Evaluation of venoms as a source of novel ligands for drug discovery

Assessing the practical challenges of venom screening to explore the hit rate & activity of a selection of venoms against a range of targets

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Over time animal venoms have evolved to be cocktails of biologically very potent molecules. Venom libraries remain a largely unexplored area for systematic screening against different types of target and assays, providing an opportunity to identify novel target validation tools, hits or leads. Unlike a member of a small molecule library, a venom will contain multiple bioactive components and are complex mixtures of mainly peptides & proteins, hence identifying the active component requires a deconvolution strategy.

In this poster we present data from a custom-made library of crude venom fractions screened against a variety of biological targets, including several GPCRs.

Crude fractions with μ opioid receptor agonist activity were purified by 2D fractionation, and then retested for biological activity and characterisation by liquid chromatography mass spectrometry.

Venomtech® custom-made libraries

- Commercially available collection of venoms from a very wide range of species. Libraries are selected based on customers' objectives.
- Supplied as first dimension Reverse Phase - HPLC fractionated venoms i.e. separation by lipophilicity, correlates naturally with some limited separation relating to MW
  - Snake venom : 30-40 fractions (single-phase pass)
  - Tarantula venom : 20-30 fractions (single-phase pass)
- Each fraction contain ~10 protein / peptide components
- Snake venom typically wide range of MW and compound classes (peptides, enzymes, etc) whereas tarantula venom has narrower range and fewer enzymes
- Fractions supplied lyophilised in 384 well plates; aqueous solubility; compatible with aqueous acoustic dispensing

Abbr.	Species	Common Name	No. of fractions
D.ang	*Dendroaspis angusticeps*	Eastern green mamba	31
D.pol	*Dendroaspis polylepis*	Common black mamba	28
P.reg	*Poecilotheria regalis*	Indian ornamental tree spider	21
N.Nig	*Naja nigricollis*	Black-necked spitting cobra	31
N.Kao	*Naja kaouthia*	Monocled cobra	30
H.gig	*Hysterocrates gigas*	Giant baboon spider	19
P.cam	*Psalmopeus cambridgei*	Trinidad chevron tarantula	7
C.atr	*Crotalus atrox*	Western diamondback rattlesnake	19
P.tra	*Parabuthus transvaalicus*	Transvaal thick-tailed scorpion	22
P.imp	*Pandinus imperator*	Emperor scorpion	19

Table 1: Custom-made library from Venomtech®
Ten different species selected based on biological targets of interest

Screening summary for custom-made 1D fraction library

A) Tested at 0.83 μg/mL in two Grow/No Grow gram negative antibacterial assays – confirmed literature reports of antimicrobial activity in *Pandinus imperator* fractions[1]

B) Tested at 0.1 μg/mL in two ion channel assays (hERG – no actives ; Nav1.8 – several active fractions from different species identified)

C) Tested at 0.1 μg/mL in ten GPCR PathHunter® β-arrestin assays (Fig.1) (receptors involved in pain, respiratory, inflammation and diabetes, plus orphan receptors) - confirmed literature reports of activity against adrenoceptors in *Dendroaspis angusticeps* fractions[2]. Identified two active *Hysterocrates gigas* fractions with μ opioid receptor agonist activity (Fig. 2B)

Testing custom-made 1D fraction library in GPCR PathHunter® β-arrestin assays

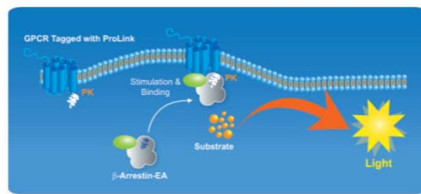

Fig.1 DiscoverX PathHunter® β-arrestin Cell-based Enzyme Fragment Complementation Assay Platform
GPCR fused in frame with small enzyme fragment ProLink™ & co-expressed in cells stably expressing a fusion protein of β-Arrestin & larger, N-terminal deletion mutant of β-gal (called enzyme acceptor or EA). Activation of GPCR stimulates binding of β-Arrestin to ProLink-tagged GPCR and forces complementation of the two enzyme fragments, resulting in formation of an active β-gal enzyme. This interaction leads to an increase in enzyme activity which can be measured using chemiluminescent PathHunter® Detection Reagents

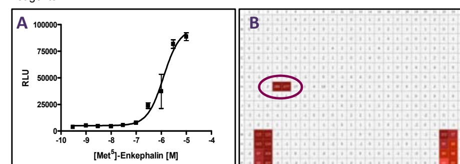

Fig.2 Identifying *H.gigas* fractions with μ opioid receptor agonist activity. PathHunter® CHO-K1 OPRM1 β-arrestin cells were plated in a 384-well plate and incubated overnight at 37°C 5% CO_2 to allow cells to adhere. Cells were then stimulated with either A) concentration response to a control agonist [Met5]-Enkephalin or B) 1 μg/mL venom fractions (wells A3 to L9) for 90 mins at 37°C 5% CO_2. Columns 1 & 24 contained neutral controls (cell medium). Stimulator controls (500nM [Met5]-Enkephalin EC_{80}) were in wells M2-P3 and M22-P23. Following stimulation, signal was detected using the PathHunter® detection kit according to the recommended protocol. Control agonist data was analysed using GraphPad Prizm. Venom fraction data was analysed using Genedata Screener and normalized to stimulator minus neutral controls.

Characterisation of *H.gigas* active fractions

Second dimension fractionation was performed on crude *H.gigas* venom fractions pooled from the two wells (H4 & H5) which showed agonist activity at μ opioid receptor. Following ion exchange chromatography and reverse phase-HPLC obtained seventeen 2D fractions for further testing.

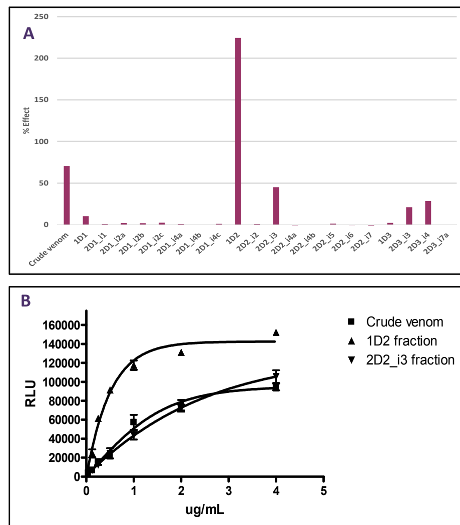

Fig.3 Testing *H.gigas* second dimension fractions for μ opioid receptor agonism. PathHunter® CHO-K1 OPRM1 β-arrestin cells were plated in a 384-well plate and incubated overnight at 37°C 5% CO_2 to allow cells to adhere. A) Cells were stimulated with 1 μg/mL venom fractions for 90 mins at 37°C 5% CO_2. Neutral (cell medium) and stimulator (500nM [Met5]-Enkephalin EC_{80}) controls were included. B) Cells were stimulated with concentration response to active fractions from (A). Following stimulation, signal was detected using the PathHunter® detection kit according to the recommended protocol. Data from (A) was normalized to stimulator minus neutral controls

LCMS profiling of *H.gigas* active fractions

Identified one 1D fraction and one 2D fraction with agonist activity at μ opioid receptor. These active fractions were profiled (with original crude venom fraction) by liquid chromatography mass spectroscopy using an Agilent QTOF and a 2 minute H_2O/Acetonitrile gradient on a 50x2mm C8 HPLC column. Analysis of the crude venom fraction showed a number of distinct peaks in the chromatogram (Fig. 4A). A combined spectra across the retention window of peptide species (0.7-1.7 mins) (Fig.4B) showed a complex mixture of multiple peptides in the fraction.

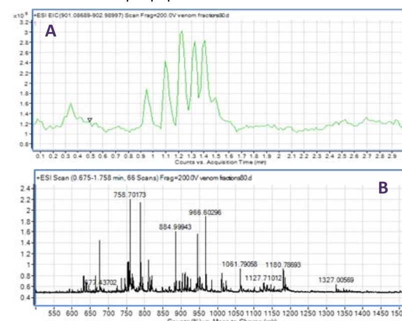

Fig.4 Representative chromatogram (A) and combined spectra (B) of crude venom showing multiple components and a complex spectra consistent with a mixture of many peptides.

A mass chromatogram of 901Da from the second dimension fractionation (2D2_i3) gave two major and two minor components with this molecular weight (Fig. 5A). The combined spectra (Fig. 5B) from across these four components shows the spectra has three major peaks 644.5((M+7H)/7), 751.8((M+6H)/6) and 901.9((M+5H)/5) corresponding to a molecular weight of the peptide of 4504.6Da. The likely reason for what appears to be multiple components in this fraction is that there are a number of di-sulphide bridges in these venoms that are not fully reduced in the analytical conditions.

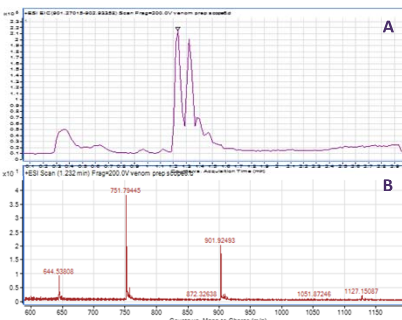

Fig.5 Representative chromatogram (A) and combined spectra (B) of 2nd dimension venom fraction showing multiple components and a simple spectra consistent with a mixture of structural isoforms of a single peptide.

The active second dimension fraction was processed further by reduction, alkylation & tryptic digestion to identify a nine amino acid peptide, only a partial sequence based on predicted mass of 4504.6Da. BLAST results link this sequence to cys-rich domains found in peptides

Summary & next steps

Using a custom array screening approach for multiple targets we have identified a putative novel active with nM potency at the μ opioid receptor. Venomtech® will perform third dimension fractionation to single entity purity. Following MS analysis & peptide sequencing of active entitiy/ies, chemical peptide synthesis will allow further profiling against the μ opioid receptor.

Acknowledgements
Thanks to Matthew Burnham and Matthew Bridgland-Taylor for testing the venom fractions in the ion channel assays, and to Hilary Lewis and Rachel Rowlinson for generating the mass spectroscopy sequencing data

References:
1. "Three new antimicrobial peptides from the scorpion Pandinus imperator" Zeng, X-C *et al*; Peptides **45** (2013) 28-34
2. "G protein-coupled receptors, an unexploited animal toxin targets: Exploration of green mamba venom for novel drug candidates active against adrenoceptors" Maiga *et al*; Toxicon **59** (2012) 487-496

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