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Structure and activity of contryphan-Vc2: Importance of the D-amino acid residue

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ABSTRACT

In natural proteins and peptides, amino acids exist almost invariably as L-isomers. There are, however, several examples of naturally-occurring peptides containing D-amino acids. In this study we investigated the role of a naturally-occurring D-amino acid in a small peptide identified in the transcriptome of a marine cone snail. This peptide belongs to a family of peptides known as contryphans, all of which contain a single D-amino acid residue. The solution structure of this peptide was solved by NMR, but further investigations with molecular dynamics simulations suggest that its solution behaviour may be more dynamic than suggested by the NMR ensemble. Functional tests in mice uncovered a novel bioactivity, a depressive phenotype that contrasts with the hyperactive phenotypes typically induced by contryphans. Trp3 is important for bioactivity, but this role is independent of the chirality at this position. The D-chirality of Trp3 in this peptide was found to be protective against enzymatic degradation. Analysis by NMR and molecular dynamics simulations indicated an interaction of Trp3 with lipid membranes, suggesting the possibility of a membrane-mediated mechanism of action for this peptide.

1. Introduction

The contryphans are a family of small disulfide-cyclised peptides found in the venoms of marine cone snails of the genus *Conus*. All contryphans investigated to date produce strong behavioural effects when administered by intracranial injection in mice (Jacobsen et al., 1999; Jimenez et al., 1997, 1996, 2001). The molecular basis for this activity is unknown, although target receptors for four contryphans have been proposed: voltage-gated Ca^{2+} channels (contryphan-Lo and contryphan-Am) (Sabareesh et al., 2006), L-type Ca^{2+} channels (glacontryphan-M) (Hansson et al., 2004) and Ca^{2+}-dependent K^{+} channels (contryphan-Vn) (Massilia et al., 2003). Contryphans range in length from 7 to 11 residues and in most cases share the consensus sequence CO(^{D}W)XPWC. A sequence alignment of the known contryphans that have been investigated at the protein level is presented in Table 1. Consensus features include two Pro residues at positions 2 and 5 of the intercystine loop, with the first proline usually modified to hydroxyproline. A C-terminal Pro-Trp-Cys tripeptide is also present in all family members characterised to date, except contryphan-Tx (in which Trp is replaced by Tyr) (Jimenez et al., 2001). C-terminal amidation is almost ubiquitous, being absent only in Leu-contryphan-P (Jacobsen et al., 1999). Several members also have

Abbreviations: BMRB, Biological Magnetic Resonance Data Bank; BSA, bovine serum albumin; DIPEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DPC, dodecylphosphocholine; Hyp, hydroxyproline; LC-MS, liquid chromatography/mass spectroscopy; MD, molecular dynamics; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; PDB, Protein Data Bank; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol; ROESY, rotating-frame Overhauser effect spectroscopy; RP-HPLC, reversed-phase high performance liquid chromatography; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy. The abbreviations for the common amino acids (L-isomers unless indicated otherwise) are in accordance with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* 1984, 138:9-37)..

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Table 1
Sequence alignment of members of the contryphan family. W = D-tryptophan, L = D-leucine, ~~W~~ = L-6-bromo-tryptophan, O = hydroxyproline, γ = gamma-carboxyglutamic acid, * = C-terminal amidation. Adapted from (Thakur and Balaram, 2007).

Designation	Sequence	Ref
Contryphan-Vc2	----CRWTPVC*	(Robinson et al., 2014)
Leu-contryphan-P	---GCVLLPWC*	(Jacobsen et al., 1998)
Leu-contryphan-Tx	----CVLYPWC*	(Jimenez et al., 2001)
Contryphan-In (In936)	---GCVLYPWC*	(Thakur and Balaram, 2007)
Glacontryphan-M	NγSγCPWHPWC*	(Hansson et al., 2004)
Contryphan-Vn	--GDCPWKPWC*	(Massilia et al., 2001)
Contryphan-Lo (Lo959)	---GCPWDPWC*	(Sabareesh et al., 2006)
Contryphan-Tx	---GCOWQPYC*	(Jimenez et al., 2001)
Contryphan-Sm	---GCOWQPWC*	(Jacobsen et al., 1998)
Contryphan-P	---GCOWDPWC*	(Jacobsen et al., 1998)
Contryphan-R	---GCOWEPWC*	(Jimenez et al., 1996)
Des(Gly1) contryphan-R	----COWEPWC*	(Jimenez et al., 1996)
Bromocontryphan-R	---GCOWEP~~W~~C*	(Jimenez et al., 1997)
Contryphan-Am (Am975)	---GCOWDPWC*	(Sabareesh et al., 2006)
Contryphan-fib	---GCOWMPWC*[a]	(Rajesh, 2015)
Unnamed[b]	-VVGCOWQPWC*	(Thakur and Balaram, 2007)

[a] Detected by mass spectrometry; isomerism of residue 3 not investigated.
[b] Detected in venom of *C. zeylanicus*, *C. betulinus* and *C. figulinus*.

N-terminal extensions to the consensus motif.

One noteworthy feature of contryphans is the presence of a D-amino acid at position 3 of the intercystine loop, which is either Trp or (rarely) Leu (Hansson et al., 2004; Jacobsen et al., 1998, 1999; Jimenez et al., 1997, 1996, 2001; Massilia et al., 2003; Sabareesh et al., 2006). The role of this D-amino acid in contryphans has not been explored in depth. The inclusion of a D-amino acid residue in a natural peptide is uncommon, but not unknown; in 2009, just over 30 examples of D-amino acid-containing peptides in animals were reported, of which nine were contryphans (Bai et al., 2009). In other peptides, D-amino acids have a range of effects. The first D-amino acid-containing peptide to be discovered in vertebrates was dermorphin, which was isolated from the skin of the frog *Phyllomedusa sauvagei* and contains D-Ala in position 2 (Montecucchi et al., 1981). The D-residue was found to be crucial for the potent opiate-like activity, which was lacking in a synthetic all-L analogue (Montecucchi et al., 1981). In the P-type Ca^{2+} channel antagonist ω-agatoxin IVB, found in spider venom, D-Ser46 conferred protection against the proteolytic effect of carboxypeptidase P (Heck et al., 1994). The excitotoxic conopeptide ι-RXIA contains D-Phe, and the analogue with L-Phe, ι-RXIA[L-Phe44], had a two-fold lower affinity and two-fold faster off rate than ι-RXIA on $Na_V1.6$ channels. In addition, the L-analogue was inactive at $Na_V1.2$ channels (Fiedler et al., 2008).

In this study, we investigate a newly-discovered contryphan, contryphan-Vc2, identified in the transcriptome of *Conus victoriae* (Robinson et al., 2014). Mouse bioassays were used to define a novel bioactivity, which is distinct from that seen previously for other contryphans. We assessed the effect of epimerising Trp3 on the activity, solution structure, proteolytic stability and lipid binding of this peptide. Homonuclear 2D ^1H NMR was used to calculate a solution structure, and molecular dynamics (MD) simulations were used to reveal further detail of the behaviour of the peptide in solution. Both NMR and MD indicated an interaction with lipid membranes.

2. Materials and methods

2.1. Chemical synthesis

Contryphan-Vc2 was identified in the venom gland transcriptome of *C. victoriae* as reported previously (Robinson et al., 2014). Contryphan-Vc2 peptides containing D-Trp, L-Trp or L-Ala in position 3 were prepared by conventional *N*-(9-fluorenyl)methyloxycarbonyl (Fmoc) chemistry on Rink amide resin at 0.1 mmol scale. Briefly, deprotection was performed in 20% piperidine (in DMF), followed by activation and elongation with 70 mL/L DIPEA (in DMF) and 3 equivalents of HCTU with Fmoc-protected amino acid for 50 min. Cleavage from the resin was performed over 2 h with a mixture of trifluoroacetic acid, triisopropylsilane, 1,3-dimethoxybenzene and 3,6-dioxa-1,8-octanedithiol (TFA:-TIPS:DMB:DODT, 92.5:2.5:2.5:2.5 by volume). The cleavage mixture was evaporated and precipitated with ice-cold diethyl ether. The crude product was lyophilised and stored at −20 °C until further purification.

Disulfide formation was achieved by stirring ~0.5 mg/mL crude peptide in 0.1 M ammonium bicarbonate (pH 8.0) for 17 h at room temperature. The cyclised peptides were purified on a Vydac 10 μm C18 (250 × 10 mm) column using a gradient of 40–70% buffer B over 30 min (buffer A: 0.1% TFA in MilliQ water; buffer B: 0.1% TFA in 80% acetonitrile). Sample purity was assessed by LC-MS to be greater than 95%. Further samples of synthetic [D-Trp3]- and [L-Trp3]-contryphan-Vc2 were purchased from Purar Chemicals (Victoria, Australia) and used for the proteolysis and DPC assays.

2.2. Proteolysis assays

Peptide resistance to proteolysis by trypsin, α-chymotrypsin and pepsin was measured by incubating a 250:1 peptide:enzyme mixture at 37 °C for 4 h. BSA was used as a positive control of protease activity. Stock solutions of trypsin and α-chymotrypsin were prepared in 1 mM HCl/2 mM $CaCl_2$ and reactions were run in 50 mM Tris, 100 mM NaCl (pH 7.4). Pepsin was prepared in 10 mM HCl and reactions run in 10 mM acetic acid/10 mM HCl (pH 2.0). Trypsin and α-chymotrypsin reactions were halted using 2.5% volume of acetic acid solution (25% v/v), and pepsin reactions were halted using 2.5% volume of 200 mM glycine-NaOH buffer (pH 11.4). The extent of digestion was analysed by LC-MS using a Jupiter 5 μm C4 300 Å column (50 × 2.0 mm) (buffer A: 0.1% formic acid in MilliQ water; buffer B: 0.1% formic acid in acetonitrile). Samples were eluted with a gradient of 0–60% B over 10 min.

2.3. Behavioural assay

Swiss Webster mice (15–21 days old; 6.6–10.1 g) were injected intracranially with different doses of synthetic peptides dissolved in 10 μL 0.9% NaCl, as described previously (McIntosh et al., 1994). Control mice were injected with 10 μL 0.9% NaCl solution. Following intracranial injection, mouse behaviour was observed for 2 h to determine differences between treated and control animals. All experiments involving the use of animals were approved by the Institutional Animal Care and Use Committee of the University of Utah.

2.4. Nuclear magnetic resonance spectroscopy

NMR spectra were acquired using a Bruker Avance III 600 MHz instrument. Lyophilised [D-Trp3]-contryphan-Vc2 was dissolved in 93% H_2O/7% ^2H_2O at pH 4.0 to a concentration of 2 mM and 300 μL samples were placed in Shigemi tubes. TOCSY and ROESY experiments were recorded at 5 °C using mixing times of 80 and 350 ms, respectively. An additional ROESY experiment was recorded with a mixing time of 50 ms to assist in χ_1 angle determination. 1D ^1H experiments were recorded at temperatures ranging from 10 to 30 °C in 5 °C steps to calculate amide proton temperature coefficients, and at 37 °C to test stability under physiological conditions. Hydrogen-deuterium exchange rates were measured by