Conantokins: from "sleeper" activity to drug development

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Abstract

The conantokins belong to a distinctive family of *Conus* peptides which are known to have sleep-inducing activity in young mice, and are found to be inhibitors of N-methyl-D-aspartate (NMDA) receptors. This paper presents an overview of the various conantokins characterized so far, with focus on the biochemistry, solution structures in the absence or presence of divalent cations, function as selective NMDA receptor antagonists, and potential as therapeutic agents for some neurological conditions associated with NMDA receptor dysfunction.

1. Introduction

The marine cone snails of the genus *Conus* (Figure 1) produce in their venoms an enormous assortment of small peptide toxins (typically ~ 10 to 40 amino acid residues) known as conotoxins or conopeptides. Based on their prey, the snails are classified into three groups: the piscivorous species that paralyze and feed on fish, the vermivorous species that feed on polychaete, echiuroid, and hemichordate worms, and the molluscivorous species that feed on other mollusks (Terlau and Olivera 2004). Analyses of venoms from various *Conus* species have revealed that the venom is a complex mixture of conopeptides which target various receptors and ion channels in the nervous system (Olivera 1997). The different predatory interests and specializations of cone snails, as well as the diversity of molecular targets of conopeptides, provide the rationale for the diversity and complexity of peptides found in the venoms (Teichert et al. 2009; Terlau and Olivera 2004).

A distinctive family of conopeptides is comprised of the conantokins. The conantokins are considered to be part of the "nirvana cabal" whose major function is to deaden the sensory circuitry of the prey, facilitating prey capture by fish-hunting cone snails using the net strategy (Terlau and Olivera 2004). The conantokins are identified by virtue of their ability to induce sleep when administered intracranially (i.c.) to young mice. A notable biochemical feature of conantokins is the presence of multiple residues of post-translationally modified amino acid, γ-carboxyglutamate (Gla), which are essential for their biological activities (McIntosh et al. 1984). The conantokins are the only peptide ligands known to inhibit the N-methyl-D-aspartate (NMDA) receptors. The function of conantokins as selective antagonists of NMDA receptors has raised interest in these peptides as potential therapeutic agents.

2. Identification of conantokins as "sleeper peptides"

Conantokins have been systematically referred to as "sleeper peptides" because of their ability to induce sleep in young mice; "conantokin" is derived from the Filipino word "antokin" which translates into "sleepy" (Haack et al. 1990). By following the sleep-inducing activity of venom fractions from the piscivorous *Conus geographus* (the geography cone), the first identified conantokin (Con)-G was purified and characterized (McIntosh et al. 1984; Olivera et al. 1985). Later, various conantokins from other fish-hunting species were discovered and characterized, either by direct purification from *Conus* venoms or by the molecular biology approach and inference from sequences of cDNAs derived from venom ducts. Con-T and Con-R were originally isolated from the venoms of *Conus tulipa* (the tulip cone) and *Conus radiatus* (the radial cone), respectively (Haack et al. 1990; White et al. 2000), while Con-Pr1, Con-Pr2 and Con-Pr3 were initially purified from the

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Figure 1. Shells of piscivorous cone snails (genus *Conus*) that produce the earlier identified and more characterized conantokins described in this paper. Left to right: *Conus geographus*, the geography cone; *Conus radiatus*, the radial cone; *Conus tulipa*, the tulip cone. The cone snails were collected in the Philippines.

venom of a single species, *Conus parius* (the parian cone) (Teichert et al. 2007). Con-L was the first conantokin originally identified from the venom duct of *Conus lynceus* (the lynceus cone) by cDNA cloning (Jimenez et al. 2002). More recently, Con-P, Con-E, and Con-Br were identified from the venom ducts of *Conus purpurascens* (the purple cone), *Conus ermineus* (the turtle cone), and *Conus brettinghami*, respectively, also by using the molecular biology approach (Gowd et al. 2008; Twede et al. 2009).

3. Multiple γ-carboxyglutamate residues in conantokins

A majority of the previously characterized conopeptides have multiple disulfide bonds. In contrast, most conantokins do not have any, while a few have only a single disulfide bond (Table 1). Biochemical characterization of Con-G reveals five Gla residues in a 17-mer peptide (McIntosh et al. 1984). Con-T, a 21-residue peptide, has four Gla residues (Haack et al. 1990). All the other characterized conantokins have three to five Gla residues (White et al. 2000; Jimenez et al. 2002; Teichert et al. 2007; Gowd et al. 2008; Twede et al. 2009).

Because of the absence of disulfide bonds in conantokins, it is the Gla residues which provide the structural framework, giving rise to stable α-helical structures in conantokins, particularly in the presence of divalent cations, as will be detailed in Section 4. Thus, aside from having multiple disulfide bonds, the presence of multiple Gla residues may be considered as an additional strategy for assuming rigid and functional conformation in small peptides. Moreover, the Gla residues contribute to the biological activities of conantokins; when Gla residues were decarboxylated, the sleep-inducing activity of Con-G was lost (McIntosh et al. 1984).

4. Solution structures of conantokins

Nuclear magnetic resonance (NMR) spectroscopy and circular dichroism (CD) spectroscopy have been employed in the elucidation of solution structures of conantokins, in free form or with bound divalent cations.

4.1. Structures of conantokin-G

Studies using CD and NMR revealed that Con-G has almost unstructured conformation in solution, with no observable α-helical structure (Chen et al. 1998; Prorok et al. 1996). On the other hand, NMR spectroscopy demonstrated that Con-G has a structured region from Gla3 to Arg13, with a partial loop focused around Gla3 and Gla4. Furthermore, there is a distorted type I turn between Gln6 and Gln9, and also two type I turns formed by Gla10 and Leu11, and Ile12 and Arg13; wherein these two turns delineate about 1.6 turns of a distorted curvilinear 3(10) helix (Rigby et al. 1997a).

The structure of Con-G is modified from a random conformation to that with an α-helix upon binding of divalent cations. The NMR and CD spectra indicated a considerable Ca^{2+}-induced conformational change in Con-G which shifts

Table 1. Amino acid sequences of previously characterized conantokins.

A. Conantokins without disulfide bond

Peptide	*Conus* species	Sequence	Ref.
Conantokin-G	*Conus geographus*	GEγγLQγNQγLIRγKSN*	McIntosh et al. 1984
Conantokin-T	*Conus tulipa*	GEγγYQKMLγNLRγAEVKKNA*	Haack et al. 1990
Conantokin-L	*Conus lynceus*	GEγγVAKMAAγLARγDAVN*	Jimenez et al. 2002
Conantokin-Pr_1	*Conus parius*	GEDγYAγGIRγYQLIHGKI	Teichert et al. 2007
Conantokin-Pr_2	*Conus parius*	DEOγYAγAIRγYQLKYGKI	Teichert et al. 2007
Conantokin-Pr_3	*Conus parius*	GEOγVAKWAγGLRγKAASN*	Teichert et al. 2007
Conantokin-Br	*Conus brettinghami*	GDγγYSKFIγRERγAGRLDLSKFP	Twede et al. 2009

B. Conantokins with a single disulfide bond

Conantokin-R	*Conus radiatus*	GEγγVAKMAAγLARγNIAKGCKVNCYP	White et al. 2000
Conantokin-P	*Conus purpurascens*	GEγγHSKYQγCLRγIRVNKVQQγC	Gowd et al. 2008
Conantokin-E	*Conus ermineus*	GEγγHSKYQγCLRγIRVNNVQQγC	Gowd et al. 2008

γ, γ-carboxyglutamate; O, 4-*trans*-hydroxyproline; *, C-terminal amidation

dramatically to a high degree of α-helix at high Ca^{2+} concentration (Prorok et al. 1996). The transformation of Con-G from a distorted curvilinear 3(10) helix into a linear α-helix upon binding of Ca^{2+} to Gla residues results in the alignment of Gla3, Gla7, Gla10, and Gla14 in a linear array on one face of the helix, exposing the hydrophobic amino acid residues on the opposite face of the helix (Rigby et al. 1997b). The NMR data suggested at least two Ca^{2+}-coordinating sites in Con-G, such as those involving Gla3 and Gla7, and Gla10 and/or Gla14 (Nielsen et al. 1999).

The CD spectra of Con-G with bound Mg^{2+} showed an α-helix spanning the whole peptide, with a Mg^{2+} ion coordinated to oxygen atoms of the γ-carboxylates of Gla3, Gla4, and Gla7; a Mg^{2+} ion bound to an oxygen atom from each of the γ-carboxylates of Gla7; and another Mg^{2+} ion chelated to three oxygen atoms of γ-carboxylates from Gla10 and Gla14. Studies using Con-G analogs showed that all the Gla residues contribute to the formation of α-helical conformation induced by Mg^{2+} in Con-G (Chen et al. 1998). The proportion of α-helix formed by Con-G was correlated with the size of the divalent cations; it ranges from ~ 7% with bigger cations, such as Ba^{2+}, to > 70% with smaller cations, such as Mg^{2+}, Mn^{2+} and Zn^{2+} (Blandl et al. 1997).

4.2. Structures of conantokin-T

Both CD and NMR spectra showed that Con-T has significant α-helical conformation; addition of high Ca^{2+} concentration leads to smaller change in α-helical structure (Lin et al. 1997; Prorok et al. 1996; Warder et al. 1997). Further studies using CD and NMR showed that the Gla residues form salt bridges which stabilize the α-helical conformation of Con-T; replacement of Gla residues lessens the α-helical content (Lin et al. 1997). Another study confirmed that Con-T has a stable α-helical structure, either in the absence or presence of various cations, such as Ca^{2+}, Mg^{2+}, Cu^{2+}, or Zn^{2+}; the structure of Con-T determined by NMR showed a mixture of 3(10) helix and α-helix, resulting in straight and curved helical conformations (Skjaerbaek et al. 1997).

The NMR spectra of Con-T revealed a hydrogen bond between Gln6 and Gla10 and an ionic interaction between Arg13 and Gla14. Moreover, Tyr5, Met8, Leu9 and Leu12 form a stable hydrophobic cluster, while the amide of Asn20 and the C-terminal amide interact with the backbone (Warder et al. 1997). Further studies showed that similar to metal-free Con-T, the Ca^{2+}-coordinated Con-T has α-helix as the major conformation, though there is reorientation of several residues, wherein Gla10 and Gla14 are more optimally positioned to bind Ca^{2+}. One Ca^{2+} ion binds two oxygen atoms each from Gla10 and Gla14, while another Ca^{2+} ion is possibly coordinated to three oxygen atoms, two from Gla3 and one from Gln6 (Warder et al. 1998). Moreover, ionic interaction between Gla3/Gla10 and Lys7 contributes to the stabilization of the α-helical structure. The use of Con-T analogs indicated that Gla4, Gla10, and Gla14 are important in stabilizing the α-helical structure or regulating the conformational change induced by Ca^{2+} (Warder et al. 1998).

Table 2. NMDA receptor selectivity of conantokins

Peptide	NMDA receptor subtype	Ref.
Conantokin-G	NR2B	Donevan and McCabe 2000
Conantokin-R	NR2B ~ NR2A	White et al. 2000
Conantokin-T	NR2B, NR2A	Klein et al. 2001
Conantokin-Pr1	NR2B	Teichert et al. 2007
Conantokin-Pr2	NR2B > NR2D	Teichert et al. 2007
Conantokin-Pr3	NR2B	Teichert et al. 2007
Conantokin-P	NR2B > NR2A	Gowd et al. 2008
Conantokin-Br	NR2D	Twede et al. 2009

4.3. Structures of other conantokins

In CD spectra, Con-R exhibited a low degree of α-helical content, with an increase in α-helical structure upon the addition of Ca^{2+}, Mg^{2+}, or Zn^{2+} (Blandl et al. 2000). Studies using CD spectroscopy showed that Con-Pr1 and Con-Pr2 are mainly unstructured in the absence of Ca^{2+} or Mg^{2+}, but exhibit α-helical conformation in the presence of either cation; while Con-Pr3 has α-helical structure even in the absence of either Ca^{2+} or Mg^{2+} (Teichert et al. 2007). The estimated α-helical content of Con-Pr1 or Con-Pr2 is greater in the presence of Mg^{2+} than Ca^{2+}, which is consistent with another study showing that Con-G has greater affinity and more definitive structure in the presence of Mg^{2+} than Ca^{2+} (Chen et al. 1998; Teichert et al. 2007). With CD spectroscopy, Con-P exhibited essentially the same degree of α-helical content in the absence or presence of Ca^{2+} (Gowd et al. 2008).

The presence of α-helical conformations in Con-P, Con-Pr3 and Con-T in the absence of Ca^{2+} is probably due to Lys7. Con-Pr1, Con-Pr2, and Con-G which have Gla residue at this locus require Ca^{2+} to stabilize the α-helical conformations (Gowd et al. 2008; Prorok et al. 1996; Rigby et al. 1997b; Teichert et al. 2007).

5. Conantokins as inhibitors of N-methyl-D-aspartate receptors

The conantokins are found to be selective inhibitors of NMDA receptor subtypes (Table 2). The NMDA receptor, a subtype of ionotropic glutamate receptor, is believed to have a tetrameric structure and is composed of two major subunit families, NR1 and NR2. There are eight splice variants of NR1 (NR1-1a and 1b, NR1-2a and 2b, NR1-3a and 3b, NR1-4a and 4b) and four subunits of NR2 (NR2A, NR2B, NR2C, NR2D). The diversity of NMDA receptors is generated by co-assembly of the NR1 and NR2 subunits (Stephenson 2006). The NMDA receptor expresses several pharmacologically distinct modulatory sites, among which are the glutamate and glycine recognition sites, and polyamine site. The receptor-linked ion channel is gated by co-agonists: glutamate, which binds to the NR2 subunit, and glycine, which binds to the NR1 subunit. The receptor is typically blocked by Mg^{2+}, which binds in the pore region and must be removed by membrane depolarization in order for glutamate and glycine to elicit currents (Stephenson 2006).

5.1. Inhibition of NMDA receptor by conantokin-G

Both competitive and noncompetitive interactions with the NMDA receptor binding site have been indicated for Con-G. The first evidence showing that Con-G is a selective antagonist of NMDA receptor was shown by using neonatal rat cerebellar preparation, wherein Con-G blocked the rise in cyclic guanosine monophosphate (cGMP) levels induced by the addition of NMDA. An increase in the NMDA concentration had no effect on this inhibition, indicating that Con-G is a noncompetitive inhibitor of NMDA receptor (Mena et al. 1990). Con-G was shown to be a noncompetitive NMDA receptor antagonist through an allosteric inhibition of the spermine- and spermidine-enhanced binding of [^{3}H]MK-801 (NMDA receptor channel blocker) to rat forebrain membranes (Skolnick et al. 1992). This is consistent with another study showing that Con-G is a noncompetitive inhibitor of spermine-stimulated binding of [^{3}H]MK-801 to NMDA receptor obtained from human brain tissue (Nielsen et al. 1999). Furthermore, in cultured mouse hippocampal neurons, with the use of the whole cell patch clamp technique, the NMDA-evoked currents were blocked by Con-G in a noncompetitive mechanism (Klein et al. 1999). On the other hand, voltage clamp recordings from cultured murine cortical neurons and from *Xenopus* oocytes expressing NMDA receptors showed that Con-G is a potent inhibitor of NMDA-induced currents which can be prevented by high concentrations of NMDA, indicating that Con-G is a competitive antagonist of NMDA receptor (Donevan and McCabe 2000).

A study using NMDA receptor expressed in mouse brain mRNA-injected *Xenopus* oocytes revealed that Con-G interacts with the glutamate binding site, but not with the glycine binding site (Hammerland et al. 1992). With the use of voltage clamp recordings from *Xenopus* oocytes expressing NMDA receptor subtypes, Con-G was shown to selectively block NMDA

receptors containing the NR2B subunit (Donevan and McCabe, 2000; Wittekindt et al. 2001). Whole cell voltage clamp recordings from human embryonic kidney 293 cells exhibited antagonist activity of Con-G in cells expressing NR1a/NR2B and NR1a/NR2A/NR2B receptors. Moreover, analyses of various conantokin analogs showed that Leu5 is an essential determinant of the selectivity of Con-G for the NR2B subunit (Klein et al. 2001).

5.2. Inhibition of NMDA receptors by conantokin-T and conantokin-R

Con-T inhibited the NMDA receptor-mediated Ca^{2+} influx in cultured rat cerebellar granule cells indicating that it has NMDA antagonist activity (Haack et al. 1990). With the use of whole cell patch clamp recordings, the NMDA-induced responses in cultured mouse hippocampal neurons were inhibited by Con-T in a noncompetitive manner (Klein et al. 1999). Con-T also showed a noncompetitive inhibition of spermine-enhanced [^{3}H]MK-801 binding to NMDA receptor derived from human brain tissue (Nielsen et al. 1999). Voltage clamp recordings from human embryonic kidney 293 cells expressing various NMDA receptor subunits showed that Con-T diminished NMDA-evoked currents in cells expressing NR1a/NR2A or NR1a/NR2B (Klein et al. 2001).

The NMDA receptor antagonist activity of Con-R was demonstrated by an assay involving inhibition of the spermine-enhanced binding of [^{3}H]MK-801 to rat brain membranes (Blandl et al. 2000). Voltage clamp recordings from *Xenopus* oocytes expressing NMDA receptor subunits showed that Con-R has nearly equal selectivity for NR2B and NR2A subunits (White et al., 2000). With the use of Con-R analogs, the substitution of Met8 and Leu12 by Ala led to ~ 20-fold and 55-fold reduction in potency, respectively, while a single Ala replacement of any of the first five N-terminal amino acid residues resulted in considerable activity losses ranging from 11-fold to > 1000-fold (Blandl et al. 2001).

5.3. Inhibition of NMDA receptors by other conantokins

The inhibition of NMDA receptor by Con-L was confirmed by using whole cell voltage clamp recordings from cultured mouse cortical neurons (Jimenez et al. 2002). Electrophysiological assays using NMDA receptors expressed in *Xenopus* oocytes showed that Con-Pr1, Con-Pr2, and Con-Pr3 inhibit the NMDA receptors, with the highest potency for NR2B. Con-Pr2 showed the least difference in the selectivity for the NR2B and NR2D subunits (Teichert et al. 2007). With the use of a similar method, Con-P blocked NMDA receptor subtypes, wherein the inhibition of NR2B is greater than NR2A subunit (Gowd et al. 2008). In contrast to the other characterized conantokins, Con-Br has a high potency and selectivity for NR2D subunit. Tyr5 appears to be a determinant of the potency for NR2D; replacement by Val results in a decrease in the potency of Con-Br (Twede et al. 2009).

6. Conantokins for drug development

The NMDA receptor has been implicated in several acute and chronic neurological conditions; thus, it has potential as a therapeutic target. The NMDA receptor-linked channel is very permeable to Ca^{2+} ions; excessive activation leads to excitotoxicity and death of neuronal cells, which may be remedied by NMDA receptor inhibitors. Hence, there have been much interest and efforts in the development of effective and safe therapeutic agents that specifically block the NMDA receptors.

The conantokins have been characterized in animal models of disease states including pain, convulsive disorder, and post-ischemia. In fact, Con-G is a potent anticonvulsant and analgesic, and has reached human clinical trials as a drug (CGX-1007, Cognetix) for both epilepsy and pain (Malmberg et al. 2003). Moreover, it has been shown to be neuroprotective in rat models of ischemia (Williams et al. 2000; Williams et al. 2002).

6.1. Conantokins for pain

Studies indicate that the analgesic activity of conantokins may be related to their NR2B selectivity, and that conantokins may be useful as therapeutic agents for the relief of pain. Non-selective NMDA receptor antagonists can alleviate injury-induced pain, while generally causing undesirable side effects (Malmberg et al. 2003).

Con-G or Con-T exhibited potent anti-nociceptive effects in mice models of pain induced by tissue injury, nerve injury, and inflammation. In the formalin test, intrathecal (i.t.) administration of Con-G or Con-T relieved pain at doses that were 17 to 27 times lower than those analgesics which impair motor function. Moreover, Con-G reduced the mechanical and thermal allodynia evoked by complete Freund's adjuvant (CFA) (Malmberg et al. 2003). The analgesic effects of Con-G in mice, determined by the hot-plate test, following stimuli using CFA, formalin, and acetic acid, were higher compared to Con-R[1-17], a non-selective inhibitor of NMDA receptor. In the formalin test, Con-G was shown to significantly block the second phase nociceptive response and suppress paw edema (Xiao et al. 2008). By i.t. injection of Con-G in rats with a spinal nerve ligation or with a spinal cord compression injury, and in the formalin test, Con-G alleviated nociceptive responses in a dose-dependent manner (Hama and Sagen 2009).

6.2. Conantokins for convulsive disorder

The NMDA subtype selectivity of conantokins offers an advantage over the noncompetitive NMDA receptor antagonists, such as MK-801 and ifenprodil. Con-R is much more highly potent, with a high protective index when examined using Frings audiogenic mice as models of epilepsy. In the audiogenic seizure-susceptible mice, Con-R prevented sound-induced tonic extension seizures at nontoxic doses, even with maximal stimulation, and partially blocked clonic seizures induced by

pentylenetetrazol (White et al. 2000). Thus, Con-R is a useful anticonvulsant without causing the undesirable side effects associated with other NMDA receptor antagonists.

6.3. Conantokins for post-ischemia

Studies present evidence for the potent effects of Con-G as a neuroprotective agent against ischemic brain injury. In cerebellar neurons, Con-G reduced the excitotoxic Ca^{2+} responses and demonstrated neuroprotection against injury induced by NMDA, glutamate, veratridine, or hypoxia/hypoglycemia. With the use of middle cerebral artery occlusion as a rat model of transient focal brain ischemia, Con-G (i.c.) caused 89% reduction in brain infarction in the core region of injury, with significant recovery, although with mild sedation (Williams et al. 2000). Further study showed that with the same animal model, the neuroprotective effect of Con-G (i.t.) resulted in a reduction in brain infarction with significant recovery, and a therapeutic window which lasted for at least 8 hours from the onset of injury (Williams et al. 2002). Post-injury treatment with Con-G during the initial 24 hours of transient middle cerebral artery occlusion in rats showed that Con-G reduced by 50% the expression of c-fos gene throughout the injured area, which can be linked to a neuroprotective relief of cerebral ischemia (Williams et al. 2003).

7. Concluding remarks

The "sleeper" activity and NMDA receptor antagonism of conantokins may be correlated with the presence of multiple Gla residues (aside from other key amino acid residues) contributing to the formation of stable and functional α-helical structures. The efficacy of conantokins in preclinical models of pain, convulsive disorder, and ischemia, and their safety profiles, indicate that these conopeptides are not only useful probes for NMDA receptor functions, but are also important natural products to be explored for their therapeutic potential. Remarkably, conantokins have NMDA receptor subunit selectivity, a pharmacological feature which is essential in the search for appropriate drug candidates.

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