Identifying novel and selective sodium channel modulators from Theraphosidae venoms

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1 ABSTRACT

Voltage-gated sodium (Na_V) channels have been extensively studied as targets for therapeutic indications such as pain, epilepsy and paralysis, where modulating these channels could be beneficial by altering action potential firing. However, small molecule interventions have been largely unsuccessful in the clinic, primarily due to challenges in developing compounds with high subtype selectivity to minimise off-target effects.

Many animal venoms have evolved as Na_V channel modulators in order to subdue prey and defend against predators. Several toxin peptides are highly potent and specific to individual Na_V subtypes, and as such have become widely-used tools in research to elucidate structure and function of the channels. These venom peptides are also inherently stable due to their biology. Increasingly, toxins have been used in the development of novel therapeutics as peptide frameworks for new bioactive molecules or targeting scaffolds for drug conjugates. However very few have moved to the clinic and thus new approaches are needed.

Ion channels are notoriously challenging to study in drug discovery, due to long and laborious manual patch clamp approaches. Recent advances in automated patch clamp technologies have allowed high quantities of meaningful data to be generated quickly.

We have successfully collected, fractionated and standardised venoms from 17 Theraphosidae species to produce a Targeted Venom Discovery Array (T-VDA). This T-VDA, containing 960 theraphosid venom fractions, was screened in duplicate against $Na_V1.4$ and $Na_V1.7$ channels on the Sophion Qube high-throughput automated patch clamp platform in 384 well format. We identified 33 fractions, which modulated the activity of one or both channels, thus generating a hit rate of 3.4% across both targets with the majority modulating $Na_V1.7$. Here we present our findings and discuss the advantages and challenges of using automated patch clamp technology to identify novel hits using a venom peptide library.

2 MATERIALS AND METHODS

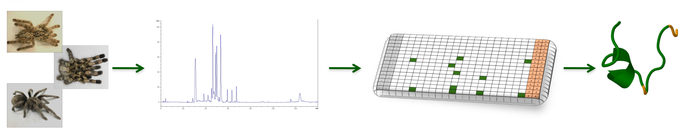


Figure 1: Schematic of methods used.

T-VDA Preparation: Dual Na_V channel Targeted-Venom Discovery Array™ (T-VDA^{Nav}) designed using Venomtech proprietary process. Venoms recoverably collected from 17 Theraphosidae species then fractionated using in-line two-dimension HPLC on UltiMate 3000 (ThermoFisher). T-VDA was standardized, assembled and lyophilized in Echo qualified 384-well plates.
Cell Culture: CHO $Na_V1.4$ and CHO T-REX $Na_V1.7$ were produced at Charles River and are commercially available. Cells were cultured according to their SOP and were kept in a serum-free medium on the Qube for up to 4 hours.
Solutions: Extracellular solution (mM): 145 NaCl, 4 KCl, 10 HEPES, 10 Glucose, 1 MgCl2, 2 CaCl2, pH 7.4. Intracellular solution (mM): 120 CsF, 20 CsCl, 10 NaCl, 10 HEPES, 10 EGTA, pH 7.3.
Test Fractions: T-VDA plates were prepared in extracellular solution containing 0.01% Pluronic Acid F-127. Final fraction concentration was 2 µg/mL for single point screening or as 10 pt CRCs, 1:3 dilution at 2 µg/mL top concentration.
Qube Experiments: Experiments were conducted with Sophion Qube software version 2.6.40 (Goldcrest) using Multi-Hole QChips. The protocol included an Online Boltzmann Fit liquid period, followed by a voltage protocol aimed to test compound effect on resting state and 50% inactivated state. Currents were sampled at 25 kHz with cut off at 5 kHz and Bessel Filtering.
Analysis: Data analysis was performed using Qube Analyzer software, GraphPad Prism (8.4) and Dotmatics Vortex (v2020)
Mass Spectrometry ID: Two samples from each hit were sent to Peak Proteins Ltd (UK): one for intact mass and the other a trypsin/chymotrypsin double digest for peptide mapping.

3 RESULTS

3.1 Sophion Qube experiments generated high quality data, with an average success rate of 92.7% against $Na_V1.4$ and 89.3% against $Na_V1.7$.

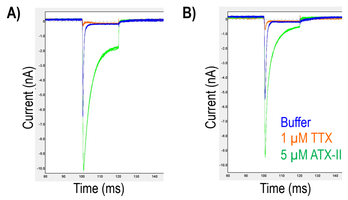


Figure 2: Representative current traces from $Na_V1.X$ experiments. Current traces from buffer control (blue) or following treatment with 1 µM TTX inhibitor control (orange) or 5 µM ATX-II activator control (green) from $Na_V1.4$ **(A)** and $Na_V1.7$ **(B)** channels.

3.2 960 fractions from theraphosid venoms were screened in duplicate against $Na_V1.4$ and $Na_V1.7$ channels, with 33 fractions identified for progression to concentration response curve (CRC) profiling.

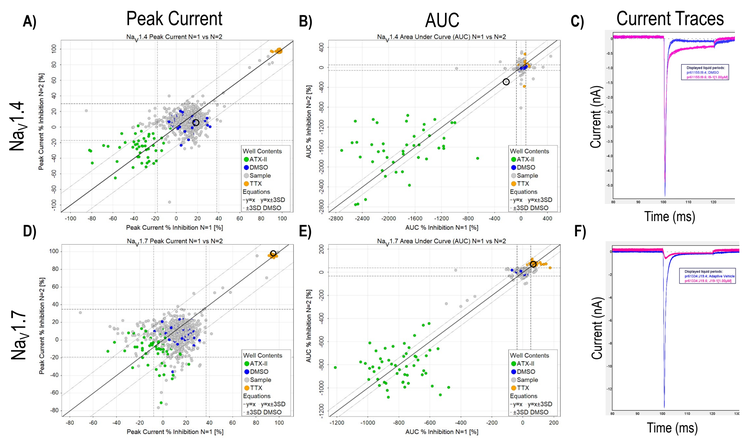
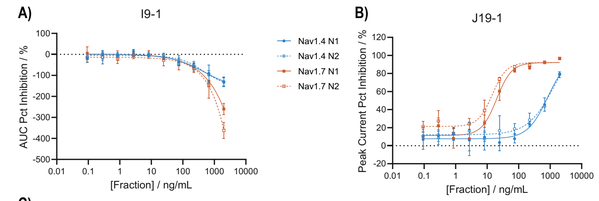


Figure 3: Hit identification from single point screening. Scatter plots showing N = 1 vs. N = 2 % inhibition of peak current **(A,D)** or area under curve (AUC) **(B,E)** of venom fractions and controls screened against $Na_V1.4$ **(A-C)** or $Na_V1.7$ **(D-F)** channels. Fractions with an average peak current % inhibition > ±30% or an average AUC % inhibition > ±50% against one or both channels were evaluated and 33 fractions were selected for progression to CRC profiling, generating a hit rate of 3.6%. Sample current traces are given for 2 fractions, with vehicle period prior to fraction addition in blue and following fraction addition in pink: **(C)** Representative $Na_V1.4$ traces for activator fraction I9-1 (circled in **A,B**); **(F)** Representative $Na_V1.7$ traces for inhibitor fraction J19-1 (circled in **D,E**).

3.3 26 fractions were confirmed as modulators of one or both channels in CRC profiling, with many demonstrating $Na_V1.7$ subtype selectivity.



Channel	IC_{50} (ng/mL)		
	N1	N2	Average
$Na_V1.4$	833	1351	1092
$Na_V1.7$	20.08	14.66	17.37

C)

Activity	$Na_V1.4$	$Na_V1.7$
Activator	1	5
Inhibitor	11	21
Inactive	20	6
Total	32	32

Figure 4: Potency determination of selected venom fractions. Concentration response curves of fractions I9-1 **(A)** and J19-1 **(B)** identified in figure 3, against $Na_V1.4$ (blue) and $Na_V1.7$ (orange) channels. AUC was selected as the analysis parameter for activators with peak current inhibition used for inhibitors. Data shown as Mean ± SD, N = 2-4, with IC_{50} values for J19-1 given in ng/mL. **(C)** Table showing number of fractions identified in CRC testing with each activity. All fractions identified as $Na_V1.4$ modulators were also identified as $Na_V1.7$ modulators.

3.4 10 fractions were selected for progression to hit identification by mass spectrometry

A)

Hit Coordinate	Inhibits	Peptide ID
C9-2	$Na_V1.7$ only	Known theraphotoxin
C14-3	$Na_V1.7$ only	Novel theraphotoxin
A18-1	$Na_V1.7$ only	Novel theraphotoxin
B22-3	$Na_V1.4$ & 1.7	Known theraphotoxin
J19-1	$Na_V1.4$ & 1.7	Novel theraphotoxin
N10-2	$Na_V1.4$ & 1.7	Variant of known theraphotoxin
O6-2	$Na_V1.4$ & 1.7	Variant of known theraphotoxin

B)

Hit Coordinate	Activates	Peptide ID
I9-1	$Na_V1.4$ & 1.7	Novel activity of known theraphotoxin
J15-2	$Na_V1.7$ only (strong)	Novel theraphotoxin
P6-2	$Na_V1.7$ only (weak)	Novel theraphotoxin

Figure 5: Mass Spec Hit ID of selected venom fractions. Tables showing fraction coordinate, channel specificity and peptide identification of inhibitors **(A)** and activators **(B)** submitted for mass spec ID. Of the 10 fractions submitted, 5 were identified as novel, while the rest were known or variants of known theraphotoxins. One fraction, I9-1, is a known theraphotoxin with previously unreported activity as a $Na_V1.4$ and 1.7 activator.

4 SUMMARY

960 fractions of theraphosid venoms were successfully screened in duplicate against $Na_V1.4$ and $Na_V1.7$ channels using the Sophion Qube high-throughput APC platform. 33 fractions were identified which significantly modulated the peak current or area under curve of one or both channels, generating an overall hit rate of 3.4%. Of the 32 fractions tested as 10pt CRCs, 26 were confirmed as modulators, with the majority identified selective for the $Na_V1.7$ subtype. This data supported our expectations from the initial library design, and the MS ID further confirmed our hypothesis that this T-VDA library contained known peptide toxins in addition to novel toxins not previously identified.

Some technical limitations of using a natural product library were observed. Firstly, seal resistance of some cells was lost during the Qube experiments following prolonged exposure to the venom fraction. Additionally, the top concentration of each CRC was limited by the quantity of product available, meaning the top plateau of the curve is not always seen. However, despite this, the assay performance and data quality were excellent, with good N = 1/N = 2 correlation for both the single point and CRC experiments.

$Na_V1.4$ and $Na_V1.7$ channels remain important drug targets that have eluded successful drug development. Identification of novel peptide sequences with desired activity against these channels can provide a new strategy for drug design by improving the power of subsequent Structure-Activity Relationship (SAR) studies, thus improving development of peptide-based therapeutics.

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