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Neurotoxicity fingerprinting of venoms using on-line microfluidic AChBP profiling

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ABSTRACT

Venoms from snakes are rich sources of highly active proteins with potent affinity towards a variety of enzymes and receptors. Of the many distinct toxicities caused by envenomation, neurotoxicity plays an important role in the paralysis of prey by snakes as well as by venomous sea snails and insects. In order to improve the analytical discovery component of venom toxicity profiling, this paper describes the implementation of microfluidic high-resolution screening (HRS) to obtain neurotoxicity fingerprints from venoms that facilitates identification of the neurotoxic components of envenomation. To demonstrate this workflow, 47 snake venoms were profiled using the acetylcholine binding protein (AChBP) to mimic the target of neurotoxic proteins, in particular nicotinic acetylcholine receptors (nAChRs). In the microfluidic HRS system, nanoliquid chromatographic (nanoLC) separations were on-line connected to both AChBP profiling and parallel mass spectrometry (MS). For virtually all neurotoxic elapid snake venoms tested, we obtained bioactivity fingerprints showing major and minor bioactive zones containing masses consistent with three-finger toxins (3FTxs), whereas, viperid and colubrid venoms showed little or no detectable bioactivity. Our findings demonstrate that venom interactions with AChBP correlate with the severity of neurotoxicity observed following human envenoming by different snake species. We further, as proof of principle, characterized bioactive venom peptides from a viperid (*Daboia russelli*) and an elapid (*Aspidelaps scutatus scutatus*) snake by nanoLC-MS/MS, revealing that different toxin classes interact with the AChBP, and that this binding correlates with the inhibition of α7-nAChR in calcium-flux cell-based assays. The on-line post-column binding assay and subsequent toxin characterization methodologies described here provide a new *in vitro* analytic platform for rapidly investigating neurotoxic snake venom proteins.

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1. Introduction

Many studies investigating the toxicity of individual venom components involve the initial activity screen of crude venoms followed by fractionation and re-screening of the fractions. Active fractions for the toxic effect under study are then structurally characterized, mainly by mass spectrometry (MS) based approaches. To characterize the full biological effect, relatively large quantities of toxins are needed, which are acquired by semi-preparative purification, by production in recombinant protein production systems, or by peptide synthesis. The purified bioactives can also be used to study their effect on pharmaceutically relevant receptors or enzymes in venom-based drug discovery, which is a growing research field providing new drug-leads from venoms as natural sources (Casewell et al., 2013; Kini and Fox, 2013; Vetter et al., 2011; Vonk et al., 2011).

High-throughput screening methods are desirable to rapidly assess bioactivity profiles of chosen venom toxicities. However, to investigate individual bioactives, a chromatographic separation of crude venom is still required prior to bioassaying in order to separate venom toxins with different, counteracting, and/or overlapping toxicities. As this is a costly and time-consuming endeavor for which large venom quantities are required, chromatographic analytics combining continuous flow post-column bioassays with parallel MS are an efficient alternative. These approaches, such as microfluidic high-resolution screening (HRS) (Heus et al., 2013), can be considered to directly measure fingerprint toxicity profiles of venoms. Microfluidic HRS uses nanoLC separations requiring very low sample quantities and is able to identify individual bioactives in on-line post column bioassay format, while parallel MS analysis directly allows accurate mass determination of the bioactives.

Recent studies using HRS methodology have focused on profiling venoms for drug discovery applications. The bioassay used in some of these studies was a fluorescence enhancement assay for AChBP profiling (Kool et al., 2010). The AChBP is a stable structural homologue of the extracellular ligand binding domain of nicotinic acetylcholine receptors (nAChRs), in particular the α7-nAChR (Smit et al., 2001), which has been widely used as an nAChR mimicking protein of nAChR relevant ligands (Bourne et al., 2014, 2015; Brejc et al., 2002; Celie et al., 2005; Inserra et al., 2013; Lin et al., 2016; Shahsavar et al., 2016; Wan et al., 2015; Xu et al., 2017). This implies that the AChBP could be used to profile neurotoxicity of individual venom components that target nAChRs. The α7-nAChR plays an important role in neuronal communication by converting neurotransmitter binding into electrical membrane depolarization. This receptor belongs to the family of the cys-loop ligand-gated ion channels (LGICs), and it combines binding sites for the neurotransmitter acetylcholine (ACh) and a cationic transmembrane ion channel (Albuquerque et al., 1997; Dani, 2001). One recent study by Ratanabanangkoon et al. already demonstrated the potential of using nAChR mimicking AChBP for measuring elapid antivenom potency (Ratanabanangkoon et al., 2017). In that study, *Torpedo californica* nAChR was utilized in a straightforward assay for testing antivenom generated against the elapid snake *Naja kaouthia*.

Here we demonstrate the potential of microfluidic HRS as a tool for obtaining neurotoxicity profiles of venoms based on the generic binding properties of neurotoxins to the nAChR mimic AChBP. These profiles can aid in research on neuropathological effects caused by specific neurotoxic peptides. In addition, this methodology could potentially be used to estimate the neurotoxic potency of venoms. Consequently, 47 venoms from the *Elapidae*, *Viperidae* and *Colubridae* snake families were screened with microfluidic HRS to obtain fingerprint profiles of individual bioactive compounds binding to *Lymnea stagnalis* (Ls) AChBP. These measurements were then sorted according to snake families, from which neurotoxicity profiles were compared. To demonstrate optional subsequent characterization of the selected bioactives, several venom toxins were purified followed by structural (i.e., full toxin identification) and biological characterization. For this, proteomic analysis and fluorescent imaging plate reader (FLIPR)-based assays containing mammalian cells over-expressing the α7-nAChR were performed.

2. Materials and methods

2.1. Chemicals and biological reagents

The mobile phases for nanoLC separation were prepared from nanoLC-MS grade 99.97% acetonitrile (ACN), 99.95% trifluoroacetic acid (TFA) and 99.95% formic acid (FA) obtained from Biosolve (Valkenswaard, the Netherlands). High-performance liquid chromatography (HPLC) grade water was produced by a Milli-Q purification system from Millipore (Amsterdam, the Netherlands). Ls-AChBP (from snail species *Lymnaea stagnalis*) was expressed and purified from Baculovirus using the pFastbac I vector in Sf9 insect cells as described elsewhere (Celie et al., 2005). (E)-3-(3-(4-diethylamino-2-hydroxybenzylidene)-3,4,5,6-tetrahydropyridin-2-yl)pyridine (DAHBA) was synthesized in house (Kool et al., 2010). Ammonium bicarbonate, sequencing grade trypsin and Glu-C were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Nicotine was obtained from Tocris Biosciences (Bristol, United Kingdom).

2.2. Snake venom samples

Venoms from the following species were profiled in this study: *Acanthophis antarcticus, Acanthophis praelongus, Acanthophis pyrrhus, Agkistrodon contortrix contortrix, Agkistrodon contortrix laticinctus, Agkistrodon contortrix mokasen, Agkistrodon bilineatus bilineatus, Agkistrodon piscivorus conanti, Agkistrodon piscivorus piscivorus, Aspidelaps scutatus scutatus, Atheris squamigera, Atropoides mexicanus, Bitis arietans, Bitis gabonica "rhinoceros", Bitis nasicornis, Boiga dendrophila, Boiga irregularis, Bothrops alternatus, Bothrops atrox, Bungarus caeruleus, Calloselasma rhodostoma, Causus rhombeatus, Cerastes cerastes, Crotalus adamanteus, Crotalus atrox, Crotalus basiliscus, Crotalus durissus culminatus, Crotalus durissus cumanensis, Crotalus durissus terrificus, Crotalus durissus vegrandis, Crotalus adamanteus, Crotalus horridus, Crotalus horridus "atricaudatus", Crotalus ruber ruber, Crotalus viridis viridis, Daboia russelii, Dendroaspis polylepis, Dendroaspis viridis, Echis carinatus sochureki, Gloydius blomhoffi, Oxyuranus microlepidotus, Pseudechis australis, Pseudechis porphyriacus, Pseudonaja affinis, Pseudonaja inframacula, Pseudonaja nuchalis, Pseudonaja textilis.* Most snake venoms were obtained commercially from Kentucky Reptile Zoo (City?, USA), Ventoxin (City?, USA), Biotoxin (City?, USA), Venom Supplies (Tanunda, Australia) and African Reptiles & Venoms (Johannesburg, South Africa). The *Boiga irregularis* and *Gloydius blomhoffii* venoms were kind gifts from Prof. Steve Mackessy (University of Northern Colorado, USA) and Prof. Sadaaki Iwanaga (Kyushu University, Japan), respectively. In case venom of a single animal is used, its major toxins will most likely be present regardless of the geographical location of the snake and/or when the venom was extracted, and/or if venom extraction was from a snake held in captivity or from the wild. Still, individual toxin quantities obtained per snake milking can vary due to these factors and therefore conclusions have to be drawn cautiously. Many of the venoms used, however, were not from a single animal. They are pools purchased commercially or provided by us and by others. In this case, the pooling of venom from multiple extractions of multiple individuals (possibly from different phylogenetic backgrounds and