Toxicon 103 (2015) 39–47

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

A sleep-inducing peptide from the venom of the Indian cone snail *Conus araneosus*

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ARTICLE INFO

Article history:
Received 9 February 2015
Received in revised form
15 June 2015
Accepted 17 June 2015
Available online 19 June 2015

Keywords:
Conus araneosus
Conotoxin
M-, T-superfamily
Mass spectrometry
Post-translational modification
Sleep-inducing peptide

ABSTRACT

The marine snail *Conus araneosus* has unusual significance due to its confined distribution to coastal regions of southeast India and Sri Lanka. Due to its relative scarceness, this species has been poorly studied. In this work, we characterized the venom of *C. araneosus* to identify new venom peptides. We identified 14 novel compounds. We determined amino acid sequences from chemically-modified and unmodified crude venom using liquid chromatography–electrospray ionization mass spectrometry and matrix assisted laser desorption ionization time-of-flight mass spectrometry. Ten sequences showed six Cys residues arranged in a pattern that is most commonly associated with the M-superfamily of conotoxins. Four other sequences had four Cys residues in a pattern that is most commonly associated with the T-superfamily of conotoxins. The post-translationally modified residue (pyroglutamate) was determined at the N-terminus of two sequences, ar3h and ar3i respectively. In addition, two sequences, ar3g and ar3h were C-terminally amidated. At a dose of 2 nmol, peptide ar3j elicited sleep when injected intraperitoneally into mice. To our knowledge, this is the first report of a peptide from a molluscivorous cone snail with sleep-inducing effects in mice. The novel peptides characterized herein extend the repertoire of unique peptides derived from cone snails and may add value to the therapeutic promise of conotoxins.

1. Introduction

Venoms are proving to be a remarkable source of novel peptides that have potential applications in human health (Sherman, 2005). Cone snails are well-known members of the hyper diverse group of marine gastropods and are appreciated for their history of rapid diversification, their tremendously diverse and complex venom termed as 'conotoxins' (Robinson et al., 2014). More than 700 species of cone snails are currently recognized (Puillandre et al., 2014) and several lines of evidence suggests that each snail species may produce more than 1000 distinct compounds in their venom (Lewis et al., 2012). Venom peptides ('conotoxins') target a wide variety of membrane-bound protein channels and receptors (Olivera et al., 1990; McIntosh et al., 2000). Hitherto, venoms of more than 100 species of Conidae have been studied and many conotoxin peptides have been discovered (Kaas et al., 2012). The relative simplicity of conotoxins has made them valuable in the advancement of neuroscience research and consequent drug development (Yao et al., 2008). Active research in the study of conotoxins typically involves isolation, identification and assessment of biological activity for individual peptides that possess the prospective to be made into potent and selective therapeutic drugs. Preceding three decades of greatly accelerated *Conus* peptide discovery has revealed several groups of conotoxins. To date, the conotoxin drug approved by the United States Food and Drug Administration for public use is Prialt™, derived from *Conus magus* to treat chronic pain, which is one of the most potent analgesics available (Atanassoff et al., 2000; Alonso et al., 2003; Skov et al., 2007). Prialt™ is the trade name for ω-conotoxin MVIIA, a calcium channel blocker that provides a non-addictive means to block

Abbreviations: TCEP, Tris (2-carboxyethyl) phosphine; NEM, N-ethylmaleimide; ACN, acetonitrile; RP-HPLC, reverse phase-high performance liquid chromatography; ESI, electrospray ionization; LC–ESI–MS, liquid chromatography–electrospray ionization-mass spectrometry; TFA, tri fluoro acetic acid; MALDI-TOF-MS, matrix assisted laser desorption ionization-time of flight-mass spectrometry; CCA, alpha-cyano-4-hydroxycinnamic acid; CID, collision induced dissociation; ETD, electron transfer dissociation.

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http://dx.doi.org/10.1016/j.toxicon.2015.06.017
0041-0101/

pain in subject patients by preventing the source of pain transmission in nerve cells of the spinal cord (Du et al., 2007; Jacob and McDougal, 2010).

Although, conotoxins can be classified in several ways, inferences regarding pharmacological activity and cysteine framework have been the primary utilized methods. For example, conotoxins have been assigned to pharmacological families based on the receptor they target and types of interactions they have with their receptors. On the other hand, conotoxins can be assigned to particular classes based on their cysteine framework independent of their actions (Robinson and Norton, 2014). Conotoxins can be classified based on the signal sequence of their protein precursors. The gene superfamily classification is based on the high sequence similarity in their signal and propeptide regions of conotoxins. Because, cDNA sequencing is widely used in conotoxin research, gene superfamily classification has become the most convenient method for classification of conotoxins (Robinson and Norton, 2014). In this work, as cDNA sequences were not achieved, we assigned the conotoxin sequences to the most commonly associated, M- and T-superfamilies.

The M-superfamily of conotoxins is likely the most diverse of all the conotoxin superfamilies so far characterized (McDougal et al., 2008; Jacob and McDougal, 2010). The M-superfamily has been further subdivided into five branches, M-1 through M-5, based on the number of residues present in the third intercysteine loop. Further they are classified as mini-M (M-1 through M-3) and maxi-M (M-4, M-5) conotoxins (McDougal et al., 2008). Mini-M conotoxins contain less than 22 residues, whereas maxi-M peptides contain more than 22 amino acids. Maxi-M-conotoxins are subgrouped as μ-conotoxins (which block voltage-gated sodium channels) (Myers et al., 1993), κM-conotoxins (which block voltage-gated potassium channels) and ψ-conotoxins (which block nicotinic acetylcholine receptors) (Corpuz et al., 2005) based on functions. Information on functions of mini-M conotoxins, however are limited or unknown (Jacob and McDougal, 2010). The peptide, LtIIIA an M-1-conotoxin from *Conus litteratus* acts on sodium channels whose pharmacological target is unidentified (Wang et al., 2009). The peptide, QcIIIA from *Conus quercinus* elicits scratching in mice (Olivera et al., 1990). To date, the peptide QcIIIA is the only member of M-2-conotoxins that has known biological activity.

The T-superfamily of conotoxins has been further subdivided into three groups based on cysteine framework. Framework V contain two pairs of adjacent Cys residues separated by four, five or six amino acids (CC———CC) and framework X have the structure CC——C–C. Of the few T-superfamily conotoxins with type V framework, most have shown behavioral effects on fish and mice (Walker et al., 1999; Aguilar et al., 2006). A few others have defined targets: TxVA targets pre-synaptic Ca^{2+} channels or G protein-coupled receptors (Rigby et al., 1999) and LtVD inhibits tetrodotoxin-sensitive Na^{+} currents in rat DRG neurons (Liu et al., 2007). Recently, synthesized peptides of *Conus consors* with framework V showed interaction with somatostatin-3 receptors (Petrel et al., 2013). Framework X conotoxins of T-superfamily was first identified in *Conus marmoreus* (Sharpe et al., 2001; McIntosh et al., 2000; Balaji et al., 2000; Robinson and Norton, 2014) and subsequently in *Conus araneosus* (Kallol et al., 2010). Recently, it has also been confirmed in *Conus victoriae* (Robinson et al., 2014). The peptides of *C. marmoreus*, mr10a (T-2- conotoxin) has shown antinociceptive activity (McIntosh et al., 2000) while, λ-CMrVIa and λ-CMrVIX produced seizure, paralysis and death when injected (IC) in mice (Balaji et al., 2000). The peptides, χ-MrIA and mr10a reversibly inhibit the neuronal noradrenalin transporters (Sharpe et al., 2001).

As a part of the comprehensive program for the identification and characterization of peptides from cone snails found in the Indian coastal seas (Sudarslal et al., 2003, 2004; Gowd et al., 2005; Sabareesh et al., 2006; Mandal et al., 2007; Kallol et al., 2010), a molluscivorous cone snail endemic to India, *C. araneosus* was undertaken for this study. This species is restricted to shallow coastal seas of the southeast India and Sri Lanka (Röckel et al., 1995; Franklin et al., 2009). Four conopeptides have been reported earlier from this species (Gowd et al., 2005; Kallol et al., 2010). In this work, we identified 14 novel peptides from *C. araneosus* venom, totally characterized by amino acid sequence determination. Amino acid sequences were achieved using high-resolution mass spectrometry based fragmentation techniques (Aguilar et al., 2006; Biemann, 1990; Kallol et al., 2010; Mandal et al., 2007; Rajesh, 2015). Two sequences (ar3h and ar3i) showed post-translationally modified residues (pyroglutamate) at the N-terminus and two others (ar3g and ar3h) were C-terminally amidated. Interestingly, the sequence ar3j induced sleep when injected intraperitoneally into mice. So far, very few peptides isolated from piscivorous cone snails that induce sleep in mice have been reported (Olivera et al., 1990, 1999; Jimenez et al., 2003; Teichert et al., 2007). To our knowledge, this is the first report of a conopeptide from a molluscivorous cone snail with sleep-inducing effect in mice.

2. Materials and methods

2.1. Materials

Tris (2-carboxyethyl) phosphine (TCEP) was procured from Thermo Fisher Scientific, Inc., United States. N-ethylmaleimide (NEM) was purchased from Sigma–Aldrich, United States. Analytical grade acetonitrile (ACN), Methanol and Tri fluoro acetic acid (TFA) were obtained from Merck Ltd., India.

2.2. Specimen collection and identification

Specimens of the molluscivorous cone snail *C. araneosus* were collected from bottom-set crab nets as by catch from the coastal regions of Vedhalai (09°16′N, 79°06′E), Gulf of Mannar, India. Because, *C. araneosus* is not listed as endangered or protected species, we collected eight adult specimens without prior consent from wild life authorities. Species identity was confirmed following standard keys (Franklin et al., 2009).

2.3. Extraction of crude venom

Venom ducts were dissected; venom was squeezed-out from ducts using a blunt-tipped forceps and extracted with 50% ACN. The extract was further filtered through Whatman No.1 filter paper. The filtrate was concentrated using Heidolph Loborota 4000 rotary vacuum evaporator (Germany) and lyophilized using Labconco Freezone Freeze Dry System 4.5 (United States) and stored at −20 °C.

2.4. Peptide purification

Venom extract was applied onto a Hewlett Packard 1100 series RP-HPLC system (United States) at room temperature, using a C18 semi preparative column (Phenomenex, 10 mm × 250 mm, 4 μm particle size, 90A° pore size). The components of the extract were eluted at a flow rate of 1 mL/min using a linear gradient from 5 to 90% solution B in 50 min. Solutions were as follows: A) 0.1% (v/v) TFA in water; B) 0.885% (v/v) TFA in ACN. Absorbance was monitored at 226 nm, and chromatographic peaks were collected manually. To characterize peptides present in the venom, the same column was employed for further purification, using a linear gradient starting with 20% solution B at a flow rate of 1 mL/min.