

# **CHARACTERISATION OF NOVEL INSECTICIDAL ION CHANNEL TOXINS FROM ARANEOMORPH AND MYGALOMORPH SPIDER VENOMS**

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## ABSTRACT

The high potency and selectivity of various peptide neurotoxins within spider venoms means these toxins are being considered as leads for the development of new environmentally-benign biopesticides that target pest insects. Currently, the  $\omega$ -HXTX family of 37-residue arthropod-selective peptide neurotoxins from Australian hexathelid spider venoms are considered a prime candidate for biopesticide development.  $\omega$ -HXTX-Hv1a, a prototypic member of the  $\omega$ -HXTX-1 family, was electrophysiologically characterised by voltage-clamp analysis using the whole-cell patch-clamp technique on cockroach dorsal unpaired median (DUM) neurons.  $\omega$ -HXTX-Hv1a exerted a reversible, concentration-dependent, voltage-independent block of barium currents ( $I_{Ba}$ ) through mid-low voltage-activated (M-LVA) and high voltage-activated (HVA) voltage-activated calcium ( $Ca_v$ ) channels without alteration in the activation or inactivation kinetics of the  $Ca_v$  channel. To improve the structural stability of this disulfide-rich peptide under biologically reducing conditions, and thereby increase its commercial viability, a synthetic  $\omega$ -HXTX-Hv1a mutant was produced with the replacement of one disulfide bond with a Sec<sup>1,4</sup> diselenide bridge. The selenocysteine mutant had comparable oral activity to the native toxin in blowflies and there was no significant difference between the native and diselenide toxin in terms of block of M-LVA and HVA  $Ca_v$  channels. This demonstrated that selenocysteine substitution had the potential to improve peptide stability without altering the biological activity of the toxin.

There is a continuous need to identify novel insecticidal peptide toxins for biopesticide development. By screening the venom of mygalomorph Sydney funnel-web (*Atrax robustus*) and Eastern mouse (*Missulena bradleyi*) spiders; two novel insect-selective peptide neurotoxins were isolated:  $\omega$ -HXTX-Ar1a from *A. robustus* is a homolog of  $\omega$ -HXTX-Hv1a, and  $\omega$ -AOTX-Mb1a from *M. bradleyi* has up to 59% homology with the  $\omega$ -HXTX family. In acute toxicity tests in house crickets, these neurotoxins induced potent neuroexcitatory symptoms followed by paralysis and death. Vertebrate nerve-muscle preparations showed that the toxins lacked overt vertebrate toxicity at

concentrations up to 1  $\mu$ M. To further characterise the molecular target of  $\omega$ -HXTX-Ar1a and  $\omega$ -AOTX-Mb1a on insects, whole-cell patch-clamp experiments were undertaken on cockroach DUM neurons.  $\omega$ -HXTX-Ar1a induced a reversible, and the  $\omega$ -AOTX-Mb1a an irreversible, block of both M-LVA and HVA  $Ca_v$  channels. The level of block was concentration-dependent and occurred in the absence of alterations in the voltage-dependence of  $Ca_v$  channel activation. The block was voltage-independent, suggesting that these toxins are  $Ca_v$  channel pore blockers rather than channel gating modifiers. Both  $\omega$ -HXTX-Ar1a and  $\omega$ -AOTX-Mb1a are promising biopesticide candidates and their activity on M-LVA and HVA  $Ca_v$  channels validates insect  $Ca_v$  channels as a novel molecular target for insecticides.

Apart from their insecticidal properties, spider venom can cause serious envenomation and death in vertebrates and invertebrates. Male *M. bradleyi* spiders are clinically important, but the toxin primarily responsible for the envenomation syndrome in humans has not previously been identified. By separating whole male *M. bradleyi* venom and testing for activity, a 42-residue peptide ( $\delta$ -AOTX-Mb1a) was isolated. In a chick biventer cervicis nerve-muscle preparation, 85 nM concentration of  $\delta$ -AOTX-Mb1a caused an increase in resting tension, muscle fasciculation and a decrease in indirect twitch tension. These effects were neutralised by *A. robustus* antivenom. The toxic effects were attributed to inhibition of peak tetrodotoxin-sensitive sodium current, a slowing of sodium current inactivation and a hyperpolarising shift in the voltage at half-maximal activation as determined by whole-cell patch-clamp analysis on rat dorsal root ganglion neurons. In acute insect toxicity bioassays,  $\delta$ -AOTX-Mb1a displayed only moderate insecticidal activity in house crickets (*Acheta domesticus*), with doses up to 2 nmol/g causing reversible neurotoxic symptoms including involuntary spasms and slight loss of coordination within 24 hours. At this dose, lethality was only observed in 60% of crickets after 48 hours.  $\delta$ -AOTX-Mb1a is highly toxic to vertebrates through its action on sodium channels, but has relatively low biological activity against invertebrates.

Spider peptide toxins that display non-selective toxicity toward both vertebrates and invertebrates can be used as molecular tools to probe the function of, and phylogenetic differences in, receptors and ion channels. Accordingly,  $\omega$ -CNTX-Cs1a, a 74-residue peptide toxin from the venom of the Central American hunting spider (*Cupiennius salei*) that displays high toxicity in mammalian and insect bioassays, was investigated. Whole-cell patch-clamp experiments showed that  $\omega$ -CNTX-Cs1a caused a voltage-independent block of mammalian L-type HVA  $\text{Ca}_v$  channels in rat neurons and neuroendocrine GH3 and GH4 cells, but had no significant effect on other types of HVA, or LVA  $\text{Ca}_v$  channels. In contrast,  $\omega$ -CNTX-Cs1a induced a slow voltage-independent, concentration-dependent block of both M-LVA and HVA  $\text{Ca}_v$  channels in whole-cell patch-clamp experiments performed on cockroach DUM neurons.  $\omega$ -CNTX-Cs1a shows high selectivity for a subset of mammalian  $\text{Ca}_v$  channels, but indiscriminate activity on invertebrate  $\text{Ca}_v$  channels, which makes this toxin useful as a molecular tool for further investigation of mammalian and insect  $\text{Ca}_v$  channels.

The incredible diversity in the phylogenetic selectivity and ion channel specificity of spider neurotoxins means there is great potential for these toxins as molecular tools, and many may become the defining pharmacology for receptor or ion channel subtypes. As a result of such investigations we now also understand the underlying basis for clinical envenomation syndromes that develop following envenoming, and many are being investigated as environmentally-friendly biopesticides and therapeutic drugs.

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## PUBLICATIONS ARISING FROM THIS THESIS

- 1) Herzig, V., Khalife, A.A., Chong, Y., Isbister, G. K., Currie, B.J., Churchill, T.B., Horner, S., Escoubas, P., Nicholson, G.M. and Hodgson, W.C. 2008. Intersexual variations in Northern (*Missulena pruinosa*) and Eastern (*M. bradleyi*) mouse spider venom. *Toxicon*, 517, 1167-77.
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## LIST OF ABBREVIATIONS AND ACRONYMS

$\tau_{\text{on/off}}$ : time constants for onset of (on) and recovery from (off) current block

3D: three-dimensional

4-AP: 4-aminopyridine

4VP: 4-vinylpyridine

ACh: acetylcholine

AChE: acetylcholinesterase

ACN: acetonitrile

AOTX: actinopoditoxin

APAF: Australian Proteome Analysis Facility

Arg: arginine

Asn: asparagine

Asp: aspartic acid

ATP: adenosine triphosphate

BCA: bicinchoninic acid

BSA: bovine serum albumin

*Bt. Bacillus thuringiensis*

Ca<sub>v</sub>: voltage-activated calcium

cDNA: complementary deoxyribonucleic acid

CMF-PBS: Calcium- and Magnesium-Free Phosphate-Buffered Saline

CNTX: ctenitoxin

Cys: cysteine

DDT: dichlorodiphenyltrichloroethane

DMEM: Dulbecco's Modified Eagle Medium

DNA: deoxyribonucleic acid

DRG: dorsal root ganglion

DTT: dithiothreitol

DUM: dorsal unpaired median

*E. coli: Escherichia coli*

EDTA: ethylenediaminetetraacetic acid

EGTA: ethylene glycol tetraacetic acid

ESI-MS: electrospray ionisation mass spectrometry

FPLC: fast-perfusion liquid chromatography

GABA: gamma-amino butyric acid

Gln: glutamine

Glu: glutamic acid  
 $G_{max}$ : maximum conductance  
 GNA: *Galanthus nivalis* agglutinin  
 GSSG: glutathione disulfide  
 GST: glutathione S-transferase  
 HEK: human embryonic kidney  
 HEPES: 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt  
 HF: hydrogen fluoride  
 HVA: high voltage-activated  
 HXTX : hexatoxin  
 $I$ : current  
 $I_{Ba}$ : barium currents  
 $IC_{50}$ : half maximal inhibitory concentration  
 ICK: inhibitory cystine knot  
 $KD_{50}$ : median knockdown dose  
 $K_v$ : voltage-activated potassium  
 $LD_{50}$ : median lethal dose  
 LJP: liquid junction potential  
 Lys: lysine  
 $m/z$ : mass/charge  
 MALDI-TOF MS: matrix-assisted laser desorption/ionisation-time of flight mass spectrometry  
 MeOH: methanol  
 mLVA: maintained low voltage-activated  
 M-LVA: mid-low voltage-activated  
 $Na_v$ : voltage-activated sodium  
 NIS: normal insect saline  
 POPs: persistent organic pollutants  
 Pro: proline  
 RNSH: Royal North Shore Hospital  
 Rp-HPLC: reverse phase high pressure liquid chromatography  
 $S$ : slope factor  
 SAR: structure-activity relationship  
 Sec: selenocysteine  
 Ser: serine  
 TAG: terminal abdominal ganglion/ganglia

TEA-Br: tetraethylammonium bromide  
TEA-Cl: tetraethylammonium chloride  
TEA-OH: tetraethylammonium hydroxide  
TFA: trifluoroacetic acid  
Thr: threonine  
tLVA: transient low voltage-activated  
Tris: tris(hydroxymethyl)aminomethane  
TTX: tetrodotoxin  
Tyr: tyrosine  
UNSW: University of New South Wales  
USEPA: United States Environmental Protection Agency  
UTS: University of Technology, Sydney  
UV: ultraviolet  
UV-VIS: ultraviolet-visible  
V: voltage  
 $V_{1/2}$ : voltage of half-maximal activation  
 $V_h$ : membrane holding potential  
 $V_{rev}$ : reversal voltage  
WHO: World Health Organisation