically encoded as propeptides which following expression are cleaved by specialized venom end proteases to produce

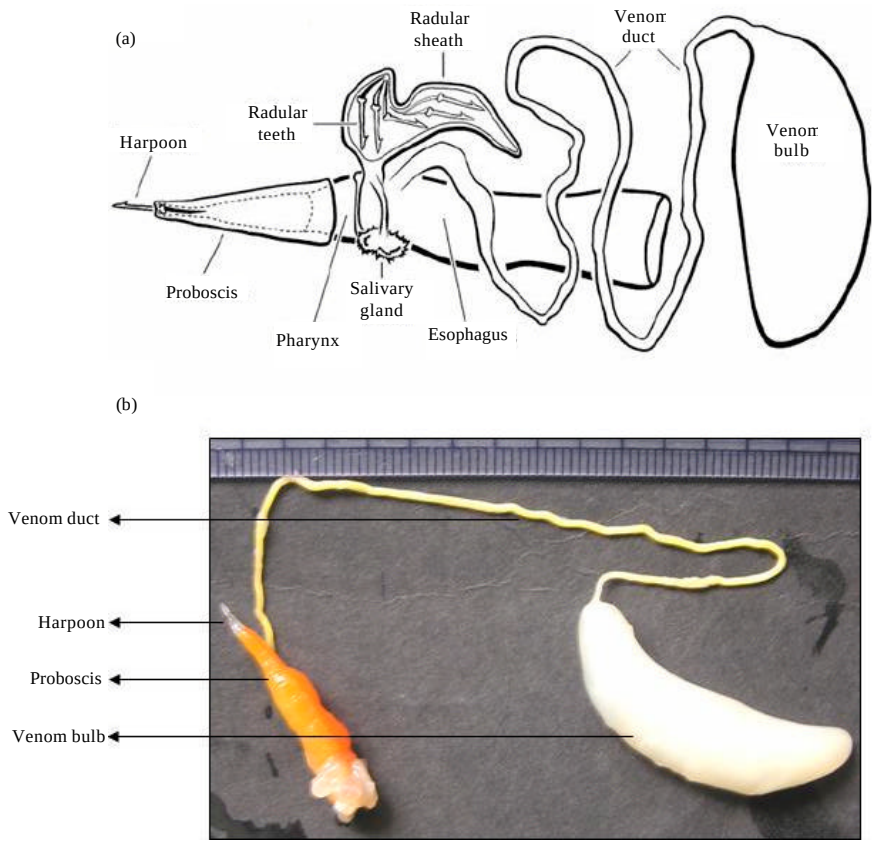

Fig. 1: (a) Cartoon diagram of venom apparatus and (b) venom apparatus of *Conus striatus*

the final mature venom peptide (Milne *et al*., 2003). Their small size and ease of synthesis, structural stability and target specificity make them ideal pharmacological probes (Adams *et al*., 1999). Interestingly, many of them are act on pain targets, allowing the specific dissection of key ion channels and receptors underlying pain and providing new ligand with potential as pain therapeutics (Lewis and Garcia, 2003). It is estimated out of >50,000 conopeptides only 0.1% characterized pharmacologically. The use of high throughput and more recent multiplexed high content screens should accelerate target discovery, although many conotoxins are expected to be selective for the prey species over related mammalian targets and may be missed in most screens.

Biosynthesis of cono peptides: Each individual *Conus* peptide is translatede from specific mRNA transcript into a precursor polypeptide with a canonical prepropeptide organization (Rappuoli and Monteucco, 1997). The conopeptide region comprises of three regions pre region or signal sequence at the N-terminus (typically 20-25 amino acids in length); mature conopeptide at the C terminus, always present single copy (from 8 to>40 amino acids in length, with the majority between 12-30 AA) and between the signal sequence and the mature conopeptide regions is an intervening region (the pro region) which for most conopeptides is 30-40 AA in length (in conotoxin families with a long N-terminal region before the first disulphiode bond, an abbreviated pro region is found.

Molecular diversity of conopeptide: Though a conopeptide precursor is encoded by a relatively small open reading frame, the 3 regions (pre, pro and mature toxin) can be considered as the separate functional domains. The N-terminal signal sequence targets the conopeptide secretory pathway. The C terminal mature region function as the final bioactive *Conus* venom component after it is processed. The propeptide region has recognition signals for post translational modification enzymes (Harvey, 1997; Menez, 1998). The rate of mutation between the signal sequence and mature toxin was observed in the three functional domains of the precursor. The signal sequence is highly conserved where as the mature toxin exhibit hypermutation. The rate of mutation within the 'pro' region appears to be in normal range although the pre and mature domains deviate dramatically from the rates expected for normal genes.

Conopeptides-biochemistry and pharmacology: The pharmacologically active peptide components of *Conus* venoms can be divided into two major groups (1) peptides which contain multiple disulphide bonds and (2) peptides that contain only single disulphide linkage or none. The former peptides are generically referred as a Conotoxins with more than 2 cysteine residues and latter group has two/or absence of cysteine residues are several distinct pharmacological targets, such as Conantokins, Conopressin and contryphans. In addition to small peptides, Conus venoms also contain large polypeptides and small molecules. The one and only polypeptide has been characterized is conodipine, a novel phospholipase A2 (Maslennikov *et al*., 1998). Non polypeptides constituents such as serotonin (McIntosh *et al*., 1993) and arachidonic acid (Nakamura *et al*., 1982). Hence, the classification of conotoxins has relied on the distribution of Cys residues in the primary structure, the nature of the disulphide pairing topology and the functional attributes of the peptides (McIntosh *et al*., 1999; Gray *et al*., 1988). The specific targets, defensive mechanism, tranquilizing effects of conotoxins are poorly defined. This broadly evolved pharmacological target provides a unique source of therapeutic agents and have become new research tool for neuro pharmacological scientists such as ω-MVIIA (Prialt/Ziconotide). These complex group of peptides, with over two thousand known members, can be classified into six structurally different classes. Individual members can be highly specific for receptor subtypes of the target molecule (Lewis, 2009). The different types and targets of conopeptides were shown in Table 1.

α-conotoxins-antagonists of nicotinic acetylcholine receptors: α-conotoxins are a large class of small peptides from cone snails that competitively inhibit specific subtypes of the nicotinic acetylcholine receptors (nAChRs) (Nicke *et al*., 2004). These alpha conotoxins were the first toxins isolated from *Conus* (Kasheverov *et al*., 2003; Hogg *et al*., 2003) venom and were designated alpha neurotoxins from snake venoms (e.g., Bungarotoxins) which inhibit the muscle type nicotinic receptor. Muscle selective α conotoxins such as GI, GIA and GII from *Conus geographus* are used as muscle relaxants during surgery. These are homologous peptides of 13 to 15 aminoacid length and two disulphide bridges in 3:5 loop configuration. These alpha conotoxins cause post synaptic inhibition at the neuromuscular junction resulting in paralysis and death. The mechanism by which paralysis is brought about by neuronally selective α conotoxins that target nAChR subtypes comprising different combinations of α3-α10 and/or β2-β4 subunits expressed as either homomeric or heteromeric receptors depending on the neuronal cell type. Interestingly, α conotoxin Vc1.1 (Sandall *et al*., 2003) and RgI (Vincler *et al*., 2006) have been identified as having potential analgesic properties of following peripheral administration to rats. Co crystal structures with the

Table 1: Conopeptide sequences with their molecular targets and their types

Name of the *Conus* species	Class	Sequence	Target/Activity	Reference
C. victoriae	α-conotoxin	GCCSDPRCNYDHPEIC[a]	nAchR inhibitor	Sandall *et al.* (2003)
C. magus	ω-conotoxin	CKGKGAKCSRLMYDCCTGSCRSGKC	N-type calcium channels	Bowersox and Luther (1998)
C. amadis (Am2766)	δ conotoxin	CKQAGESCDIFSQNC-CVGTCAFICIE-NH_2	Voltage gated Na^+ channel	Sudarslala *et al.* (2003)
C. amadis	Contryphans	GCOWDPWC[a]-NH_2	Voltage gated Ca^{2+} Channels	Sabareesh *et al.* (2006)
C. achatinus	δ conotoxin	DECFSPGTFCGIKPGLCCSAWCYSFFCLTLTF	Voltage gated Na^+ channel	Gowd *et al.* (2008)
C. marmoreus	μO conotoxin	ACSKKWEYCIVPIIGFIYCCPGLICGPFVCV	Act as reversible noncompetitive inhibitor of the neuronal nor adrenaline transporter	Sharpe *et al.* (2003)
C. purpurescens	κ-conotoxin	CRIONQKCFQHLDDCCSRKCNNRFNKCV	Potassium channel inhibitor	Shon *et al.* (1998)
C. tulipa	δ-conotoxin	FNWRCCLIPACRRNHKKFC[a]	α- adrenoreceptor	Sharpe *et al.* (2003)
C. ermineus	δ conotoxin	DDCIKOYGFCSLPILKNGGLCCSGACVGVCADL[a]	Enhances Sodium current	Barbier *et al.* (2004)
C. striatus	μ-conotoxin	ZNCCNGGCSSKWCRDHARCC[a]	Voltage gated Na^+ channel (Na_v- inhibitor)	Schroeder *et al.* (2008)
C. catus	ω-conotoxin	CKSKGAKCSKLMYDCCSGSCSGTVGC[a]	Voltage gated Ca^+ channel ($Ca_v2.2$- inhibitor)	Adams *et al.* (2003)
C. geographus	Contulakin-G	ZSEEGGSNAT$_\Psi$KKPYIIL	Nurotensin R antagonist	Malmberg *et al.* (2003)
C. geographus	Conopressin	CFIRNCPKG[A]	Vasopressin antagonist	Dutertre *et al.* (2008)
C. marmoreus	χ-conotoxin	Z[c]GVCCGYKLCHOC	NET inhibitor (Norepinephrine transportor inhibitor)	Sharpe *et al.* (2003)
C. tulipa	Conantokin-T	GEγγLQγNQγLIRγKSN	Selective inhibitor of the NMDA receptor	Malmberg *et al.* (2003)

[a]Amidated C termini, T$^\Psi$- glycosylated threonine, Z[c]- Pyroglutamate, γ-γ Carboxyglutamic acid

acetylcholine binding protein (AChBP) are revealing precisely how α conotoxins bind at this important therapeutic target. Interestingly, certain vermivorous cone snail species utilize larger 11 kD a dimeric αD-conotoxins to target α7 and β2 not as like as other snails used smaller α-αA and αS-conotoxins to target the same nAChR subtypes.

ω-conotoxins-calcium channel inhibitors: ω-conotoxins are peptides (Adams *et al.*, 2003) with 24-30 aminoacid residues with three disulphide bonds. GVIA from *Conus geographus* and MVIIA, MVIIC and MVIID from *Conus magus* venom are the best defined ω-Conotoxins. ω-Conotoxins are acting on N type calcium channels which play a key role in pain transmission by controlling neurotransmitter release at spinal synapse and thus can act as a gate keeper for responses to sensory pathway activation. ω-Conotoxins are specific and directly act on N type channels might be analgesic whereas the opiates inhibit the N type calcium channels at the spinal level indirectly via G proteins. ω-Conotoxin MVIIA and ω-CVID (Lewis *et al.*, 2000) produce analgesia for up to 24 h in rats while injected intrathecally (Smith *et al.*, 2002). The discovery of new ω-Conotoxins with selectivity profiles that produce fewer side effects may lead to the development of better analgesics in this peptide class. Extensive structure activity relationship studies have allowed the development of a novel pharmacophore model for ω-Conotoxins (Nielsen *et al.*, 2000), combinatorial development of specific N type receptors which includes cyclic peptides and peptidomimetics as well.

Sodium channel modulators:

μ-Conotoxins: GIIIA-C from *Conus geographus* very first identified in this μ-conotoxin which targets skeletal muscle sodium channel $Na_v1.4$. In addition, PIIIA and TIIIA which inhibit $Na_v1.2$ (Lewis *et al.*, 2007) and KIIIA which inhibits Na_v 1.2, 1.6, 1.7 (Zhang *et al.*, 2007). However, little progress has been made towards the development of μ conotoxin inhibitors for specific therapeuticall relevant VGSCs, including Na_v 1.3, 1.6, 1.7 and 1.8. M-O Conotoxins were found to preferentially (ten fold selective) inhibit mammalian Na_v 1.8 over other neuronal VGSC subtypes (Wood and Boorman, 2005). MrVIB was assessed for analgesic activity in animal models of pain (Ekberg *et al.*, 2006). The analgesic activity of MrVIB was again found by Bulaj *et al.* (2006) when it was injected subcutaneously to rats.

δ-Conotoxins: δ-Conotoxins are one of the most intriguing families of peptides derived from molluscivorous cone snails. They target Na^+ channels but do not compete with tetrodotoxin and μ Conotoxins. King Kong peptide isolated from TxVIA from the snail hunter *Conus textile* was the first δ conotoxin (Hillyard *et al.*, 1989). Chemically, δ Conotoxins are very unusual, with a core of disulphides that leaves no room for the usual burying of hydrophobic aminoacids in the interior but are instead forced into the surrounding solvent. The solution structures of TxVIA (Kohno *et al.*, 2002) and EvIA (Volpon *et al.*, 2004) again provide insights into the structural determinants important for their activity at VGSCs, although the specific residues remain to be identified.

κ-Conotoxins-potassium channel inhibitors: Compare to the diversity K^+ channel inhibitors produced by scorpion and sea anemones, the cone snails appear to have evolved relatively few peptides active at this physiologically important target. Kappa conotoxin PVIIA is the first *Conus* peptide identified to target K^+ channels. PVIIA from *C. purpurascens* acts in the pore of the Shaker K^+channel (Shon *et al.*, 1998) at position that confers voltage sensitivity (Scanlon *et al.*, 1997) and state dependence (Terlau *et al.*, 1999) confers to its block. The κA Conotoxins from the venom of *C. striatus* (piscivorous) was the first identified κ-A conotoxin which block the K^+ channels. In addition to these inhibitors of K^+ channels κ-BtX was identified as a novel peptide in the venom of the vermivorous *C. betulinus* that selectively enhances large conductance calcium activated K^+ (BK) currenbt in chrommaffin cells (Fan *et al.*, 2003).

χ-Conotoxins-norepinephrine transporter inhibitors: The norepinephrine transporter (NET) plays a key role in reducing levels of neuronally released norepinephrine (NE = noradrenaline). The tricyclic antidepressants inhibit NET and appear to produce analgesia by enhancing the descending inhibitory pathway controlled by norepinephrine release. Due to antidepressant side effects these compounds as well as significant off target pharmacologies limit their usefulness in the treatment of pain. χ conopeptides first isolated from *C. marmoreus* are highly specific, non competitive inhibitors of norepinephrine uptake through the NET (Sharpe *et al.*, 2003) that produce potent analgesia in rats.

ρ-Conotoxins-adrenoreceptor antagonists: TIA is the one and only ρ-Conotoxin isolated from cone snails to date which has a cysteine and structure reminiscent of α Conotoxins selectively target mammalian α_{1A}-α_{1b} and α_{10} adrenoreceptors (Sharpe *et al.*, 2003). TIA is competitively inhibit α_{1B} adreno receptors but competitively inhibits α_{1A} and α_{1D} adrenoreceptors (Chen *et al.*, 2004).

Conantokins: Conantokins are specific inhibitors of the N-methyl D-aspartate (NMDA) receptor. (Layer *et al.*, 2004). Conantokins competitively inhibit glutamic activation, which are ligand gated Ca+ channels involved in seizures in intractable epilepsy (Jimenezab *et al.*, 2002). Unlike the Conotoxins, the conantokins have no disulphide bonds but derive their structural stability from Post tranlationally modified glutamic residues present as γ carboxy glutamate (Gla).

Conopressins-vasopressin receptor modulators: Conopressins are the having the sequence similarity with oxytoxin and vasopressin. Oxytoxin and vasopressin act at the vasopressin receptors (GPCR). Con-T a recently discovered conpressins was found to be a selective V_{1a} antagonist with partial agonist activity at the OT receptor and no detectable activity at V_{1b} and V_2 receptors (Dutertre *et al.*, 2008). Therapeutic applications for this class of Conotoxins, beyond the potential in cardiovascular disorders and pre term child birth, may relate to their central actions where effects on mood have been observed in animals.

Contulakin-G-neurotensin receptor agonists: Cone snails produce a second endogenous peptide analogue, a glycosylated neurotensin analogue named contulakin-G (Craig *et al.*, 1998). This peptide is a potent analgesic in a wide range of animal models of pain (Allen *et al.*, 2007). Interestingly ContulakinG is 100 fold less potent than neurotensin receptor I, but 100 fold more potent as an analgesic. Based on its potency and wide therapeutic window, contulakin G (GGX-1160) went into early stage clinical development for the treatment of pain.

Contryphans and other cono peptides: Contryphans are the smallest peptides 8-9 aminoacid residues with one disulphide bond being developed for neuropathic pain. Only very few number of contryphans were characterized till now. For example, Contryphans from *Conus loroisii* (959) and *Conus amadis* (Am975) (Sabareesh *et al.*, 2006) target Ca^+ channel.

CONCLUSION AND FUTURE DIRECTION

As a consequence of their high selectivity, conus peptides have proved particularly useful for *in vitro* and *in vivo* proof-of-concept studies. The vast array of combinatorial library of peptides remain pharmacologically uncharacterized enormous opportunity remains to identify new research and potential therapeutics from amongst the highly diverse venoms of Conidae. The exploration of cone snails for the novel drug development is very scanty. Hence, the high throughput screening strategies are expected to accelerate the drug discovery process of new conotoxin. However, for therapeutic applications, a number of issues associated with safety, pharmacokinetics and delivery need to be addressed.

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