General and Comparative Endocrinology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

# Hormone-like peptides in the venoms of marine cone snails

Samuel D. Robinson [a,*], Qing Li [b], Pradip K. Bandyopadhyay [c], Joanna Gajewiak [c], Mark Yandell [b], Anthony T. Papenfuss [d], Anthony W. Purcell [e], Raymond S. Norton [a], Helena Safavi-Hemami [c,f,*]

[a] Medicinal Chemistry, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria 3052, Australia
[b] Eccles Institute of Human Genetics, University of Utah, and School of Medicine, Salt Lake City, UT 84112, USA
[c] Department of Biology, University of Utah, Salt Lake City, UT 84112, USA
[d] Bioinformatics Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia
[e] The Department of Biochemistry and Molecular Biology, School of Biomedical Sciences, Monash University, Clayton, Victoria 3800, Australia
[f] Department of Biology, Copenhagen Biocenter, Ole Maaløes Vej 5, DK-2200 Copenhagen, Denmark

ARTICLE INFO

*Article history:*
Received 30 April 2015
Revised 30 June 2015
Accepted 4 July 2015
Available online xxxx

*Keywords:*
Prohormone-4
Elevenin
Conotoxin
Venom
Neuropeptide
Hormone

ABSTRACT

The venoms of cone snails (genus *Conus*) are remarkably complex, consisting of hundreds of typically short, disulfide-rich peptides termed conotoxins. These peptides have diverse pharmacological targets, with injection of venom eliciting a range of physiological responses, including sedation, paralysis and sensory overload. Most conotoxins target the prey's nervous system but evidence of venom peptides targeting neuroendocrine processes is emerging. Examples include vasopressin, RFamide neuropeptides and recently also insulin. To investigate the diversity of hormone/neuropeptide-like molecules in the venoms of cone snails we systematically mined the venom gland transcriptomes of several cone snail species and examined secreted venom peptides in dissected and injected venom of the Australian cone snail *Conus victoriae*. Using this approach we identified several novel hormone/neuropeptide-like toxins, including peptides similar to the bee brain hormone prohormone-4, the mollusc ganglia neuropeptide elevenin, and thyrostimulin, a member of the glycoprotein hormone family, and confirmed the presence of insulin. We confirmed that at least two of these peptides are not only expressed in the venom gland but also form part of the injected venom cocktail, unambiguously demonstrating their role in envenomation. Our findings suggest that hormone/neuropeptide-like toxins are a diverse and integral part of the complex envenomation strategy of *Conus*. Exploration of this group of venom components offers an exciting new avenue for the discovery of novel pharmacological tools and drug candidates, complementary to conotoxins.



## 1. Introduction

Marine cone snails (genus *Conus*) are venomous. They use their venoms for prey capture, self-defense and possibly intraspecific competition (Dutertre et al., 2014; Olivera, 1999). Cone snail venoms are remarkably complex cocktails containing hundreds of small cysteine-rich peptide toxins (conotoxins) (Olivera et al., 1990; Robinson and Norton, 2014). Many conotoxins have unmatched potency and selectivity profiles for their respective targets, including specific subtypes of voltage- and ligand-gated ion channels, G protein-coupled receptors and neurotransmitter transporters. As such, conotoxins represent a rich source of valuable pharmacological tools and drug candidates.

Conotoxins are produced in a specialized venom gland, where they are translated as precursor peptides. As a general rule, conotoxin precursor peptides are comprised of an N-terminal signal sequence for targeting to the cellular secretory pathway, an intermediate pro-region that plays a role in vesicular transport (Conticello et al., 2003), post-translational modification (Bandyopadhyay et al., 1998) and folding (Buczek et al., 2004) and is presumably cleaved following secretion, and a single copy of the mature toxin peptide at the C-terminus. In many ways this process mirrors the production of endogenous hormones/neuropeptides.

While conotoxins are the major, and by far the most studied, constituent of *Conus* venom, several cases of hormone/neuropeptide-like components have been reported (Table 1). Examples include the conopressins (vasopressin analogues) from

*Abbreviations:* MeCN, acetonitrile; MS, mass spectrometry; TFA, trifluoroacetic acid.

* Corresponding authors at: Department of Biology, University of Utah, 257 South 1400 East, Salt Lake City, UT 84112, USA.

*E-mail addresses:* sam.robinson@utah.edu (S.D. Robinson), safavihelena@gmail.com (H. Safavi-Hemami).

http://dx.doi.org/10.1016/j.ygcen.2015.07.012
0016-6480/

**Table 1**
Hormones/neuropeptides identified in *Conus* venoms.[#]

| | | |
|---|---|---|
| **Conopressins** | | |
| Conopressin-S | **C**IIRN**C**PRG[*] | Cruz et al., 1987 |
| Conopressin-G | **C**FIRN**C**PKG[*] | Cruz et al., 1987 |
| Conopressin-Vil | **C**LIQD**CP**γG[*] | Möller and Marí (2007) |
| Conopressin-T | **C**YIQN**C**LRV[*] | Dutertre et al. (2008) |
| **Contulakin-G** | ZSEEGGSNA<u>T</u>KKPYIL | Craig et al., 1999 |
| **Conorfamides** | | |
| CNF-Sr1 | GPMGWVPVFYRF[*] | Maillo et al., 2002 |
| CNF-Sr2 | GPMγDPLγIIRI[*] | Aguilar et al. (2008) |
| CNF-Vc1 | HSGFLLAWSGPRNRFVRF[*] | Robinson et al., 2015 |
| **Conomap-Vt** | AfVKGSAQRVAHGY[*] | Dutertre et al., 2006 |
| **ConoCAP-a** | PF**C**NSFG**C**YN[*] | Möller et al., 2010 |
| **Neuropeptide-F/Y** | TVSDPPARPAVFHSREELMNYVRELNRYFAIVGRPRF[*] | Wu et al. (2010) |
| | TVSDPPARPAVFHSREELMNYVRELNLYFAIVGRPRY[*] | Wu et al. (2010) |
| **Insulin** | | |
| Con-Ins G1 A-chain | GVVγH**CC**HRP**C**SNAEFKKY**C**[*] | Safavi-Hemami et al. (2015) |
| B-chain | TFDTOKHR**C**GSγITNSYMDL**C**YR | |
| **Elevenin-Vc1** | RRID**C**KVFVFAPI**C**RGVAA | This study |
| **PH4-Vc1** | IGFPGFSTPPR | This study |

[#] Only those confirmed, in their entirety, at the peptide level are shown.
[*] C-terminal amidation, γ; gamma-carboxyglutamate, Z; pyroglutamic acid, <u>T</u>; O-glycosylated threonine, f; D-phenylalanine, O; hydroxyproline, cysteines are bolded.

*Conus geographus* and *Conus striatus* (Cruz et al., 1987), contulakin-G (a neurotensin analogue) from *C. geographus* (Craig et al., 1999), RFamide neuropeptides from *Conus spurius* and *Conus victoriae* (Maillo et al., 2002; Robinson et al., 2015), conomap (a myoactive tetradecapeptide) from *Conus vitulinus* (Dutertre et al., 2006), conoCAPs (analogues of crustacean cardioactive peptide) from *Conus villepinii* (Möller et al., 2010) and neuropeptide-F/Y from *Conus betulinus* (Wu et al., 2010). Furthermore, we recently demonstrated that specialized insulins are an abundant and active component of some *Conus* venoms (Safavi-Hemami et al., 2015). When injected into fish, the venom insulin elicits hypoglycemic shock, thus facilitating capture of the physiologically impaired prey. It is thus becoming clear that *Conus* venoms are not limited to conotoxins targeting neuronal ion channels, but that other fascinating strategies are also being employed.

To systematically explore the full extent of hormone/neuropeptide-like venom components expressed, secreted and ultimately injected into the prey by cone snails, we used a combined transcriptomics/proteomics-based strategy (mass spectrometry (MS)-matching) on the extruded as well as the injected venom (venom collected from live snails during an envenomation event) of *C. victoriae*, a mollusc-hunting species endemic to the coast of north-western Australia. This combined approach led to the identification of several novel peptides that share high similarity with endogenous hormones/neuropeptides from other organisms. Furthermore, interrogation of venom gland transcriptomic data from additional cone snail species revealed that these peptides are widely distributed in the genus *Conus*.

## 2. Materials and methods

### 2.1. Venom gland transcriptome

Specimens of *C. victoriae* were collected from Broome, Western Australia. Specimens were collected specifically for research use, under a commercial fishing license of the Western Australian Specimen Shell Managed Fishery (license number 2577). Ethics approval is not required, in Australia, for taking samples from *Conus*.

Preparation of the venom gland transcriptome of *C. victoriae* has been described previously (Robinson et al., 2014). Briefly, whole venom glands of live specimens were removed, snap-frozen in liquid nitrogen and stored at −80 °C. Frozen venom glands were pulverized and homogenized prior to extraction of total RNA with Trizol (Invitrogen, Life Technologies). cDNA library preparation, normalization and 454 sequencing were performed by Eurofins, MWG Operon (Budendorf, GER). *De novo* transcriptome assembly was performed using MIRA3 (Chevreux et al., 2004) and annotated using BLAST+ (version 2.2.27+) (Altschul et al., 1990; Camacho et al., 2009).

The venom gland transcriptomes of *Conus marmoreus*, *C. geographus*, *C. bullatus*, *Conus tessulatus*, *Conus varius* and *Conus virgo* were sequenced on an Illumina HiSEq. 2000 platform at Cofactor Genomics as described previously (Safavi-Hemami et al., 2015). Reads were *de novo* assembled using Trinity (Grabherr et al., 2011) and annotated using BLASTx.

### 2.2. Venom extraction & preparation

Extruded venom samples were obtained by manually squeezing freshly dissected *C. victoriae* venom glands, then snap-frozen in liquid nitrogen and stored at −80 °C. Extruded venom (from several specimens) was reconstituted in 0.1% trifluoroacetic acid (TFA), pooled and homogenized using a glass Dounce tissue grinder. Insoluble material was pelleted by centrifugation, supernatant collected and lyophilized. Pellets were resuspended in 0.1% TFA/20% acetonitrile (MeCN), centrifuged, supernatant collected and lyophilized. This process was repeated with 40% and 60% MeCN. Lyophilized venom was resuspended in 2% MeCN, 0.1% TFA and pooled. Protein concentration was determined using a Bradford assay with ovalbumin as the standard. An aliquot of the venom was reduced in 20 mM tris(2-carboxyethyl)phosphine (pH 8) for 30 min at 60 °C, then alkylated by incubating in 40 mM iodoacetamide for 30 min. An aliquot of reduced and alkylated venom was further processed by tryptic digestion, essentially according to the manufacturer's instructions (Sigma–Aldrich, St. Louis, MO, USA), with an incubation time of 4 h at 37 °C.

Lyophilized injected venom of *C. victoriae* was purchased from BioConus (www.bioconus.com). These specimens were also sourced from Broome, Western Australia and maintained in captivity where injected venom was collected using a procedure adapted from Hopkins et al. (1995). The injected venom samples were

Please cite this article in press as: Robinson, S.D., et al. Hormone-like peptides in the venoms of marine cone snails. Gen. Comp. Endocrinol. (2015), http://dx.doi.org/10.1016/j.ygcen.2015.07.012