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Analytical workflow for rapid screening and purification of bioactives from venom proteomes

Reka A. Otvos [a,b], Ferry Heus [a], Freek J. Vonk [c,e,f], Jenny Halff [a], Ben Bruyneel [a], Iryna Paliukhovich [b], August B. Smit [b], Wilfried M.A. Niessen [a,d], Jeroen Kool [a,*]

[a] AIMMS Division of BioMolecular Analysis, Faculty of Sciences, De Boelelaan 1081, 1083 HV Amsterdam, Amsterdam VU University, The Netherlands
[b] Department of Molecular and Cellular Neurobiology, Center for Neurogenomics and Cognitive Research, Neuroscience Campus Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam, VU University Amsterdam, The Netherlands
[c] Leiden University, Institute of Biology, Sylvius Laboratory, Sylviusweg 72, 2333 BE Leiden, The Netherlands
[d] Hyphen MassSpec, de Wetstraat 8, 2332 XT Leiden, The Netherlands
[e] School of Biological Sciences, University of Wales, Bangor L57 2UW, UK
[f] Naturalis Biodiversity Center, Darwinweg 2, 2333 CR Leiden, The Netherlands

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ABSTRACT

Animal venoms are important sources for finding new pharmaceutical lead molecules. We used an analytical platform for initial rapid screening and identification of bioactive compounds from these venoms followed by fast and straightforward LC–MS only guided purification to obtain bioactives for further chemical and biological studies. The analytical platform consists of a nano-LC separation coupled post-column to high-resolution mass spectrometry and parallel on-line bioaffinity profiling for the acetylcholine binding protein (AChBP) in a chip based fluorescent enhancement based bioassay. AChBP is a stable structural homologue of the extracellular ligand binding domain of the α7-nicotinic acetylcholine receptor (α7-nAChR). This receptor is an extensively studied medicinal target, previously associated with epilepsy, Alzheimer's, schizophrenia and anxiety.
The workflow is demonstrated with the venom of the *Naja mossambica mossambica*. Two medium affinity AChBP ligands were found. After subsequent LC–MS guided purification of the respective venom peptides, the purified peptides were sequenced and confirmed as Cytotoxin 1 and 2. These peptides were not reported before to have affinity for the AChBP. The purified peptides can be used for further biological studies.

Abbreviations: ACN, acetonitrile; AChBP, acetylcholine binding protein; CNS, central nervous system; DAHBA, (E)-3-(3-(4-diethylamino-2-hydroxybenzylidene)-3,4,5,6-tetrahydropyridin-2-yl)pyridine; EDA, effect directed analysis; EICs, extracted ion chromatograms; ESI, electrospray ionization; ELISA BR, enzyme-linked immunosorbent assay blocking reagent; HRS, high-resolution screening; HPLC, high-performance liquid chromatography; i.d., internal diameter; IT TOF, ion-trap-time-of-flight; Ls-AChBP, *Lymnaea stagnalis* acetylcholine binding protein; LC, Liquid chromatography; MALDI-TOF/TOF, matrix assisted laser desorption ionization with tandem time of flight detection; MS, mass spectrometry; α7-nAChR, α7-nicotinic acetylcholine receptor; PLA2, phospholipase A2; Q TOF, quadrupole-time-of-flight; TFA, trifluoroacetic acid; TFX3, three-finger toxin; TIC, total ion chromatogram.

* Corresponding author. VU University Amsterdam, Faculty of Science BioMolecular Analysis, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. Tel.: +31 205987542; fax: +31 205987543.
E-mail addresses: r.a.otvos@vu.nl (R.A. Otvos), f.a.h.heus@vu.nl (F. Heus), freek.vonk@naturalis.nl (F.J. Vonk), b.bruyneel@vu.nl (B. Bruyneel), i.paliukhovich@vu.nl (I. Paliukhovich), guus.smit@vu.nl (A.B. Smit), w.m.a.niessen@vu.nl (W.M.A. Niessen), j.kool@vu.nl (J. Kool).

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1. Introduction

Great advances have been made in protein-based pharmaceuticals during the last ten years (Ponzio et al., 2011; Ratner, 2010; Reichert, 2000; Reichert and Healy, 2001). Traditional problems with the use of 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, administration routes (Bailon and Won, 2009; Dinarvand et al., 2004; Hamidi et al., 2007; Orive et al., 2004; Shoyele and Cawthorne, 2006; Takahashi et al., 2007), protein engineering and production techniques (Daniell et al., 2009; Decker and Reski, 2008; Komarova et al., 2010; Wurm, 2007), as well as current knowledge and available technologies dealing with immunogenicity and immunotoxicity (Bhogal, 2010; Bussiere et al., 2009; Giezen et al., 2009; Kessler et al., 2006; Martin and Weinbauer, 2010) have now positioned protein based biopharmaceuticals solidly into the Pharma pipelines. In this regard, venom peptides also gained interest, particularly for their use in neuroscience research and their potential applicability in neurological diseases. However, limitations in venom peptide drug discovery are related to the difficulty of identifying bioactive peptides from the complex venom proteomes and their subsequent purification. The traditional workflow for screening venoms is sometimes called effect-directed analysis (EDA), as has also been used for decades in research directed at identification of bioactive toxicants in the environment (Koehn and Carter, 2005). With this approach, the venom is separated by liquid chromatography (LC) into fractions, often in the minute range, and the collected fractions are then tested for bioactivity. As pure compounds are not collected, often several iterative fractionation rounds guided by the bioactivity are needed. During this process, bioactives might get lost due to degradation, adsorption, denaturation and/or other reasons. The process often results in some of the intermediate bioactives eventually being processed for identification with mass spectrometry (MS). However, at this stage the successful interpretation and identification of the bioactives often fails for those with high affinity that are present in a low abundance due to sensitivity issues in MS. Furthermore, compounds of interest are usually present in the same fraction with high-abundant non-bioactive compounds, which further impair their detection and/or results in misinterpretation. Nerve growth factors, for example, are only present in an abundance of 0.1–0.5% in snake venom and are easily missed. Nowadays, the success rate of identification of novel compounds with classical methods is improved by using HPLC for separation (Roy et al., 2010). However, classical approaches are still very elaborate and even for the bioassays which consume only low amount of sample, much larger amounts of precious venom sample are needed from the start to isolate sufficient amounts of low abundant toxin for the bioassays. Although some snake species produce relatively large amounts of venom, most often venoms (and in there the potential peptides of interest) are of low abundance (Vonk et al., 2011). All this severely hampers efficient venom profiling.

Nano-LC–MS is the analytical technique of choice in proteomics approaches due to its mass sensitive detection and low sample consumption, and is well suited for analysis of venom proteomes as most snake venoms comprise predominantly peptides and proteins. Many (snake) venomics studies nowadays apply nano-LC–MS in their analytical strategies (Fox and Serrano, 2008). These proteomics studies, however, are only aimed at identification of peptides and proteins in venom, and cannot assess their bioactivity or bioaffinity towards chosen targets. Due to current advances in analytical strategies and microfluidics technologies, effective screening for some drug targets is now possible with so-called on-line high-resolution screening (HRS) approaches, i.e., chemical analysis and biological screening integrated in a single instrument platform. In this approach, post-LC continuous-flow biochemical detection with parallel MS analysis is performed (Kool et al., 2011). Analysing natural extracts with an HRS platform can reveal the number and chemical nature of the majority of bioactive compounds in a single measurement, with their affinity towards the drug target of interest estimated (de Jong et al., 2006; van Elswijk et al., 2004). Unfortunately, the low sample amounts from animal venoms are often not compatible with these traditional HRS approaches.

Bioactive peptides found in snake venoms can be active towards multiple targets and the many peptides present in venom ensure that the venom components interact with a myriad of relevant receptors and enzymes for pray immobilization. Some of these receptors and enzymes are of interest as drug target. For example, there are numerous peptides found to act on the nervous system and the haemostatic system (Vonk et al., 2011). Examples of medicinal products derived from snake venom compounds are the antiplatelet drug Eptifibatide (Integrilin) derived from southeastern pygmy rattlesnake (*Sistrurus miliarius barbouri*) venom and the analgesic toxin Hannalgesin from the venom of the King cobra (*Ophiophagus hannah* (Pu et al., 1995)). The latter is now in clinical trials. Snake venom toxins also have applications in clinical diagnostics, for example for the diagnostics of blood-clotting disorders and for the autoimmune disorder Myasthenia gravis (Chu, 2005; Vincent, 2002). The latter is based on the affinity of α-bungarotoxin (neurotoxin from the venom of the Taiwan krait *Bungarus multicinctus*) to the acetylcholine receptor.

Nicotinic Acetylcholine receptors (nAChRs) are associated with many CNS diseases like migraine, epilepsy and pain (Celie et al., 2005; Dutertre et al., 2007; Hogg and Bertrand, 2004; Hogg et al., 2003; Smit et al., 2001). During the last ten years, drug discovery directed at the α7-nAChR experienced a leap forward by using the acetylcholine binding protein (Smit et al., 2001) as drug model. Ls-AChBP (from *Lymnaea stagnalis*) is a stable structural homologue of the extracellular ligand binding domain of the α7 nAChR. It was first crystallized and validated as model for nAChRs, especially the α7 nAChR (Brejc et al., 2001) and has been used as nAChR model since then (de Kloe et al., 2010; Edink et al., 2011). We recently developed an HRS approach using AChBP as target (Kool et al.,