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# Conotoxin Mr1.1, a Potential Peptide Analgesic

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**Abstract** - Peptide therapeutics are becoming a promising addition to the pharmaceutical arena. The huge biodiversity offered by C*onus* venom peptides, isolated from predatory marine snails, hold enormous promise for development of future peptide-based drugs. Prialt, a synthetic version of the ω-MVIIAconotoxin, was the first conotoxin to gain FDA approval in 2004 for treatment of chronic pain. Its introduction onto the market not only demonstrated the therapeutic potential of conotoxins but also stimulated more interest from biotechnology companies into conotoxin research.

Alpha-conotoxins present in Cone snail venom antagonise the nicotinic acetylcholine receptors (nAChRs), producing both acute and long lasting analgesia. The α9α10 subtype of nAChRs are implicated in the pathophysiology of chronic pain, as well in the development of breast and lung cancers. Though a number of alpha conotoxins have been identified to date, only a few have been found to target the α9α10 nAChRsubtype. The present study reports structural and functional relationship (SAR) of the novel alpha-conotoxins Mr1.1 which exclusively targets the α9α10 nAChR subtype. with an $IC_{50}$ of 57 nM. Thus this peptide is a potential candidate for further expoitation as a pain therapeutic and also as a research tool for investigating the complex pathways of pain physiology.

**Index Terms** - Alpha-conotoxins, nicotinic acetylcholine receptor, peptide therapeutics, antagonists, Mr1.1, Fmoc solid phase peptide synthesis, structure activity relationship (SAR)

## 1 Introduction

Cone snail venom is a veritable treasure trove of peptides fine-tuned by millions of years of evolution to incapacitate and kill their prey [1]. The peptide toxins are injected into the prey *via* an envenomation apparatus [2]. The conopeptides have been found to block a variety of receptors and ion channels, shutting down or subverting their function. Their high potency and specificity make them promising drug candidates and tools for investigating receptor pharamacology and localization [3]. As peptides are known for fewer concerns with toxicology, drug to drug cross-reactions, and tissue accumulation conotoxins attract much interest as drug candidates for treatment of many diseases from pain, Arthritis, Alzheimers disease to cancer [4]. The first and so far the only approved cone snail venom-derived drug is the painkiller Prialt, which is a calcium ion channel blocker [5].

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Though less than 0.1% of the conotoxin repertoire has been characterized so far, the conotoxins have been found to target a wide variety of receptors including the sodium channels, calcium channels, acid-sensing ion channels, nicotinic acetylcholine receptors (nAChRs) etc [6]. nAChRs are integral membrane proteins belonging to the Cys-loop ligand-gated ion channel superfamily. Based on their physiological localization, nAChRs are divided into two main classes– muscle type and neuronal type. They mediate fast synaptic transmission and are distributed across both the central and peripheral nervous systems [7],[8]. nAChRs comprise five homologous subunits that are arranged symmetrically to form a central hydrophilic pore (Figure 1). Each subunit consists of an extracellular N-terminal domain (ligand binding domain), four transmembrane $\alpha$-helical domains (M1-M4), an intracellular loop and an extracellular C-terminus [9]. $\alpha$-Conotoxins are small disulfide rich conopeptides with known antagonistic activity against nAChRs. Although $\alpha$-conotoxins are the smallest among the conopeptides, they have a rigid backbone conformation and well-defined 3Dstructures [10]. Secondary structural elements such as $\alpha$-helices, 310 helices and β turns usually found in proteins, are observed in $\alpha$-conotoxins [11]. They have four cysteine residues forming two disulfide bonds with the native disulfide bond connectivity observed to be

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between cysteines I-III and II-IV. The disulfide bonds stabilize this rigid backbone scaffold and aid in the formation of their characteristic fold with a central helix flanked by turns on either side [10]. $\alpha$-Conotoxins are divided into different subfamilies depending on their loop sizes (i.e. number of residues between the cysteines).

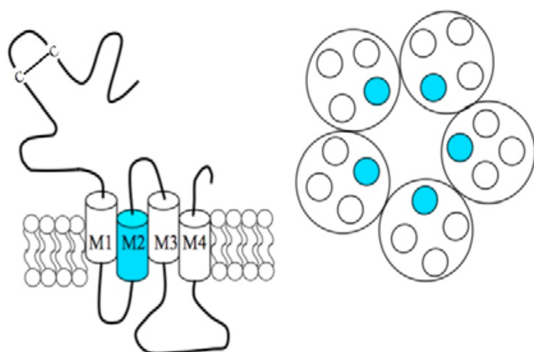


Fig 1. A schematic representation of nAChR. Each subunit comprises of five $\alpha$-helical domains(left). The M2 domain (highlighted in blue) of each subunit lines the hydrophilic receptor pore when the pentameric nAChR receptor is formed (right).

All neuronal nAChR antagonizing $\alpha$-conotoxins belong to 4/3, 4/4, 4/6, 4/7 subfamilies with four residues in loop 1 and three to seven residues in loop 2. Variations in loop size significantly influence selectivity and potency of the toxin towards different nAChR subtypes.These $\alpha$-conotoxins have a highly conserved Pro residue in the third position and a conserved Ser at position one of loop 1 forming the Ser-X-Pro (X= any amino acid) motif. The Pro residue was found to be essential for receptor binding and structural integrity [12]. Considering a large repository of potential nAChR ligands still remain to be characterized in this family, this present study reports the synthesis and characterization of the alpha conotoxin Mr1.1 which was found to target the $\alpha9\alpha10$ nAChR subtype with high potency.

## 2 MATERIALS AND METHODS:

**2.1 Peptide Assembly**- The peptides were assembled by manual Fmoc solid-phase peptide synthesis on MBHA-Rink-Amide resin (Novabiochem), with the *in situ* neutralisation/HBTU protocol [13],[14]. L-amino acids for Fmoc SPPS were purchased from Novabiochem (San Diego, CA, USA). Rink amide MBHA resin was obtained from Peptides International (Kentucky, USA). 1-[bis(dimethylamino)methylene]-1H-benzotriazoliumhexafluoro phosphate3- oxide (HBTU) was purchased from Iris Biotech (Marktredwitz, Germany). Peptide synthesis grade N,N-dimethylformamide (DMF) (filtered before use), dichloromethane (DCM), trifluoroacetic acid (TFA) and N,N-diisopropylethylamine (DIEA) were obtained from Auspep (Melbourne, Australia). All other reagents/solvents used for the experiments were from Sigma Aldrich (Sydney, Australia).

During the peptide assembly, all the Cys residue side chains present in the peptides were protected using trityl groups, so that subsequent oxidation of the reduced peptides could be performed non-selectively. For regioselective disulfide bond formation, Acm side chain protection group was used to protect Cys3 and Cys16 side chains. Peptides were cleaved from the resin using trifluoroacetic acid (TFA), with tri-isopropylsilane and water as scavengers (9:0.5:0.5 (v/v) TFA:tri-isopropylsilane:water) at 22°C for 2 h. The TFA was then removed under vacuum and peptides precipitated with ether, filtered, dissolved in 50% acetonitrile containing 0.05% TFA, and lyophilized. Crude peptides were purified by reversed phase high-performance liquid chromatography (RP-HPLC) on a $C_{18}$ column using a gradient of 0–80% B (A – $H_2O$/0.05% TFA, B – 90% $CH_3CN$/10% $H_2O$/0.045% TFA) in 80 min. Analytical RP-HPLC and electrospray mass spectrometry confirmed the purity and molecular mass of synthesized peptides.

**2.2 Disulfide bond formation**— Regioselective disulfide bond formation was carried out on the Acm protected reduced peptide. The first disulfide bond in the peptide was formed by oxidizing the peptide in 0.1 M $NH_4HCO_3$ (pH 8.2, 0.1 mg/mL) overnight at 22°C, then purifying them by RP-HPLC [15],[16]. The second disulfide bond formation was facilitated by iodine in acidic conditions. The singly disulfide bonded peptide was dissolved in 90% acetonitrile/$H_2O$, 10% acetic acid solution to which 20 equivalents of $I_2$ was added for removal of Acm protecting groups and formation of the second disulfide bond. The reaction was monitored by analytical HPLC and quenched upon completion by adding 1 M ascorbic acid until the mixture became colourless. The peptide was isolated by preparative RP-HPLC.

Non-selective disulfide bond formation was carried out on the reduced peptide which had the thiol groups exposed on all the four Cys residues. The

reduced peptide was incubated in the selected buffer solution overnight at 22°C, then purifying them by RP-HPLC. The reaction was monitored by analytical HPLC and Electrospray ionization mass spectrometry (ESI-MS) and the oxidized peptide was isolated using preparative RP-HPLC. Analytical RP-HPLC and ESI-MS were used to confirm identity and purity of the final peptides [15],[16].

**2.3 Structural Analysis**— Spectra were acquired on a Jasco J-810 spectropolarimeter, which was routinely calibrated using 0.6% (w/v) ammonium-D-camphor-10-sulfonate. For circular dichroism (CD) spectroscopy experiments, 70 μM of each peptide was dissolved in 20 mM sodium phosphate buffer at pH 7. To examine the helical propensity of each isomer, CD data was also obtained for each product after 30% tetrafluoroethene was added to the solution. All experiments were conducted at room temperature (21–23°C) under a nitrogen atmosphere (15 mL/min). The experimental parameters were set to a scanning speed of 50 nm/min, response time of 1 sec, sensitivity range of 100 mdeg and a step resolution of 1 nm. Absorbance was measured in the far UV region (185–260 nm) using a 1 mm path length quartz cuvette. Each recording was an accumulation of four scans. To eliminate any possible interference from the solvent, cuvette and spectropolarimeter optics, the CD spectra of the pure solvents were subtracted from each sample. CD spectra were also obtained upon addition of 30% TFE, to examine peptide helical propensity [17],[18].

**2.4 Electrophysiological recordings and data analysis**— Two-electrode voltage-clamping technique was utilized to carry out the electrophysiological experiments [19]. In this procedure, one electrode measured the membrane potential while the other injected current into the oocyte. The voltage recording and current-injecting electrodes were pulled from borosilicate glass (GC150T-15, Harvard Apparatus Ltd.) and had resistances of 0.3–1.5 MΩ when filled with 3 M KCl. These electrodes were impaled into the oocytes, which were voltage-clamped at a holding potential of −80 mV. During recordings, the oocytes were continuously perfused at a rate of 1.5 ml/min, with 300 s incubation intervals for each conotoxin. Acetylcholine (200 μM for $\alpha$7 and 30 μM for all other nAChR subtypes) was applied for 2 s at 5 ml/min, with 360 s washout periods between applications.Single oocyte recordings were made in a standard voltage clamp setup using a GeneClamp 500B amplifier (Molecular Devices, California, USA) while multiple experiments (eight channels in parallel) were carried out in parallel using an automated workstation (OpusXpressTM 6000A workstation, Axon Instruments Inc.), which handled both conotoxin delivery and on-line analysis. All recordings were conducted at room temperature (20–23°C) in a bath of ND96 solution. Data was collected at 500 Hz and filtered at 5 Hz. Peak current amplitude was measured before and following incubation with the toxin peptide [19, 20]. Concentration-response curves for antagonists were fitted by unweighted nonlinear regression to the logistic equation

$$Ex = E_{max}\, X^{nH}/(X^{nH} + IC50^{nH})$$

where $Ex$ is the response, $X$ is the antagonist concentration, $E$max is the maximal response, $n$H is the slope factor and IC50 is the concentration of antagonist that gives 50% inhibition of the agonist response. All the electrophysiological data was pooled ($n$ = 4–8 for each data point) and represented arithmetic means ± standard error of the fit. Computation was done using SigmaPlot 11.0 (Systat Software, California, USA).

## 3 Results and Discussion

Mr1.1 is a 16 amino acid long $\alpha$4/7 conotoxin with a C-terminal amide. This peptide was isolated from *C. marmoreus* venom duct homogenate by Yuan *et al.*, 2007 *via* cDNA cloning. This species mostly populates the Indo-Pacific marine region and feeds on other molluscs. The sequence of Mr1.1 was determined to be GCCSHPACSVNNPDIC* (*=C-terminal amide) [21]. The peptide was assembled by Fmoc-SPPS on MBHA resin at 0.5 mmol scale,with a coupling efficiency of over 99.7%. Once the assembly was complete, the peptides were cleaved from the resin using a cocktail of 9:0.5:0.5 TFA:Triisopropylsilane:$H_2O$.

Non-selective oxidation was chosen, as it allows formation of multiple isomers. Studying the variations in structure, number, ratio and hydrophobicity of the isomers generated, can provide insight into conotoxin folding characteristics. Oxidation was initially done under two trial conditions: 1) 0.1M $NH_4HCO_3$ (pH 8.2) and 2) 0.1M $NH_4HCO_3$ (pH 8.2) in 30% isopropanol. These two conditions were preferred as they are found to be optimal for $\alpha$-conotoxin folding and have been extensively used in a number of previous studies [15], [16]. The HPLC traces for the two trial conditions are shown in Fig 2a. The first condition (0.1 M $NH_4HCO_3$ pH 8.2) resulted in one isomer,

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which eluted earlier than the reduced peptide indicating a reduction in hydrophobicity upon oxidation. The second condition having 30% isopropanol in 0.1M $NH_4HCO_3$ buffer, gave rise to one primary isomer (eluting at the same point as the isomer from condition one) and two minor isomers. Mass spectrometric analysis of samples from both the conditions, showed a 4 Da decrease in molecular weight compared to the reduced peptide. This indicated the formation of two disulfide bonds. The mass of the fully oxidized peptide is shown in Fig 2c.

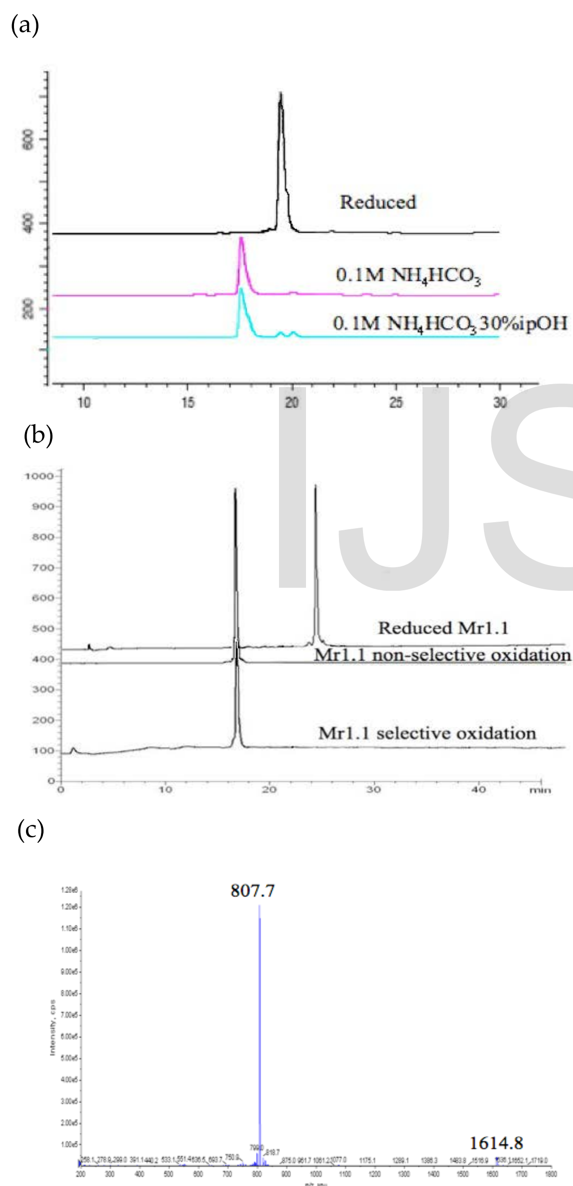



Fig 2: Mr1.1 HPLC-MS profile. Analytical RP-HPLC profiles of (a) reduced and non-selectively oxidized Mr1.1 under two trial conditions; (b) Comparison of selectively and non-selectively oxidized Mr1.1; (c) ESI-MS data showing the [M+H]+ of fully oxidized Mr1.1.

In order to further identify the native (globular) disulfide bonded isomer the Acm protected reduced peptide was oxidized in two-steps to form disulfide bonds between Cys I and Cys III, Cys II and Cys IV (globular disulfide bond isomer). The HPLC traces of products from selective and non-selective disulfide bonded peptides were identical indicating that both conditions gave rise to oxidized Mr1.1 possessing the native fold. The HPLC profiles of the selectively synthesized Mr1.1 was similar to the non-selectively synthesized products (Fig 2b), indicating that the non-selectively oxidized products had globular disulfide bond connectivity. Condition 1 yielding only the globular isomer was chosen for large-scale oxidation and the product was purified for further characterization.

CD spectra of Mr1.1 was obtained at a concentration of 70 μM with and without addition of 30% TFE (Figure 3). The peptide solution in phosphate buffer showed two local minima near 208 nm and 222 nm and a maxima at 185 nm [17]. This type of spectrum is typically observed for α-conotoxins (or other peptides and compounds containing an α-helix in their structure). The data thus suggests that Mr1.1 contains the conserved α-conotoxin structural scaffold having a helical region. The spectrum obtained after addition of 30% TFE did not induce any additional helicity [18].

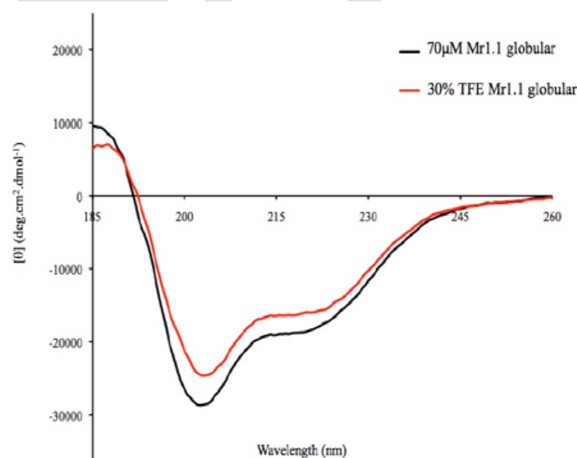


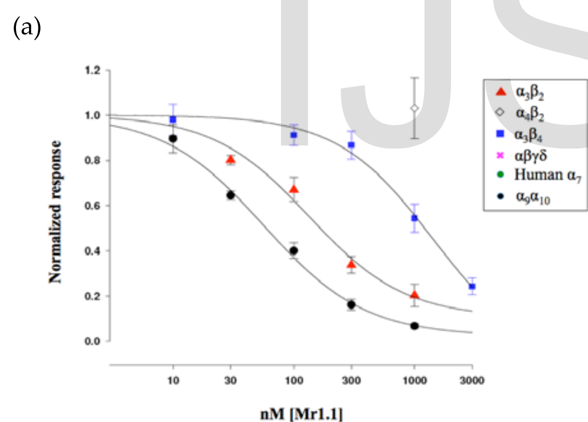

Fig 3. Mr1.1 CD Spectra. CD spectra of 70 μM globular Mr1.1 in 20 mM phosphate buffer pH 7 (black) and after addition of 30% TFE (red).

The peptides thus obtained were assayed for activity on rat (α1)2β1γδ, α3β2, α4β2, α3β4, α9α10 and human α7 nAChR subtypes heterologously expressed on *Xenopus* oocytes. Concentration response curves generated of Mr1.1 are shown in Fig

4a. The electrophysiological studies showed that the peptide targets specific neuronal nAChR subtypes and does not act on the muscle type nAChR when tested up to 1 μM of the peptide. Among the neuronal nAChR subtypes tested, Mr1.1 showed highest binding affinity towards $\alpha9\alpha10$ followed by $\alpha3\beta2$ and $\alpha3\beta4$. An $IC_{50}$ of 56.5 nM was observed at $\alpha9\alpha10$ subtype. A 10-fold decrease in affinity was found when the peptide was tested on $\alpha3\beta2$ ($IC_{50}$=133.6 nM) subtype. The concentration response curve obtained for $\alpha3\beta4$ subtype was broader. The peptide was least effective towards this subtype with an $IC_{50}$ value ~10 to 25-fold higher ($IC_{50}$ = 1408 nM) compared to $\alpha3\beta2$ and $\alpha9\alpha10$ respectively (Fig 4b).

Comparison of Mr1.1 with other $\alpha9\alpha10$ targeting $\alpha$-conotoxins sharing the same specificity profiles shows that Vc1.1 has lower potency (109 nM) while PeIA is more potent towards $\alpha_9\alpha_{10}$ subtype (6.7nM) compared to Mr1.1 (56.5 nM), [22],[23]. Sequence comparison between Mr1.1 and PeIA shows that they vary by three loop 2 residues while maintaining identical loop 1 composition. The Asn (polar, neutral, aliphatic), Asp (polar, negative) and

(a)




(b)

| nAChR subtype | $IC_{50}$ (nM) | Std. Error | Hill Slope |
|---|---|---|---|
| $\alpha_9\alpha_{10}$ | 56.5 | 7.3 | -1.04 |
| $\alpha_3\beta_2$ | 133.6 | 15.9 | -1.09 |
| $\alpha_4\beta_2$ | >1000 | - | - |
| $\alpha_3\beta_4$ | 1408.2 | 91.3 | -1.1 |
| Human $\alpha_7$ | >1000 | - | - |
| $\alpha\beta\gamma\delta$ (muscle) | >1000 | - | - |

Fig 4. Functional characterization of Mr1.1. (a) The concentration response curves obtained for Mr1.1 at different nAChR subtypes along with a table showing the mean $IC_{50}$ values and standard errors obtained from recordings on 3 separate oocytes

Ile (hydrophobic) residues at positions 12, 14 and 15 in Mr1.1 are substituted by His (polar, positive, aromatic), Glu (polar, negative) and Leu (hydrophobic) residues respectively in PeIA [22],[24]. Considering Leu and Ile have similar properties, the differences in affinity observed between PeIA and Mr1.1 can be attributed to the residue property variations at positions 12 and 14. The sequence of Vc1.1 is more diverse compared to Mr1.1 with six amino acid variations within their 16 residue long sequences. Although a considerable drop in overall potency is observed for Vc1.1, its selectivity profile remains similar to Mr1.1. The three peptides also targeted $\alpha3\beta2$ and $\alpha3\beta4$ subtypes, thus exhibiting similarity in selectivity profiles [22], [23]. Thus, Mr1.1 exhibiting a broad nAChR specificity spectrum has the potential to be exploited as a general nAChR binding scaffold.

## 5 4 Conclusion

The present study reports structural and functional relationship (SAR) analysis of the novel alpha-conotoxins Mr1.1 which targets the $\alpha9\alpha10$ nAChR among two other subtype. Structural analysis of the peptide synthesized using Fmoc solid phase peptide synthesis approach, indicated the presence of an alpha helix in the peptide structure. Electrophysiological studies on a range of nAChR subtypes showed that Mr1.1 was highly specific to $\alpha9\alpha10$ nAChR subtype with an $IC_{50}$ of 57 nM and this peptide is a potential candidate for further exploitation as a pain therapeutic and also as a research tool for investigating the complex pathways of pain physiology.

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