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An efficient analytical platform for on-line microfluidic profiling of neuroactive snake venoms towards nicotinic receptor affinity

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ABSTRACT

Venomous snakes have evolved their efficient venomous arsenals mainly to immobilize prey. The highly variable toxic peptides in these venoms target a myriad of neurotoxic and haemotoxic receptors and enzymes and comprise highly interesting candidates for drug discovery. Discovery of bioactive compounds from snake venoms, however, is a challenge to achieve. We have developed and applied a methodology to rapidly assess bioactives in a snake venom proteome. Our microfluidic platform opens up efficient and rapid profiling of venomous anti-cholinergic receptor compounds. The key advantages of our methodology are: (i) nano amounts of venom needed; and (ii) a direct correlation of selected bioaffinities with accurate mass. To achieve this, we have for the first time successfully constructed a functional post nano-LC split to MS and bioaffinity profiling. In our method, comprehensive venom profiles with accurate masses and corresponding bioaffinities are obtained in one analytical run and will subsequently allow immediate purification of bioactive peptides with LC–MS, guided by accurate masses of the bioactives only. We profiled several neurotoxic Elapidae snake venoms using our methodology in combination with the acetylcholine binding protein (AChBP) as biological target protein. The latter is a homologue of nicotinic acetylcholine receptors (nAChRs), a drug target in neurodegenerative diseases and cognitive decline such as Parkinson's and Alzheimer's, and in pain related diseases. Our methodology was evaluated and validated with high-affinity α-bungarotoxin and haemotoxic/proteolytic *Vipera ammodytes* venom spiked with α-bungarotoxin. Thereafter, the methodology was applied to profile the venom proteomes of *Dendroaspis jamesoni kaimosae*, *Naja annulifera* and *Naja nivea*. Gathering comprehensive profiling data took less than 2 h per snake venom measured. The data yielded 20 AChBP ligands of which the corresponding accurate masses were used to retrieve information from literature regarding their function and targeting specificity. We found that from these 20 ligands, 11 were previously reported on, while information on the others could not be

Abbreviations: AChBP, Acetylcholine binding protein; α BTX, α-Bungarotoxin; CTX, Cardiotoxin; DAHBA, (E)-3-(3-(4-diethylamino-2-hydroxybenzylidene) 3,4,5,6-tetrahydropyridin-2-yl)pyridine; EDA, Effect-Directed Analysis; ESI, Electrospray Ionization; EICs, Extracted Ion chromatograms; HRS, High-Resolution Screening; i.d., Internal diameter; IT TOF, Ion-Trap–Time-of-Flight; LOD, Limit Of Detection; Ls, *Lymnaea stagnalis*; LC, Liquid Chromatography; MS, Mass Spectrometry; mAChRs, Muscarinic receptors; α-NTXs, α-Neurotoxins; α7-nAChR, α7-nicotinic Acetylcholine receptor; PLA_2s, Phospholipases A_2; 3FTX, Three-finger toxins; TIC, Total Ion Chromatogram; TFA, Trifluoroacetic acid; Uniprot, Universal Protein Resource Knowledgebase.

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found. From these 11 peptides, five have been reported to have nAChR affinity, while the others are reported as cytotoxic, cardiotoxic or as orphan toxin. Our methodology has the potential to aid the field of profiling complex animal venoms for drug discovery.

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1. Introduction

Recent advances in the scientific understanding of diseases have raised hopes of an increase in the number of novel drugs reaching the market. Further, many potential disease-related orphan receptors are now being investigated. It is therefore disappointing to realise that the number of drugs reaching the market is actually dwindling. One of the solutions to this problem is the exploration of natural sources, such as animal venoms, using current analytical technologies to increase the numbers of potential new lead compounds. Why? Possible reasons: venoms are highly potent because they are under strong natural selection. Venoms contain multiple novel compounds, free from IP restrictions. They have also provided many new drugs or even new classes of drugs, in the past. In order to exploit this great natural resource, several challenges have to be resolved. These include bioanalytical challenges such as: 1) the complex nature of the samples and the intrinsically time-consuming process of elucidation, 2) the occurrence of synergistic actions between components in a complex natural extract, and 3) sourcing adequate quantities, especially when screening animal venoms. For these and other reasons, pharmaceutical companies scaled down or even abandoned their natural extract drug discovery pipelines. Nevertheless, many drugs approved during the last decades are based on natural products (Harvey, 2008).

Another potential reason for abandoning natural extract product pipelines is that most drug companies focused on plant and fungal derived bioactives (Kapoor, 2010), because these mostly small-molecule compounds fitted well in small-molecule drug discovery pipelines. Traditional problems with peptide and protein derived drugs are amongst others due to issues with (oral) drug administration, bodily distribution, e.g., limited crossing of the blood brain barrier, and immunogenicity issues, e.g., due to the difficulty of preparation. Advanced formulation methodologies and administration routes as well as current knowledge on immunogenicity and immunotoxicity have brought venom peptides back into consideration as candidate drugs. Some successful examples are Prialt (Ziconotide) derived from a predatory cone snail (*Conus textilus*) and used to treat severe chronic pain; Byetta (Exenatide) derived from the venom of the Gila monster (*Heloderma suspectum*) and used against diabetes type II; ACE inhibitors derived from the venom of the South American Lancehead snake (*Bothrops jararaca*) for the treatment of hypertension; and the antiplatelet drug Integrillin derived from the venom of the Saw scaled viper *Echis carinatus* (Vonk et al., 2011) These examples justify the screening of venoms, including snake venoms, for compounds that might be a source for biopharmaceutical drug candidates. Furthermore, from a pharmacological point of view, venom peptides are also interesting. The acetylcholine receptor, for example, was discovered in 1970 by use of bungarotoxin as molecular probe.

One of the specific difficulties of effective natural-extract screening is the accurate and rapid bioactivity/identity correlation of bioactives with current traditional effect-directed analysis (EDA) methodologies (Koehn and Carter, 2005). EDA approaches are today's standard for bioactive mixture analysis and are widely applied in environmental (Brack, 2003; Brack et al., 2007; Houtman et al., 2007) and natural-product screening (Ebada et al., 2008; Nyila et al., 2009; Yu et al., 2009), as well as in animal-venom profiling (Fernandez et al., 2011). In this approach, bioactive compounds are separated and subsequently fractionated, e.g., with liquid chromatography (LC), into a number of fractions, after which each fraction is tested for bioactivity. Ultimately, the bioactives are analysed with mass spectrometry (MS) to identify the active compounds. However, EDA studies often fail to identify the bioactive compounds, because even after repeated fractionation, biologically active fractions remain too complex for chemical identification, and in case of peptides also denaturation might occur. Additionally, the EDA workflow consumes large amounts of precious sample, especially in the case of venoms.

Even though some snake venom proteomes have been completely elucidated and sequenced, many toxins have unknown functions and protein targets. Of these "orphan toxins", only a hypothesised functionality is known by sequence homologies with toxins having known bioactivities. Based on the large functional diversification followed by minor mutations in some of the toxin families, it is difficult to estimate function based on sequence similarity. These toxins are often only classified broadly as cytotoxic, cardiotoxic or weak neurotoxic (Fry et al., 2003), with a myriad of different (unknown) receptors and proteins recognized or bound.

We recently developed a miniaturized screening methodology based on post-column on-line screening, sometimes called High Resolution Screening (HRS), for analysis of small compound libraries where nano-LC was hyphenated on-line to a microfluidic biochemical detector (Heus et al., 2010). This methodology needed two analytical runs for analysis: the first run with nano-LC connected to the biochemical detector for assessing bioactivity of individual ligands, and the second run with nano-LC connected to MS for determination of the accurate masses of the eluting compounds. However, although this methodology would in theory allow for analysis of intrinsically low venom amounts, it is unsuited for profiling snake venoms as the complexity in this case overwhelms the analytical system. The main issue here is that the system is able to only assess bioaffinity in one run therefore necessitating a second nano-LC–MS analysis for peptide identification.

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