Toxicon 152 (2018) 1–8

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Rapid ligand fishing for identification of acetylcholinesterase-binding peptides in snake venom reveals new properties of dendrotoxins

Kenia Lourenço Vanzolini[a], Stuart Ainsworth[b], Ben Bruyneel[c], Volker Herzig[d], Mitchell G.L. Seraus[c], Govert W. Somsen[c], Nicholas R. Casewell[b,f], Quezia Bezerra Cass[a,**], Jeroen Kool[c,*]

[a] SEPARARE – Núcleo de Pesquisa em Cromatografia, Departamento de Química, Universidade Federal de São Carlos, Caixa Postal 676, 13565-905, São Carlos, Brazil
[b] Alistair Reid Venom Research Unit, Parasitology Department, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, L3 5QA, UK
[c] Vrije Universiteit Amsterdam, Faculty of Sciences, Department of Chemistry and Pharmaceutical Sciences, Division of BioAnalytical Chemistry, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands
[d] The University of Queensland, Institute for Molecular Bioscience, Building 80, St. Lucia, QLD 4072, Australia
[f] Research Centre for Drugs and Diagnostics, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, L3 5QA, UK

ARTICLE INFO

Keywords:
Ligand fishing assay
Affinity-based protein assay
Snake venoms
Acetylcholinesterase-binding peptides
Mass spectrometry
Dendrotoxin
Mamba

ABSTRACT

Acetylcholinesterase (AChE) from *Electrophorus electricus* (*eel*) was immobilized on the surface of amino-modified paramagnetic beads to serve as a model for the development, validation and application of a new affinity-based ligand-fishing assay for the discovery of bioactive peptides from complex protein mixtures such as venoms. Nano liquid chromatography-mass spectrometry (nanoLC-MS) was used for the analysis of trapped peptides. Using enzyme-functionalized beads, the ligand-fishing assay was evaluated and optimized using a peptide reference mixture composed of one acetylcholinesterase binder (fasciculin-II) and five non-binders (mambalgin-1, angiotensin-II, bradykinin, cardiotoxin and α-bungarotoxin). As proof of concept, snake venom samples spiked with fasciculin-II demonstrated assay selectivity and sensitivity, fishing the peptide binder from complex venom solutions at concentrations as low as 1.0 μg/mL. As negative controls for method validation, venoms of four different snake species, not known to harbor AChE binding peptides, were screened and no AChE binders were detected. The applicability of the ligand fishing assay was subsequently demonstrated with venom from the black mamba, Jameson's mamba and western green mamba (*Dendroaspis* spp.), which have previously been reported to contain the AChE binding fasciculins. Unknown peptides (i.e. not fasciculins) with affinity to AChE were recovered from all mamba venoms tested. Tryptic digestion followed by nano-LC-MS analysis of the material recovered from black mamba venom identified the peptide with highest AChE-binding affinity as dendrotoxin-I, a pre-synaptic neurotoxin previously not known to interact with AChE. Co-incubation of AChE with various dendrotoxins *in vitro* revealed reduced inactivation of AChE activity over time, thus demonstrating that these toxins stabilize AChE.

1. Introduction

The large biomolecular diversity observed in nature is the result of billions of years of evolution that has resulted in enormous numbers of secondary metabolites in plants, microorganisms and animals (Berkov et al., 2014). Many of these compounds have had their biological activities characterized, and some exhibit medicinal potential. Modern medicinal chemistry originated in the 20th century with the use of plant-derived structures as templates to synthesize derivatives. Advances in biotechnology enabled microorganisms to be grown more efficiently and allowed further advances in efficient, large-scale production and isolation of biologically active compounds of interest.

During the last few decades, venoms have gained considerable interest as sources of compounds for drug discovery (King, 2011, 2015). In contrast to small secondary metabolites as the starting point for drug discovery, venomous animals produce mainly peptide and protein toxins. These peptides/proteins are highly selective and act potently on their targets. As these targets are often drug targets, venom components encompass interesting candidates for drug discovery (Harvey, 2014; King, 2011; Norton, 2017; Saez et al., 2010). Although venoms are a

* Corresponding author.
** Corresponding author.
E-mail addresses: quezia@pq.cnpq.br (Q.B. Cass), j.kool@vu.nl (J. Kool).

https://doi.org/10.1016/j.toxicon.2018.06.080
Received 10 April 2018; Received in revised form 16 June 2018; Accepted 25 June 2018
Available online 07 July 2018
0041-0101/ © 2018 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/BY-NC-ND/4.0/).

rich source for finding new bioactive compounds for drug discovery, maintaining the biological activity of the peptides or proteins during isolation is a challenge. Furthermore, venoms are highly bioactive, complex mixtures with many different (often interfering) bioactivities. Therefore the identification of desired bioactives in the presence of many other compounds makes venom-based drug discovery a challenging process. Nonetheless, advances in analytical chemistry have resulted in current state-of-the-art analytics allowing separation and purification of many types of venom peptides/proteins in their active form for assessment as potential bioactives that act on selected drug targets (Koh and Kini, 2012).

The majority of described animal toxins that exhibit properties amenable to medical and biochemical applications exert cardiovascular and/or neuroactive properties (Jumblatt et al., 1981; Koh and Kini, 2012; Miljanich, 2004; Monge-Fuentes et al., 2012). An example of a successful drug derived from venom is exenatide, a 39 amino acid peptide from *Heloderma suspectum* (the Gila monster - a venomous lizard) that was approved by the FDA for treatment of type 2 diabetes (Berkov et al., 2014; Harvey, 2014), and also shows potential for the management of Parkinson's disease (Athauda et al., 2017). Other examples of notable successes in this area have resulted from research on snake venoms from the vipers *Bothrops jararaca*, *Sistrurus miliarius barbouri* and *Echis carinatus*, resulting in the clinical peptide-based medicines captopril, eptifibatide and tirofiban (King and Hardy, 2013).

The generic process involved in screening complex mixtures such as venoms usually involves bioactivity-guided separations and fingerprint chromatogram analysis; both time-consuming and high cost methodologies (Qing et al., 2011; Weller, 2012; Zhu et al., 2015). Hence the research community is constantly seeking to increase the range and scope of analytical screening strategies (Kool et al., 2011b; Vetter et al., 2015).

Recent advances in separation science and detection technologies have fueled research into adjusted, advanced and/or new bioactivity screening formats for venom profiling. High-resolution screening, for example, uses a continuous flow bioassay that is coupled directly online after a liquid chromatographic (LC) separation with mass spectrometry (MS) in parallel. This way, the direct characterization of bioactive compounds identified in the bioassay is possible (Iyer et al., 2016; Kool et al., 2011a; Otvos et al., 2013). Other affinity-based screening assays for complex mixture analysis are pre-column and on-column based (Cieśla and Moaddel, 2016; Jonker et al., 2011; Moaddel and Wainer, 2009). A very straightforward approach is the use of off-line ligand-fishing assays (Marszałł et al., 2008; Moaddel et al., 2007; Oujji et al., 2012; Pochet et al., 2011; Wubshet et al., 2015; Yasuda et al., 2011). In this technique, target-coated beads are immersed directly into the natural extracts under study. Compounds with affinity for the immobilized target will be retained while non-binders will remain in solution. The bound compounds can then be eluted to identify them using LC-MS (Hu et al., 2008; Marszałł et al., 2008; Moaddel et al., 2007; Oujji et al., 2012; Vanzolini et al., 2013; Yasuda et al., 2011). The principle of ligand fishing is well known, and is established in the area of immuno-precipitation assays, but it is relatively unexplored as a technique for identifying peptide and protein ligands. In this regard, the technique can be valuable for venom profiling. As many venoms contain a multitude of proteases and other enzymes, first the potential effects of these components must be evaluated and assay conditions have to be optimized and validated for screening purposes.

Herein we report a new label-free affinity screening assay based on paramagnetic beads with immobilized acetylcholinesterase (AChE) for identifying AChE ligands from venoms. The only known AChE-binding venom peptides are potent (for mammalian AChEs) inhibitors known as fasciculins (belonging to the three finger toxin family [3FTx]), primarily found in venom of the eastern green mamba, *Dendroaspis angusticeps* (Karlsson et al., 1984). However, literature also demonstrates that fasciculin affinity for *eel*AChE is much lower in comparison to AChEs from mouse or human erythrocytes (Gonicnik and Stojan, 2002). Evaluation and development of our AChE-affinity fishing method was started using a reference peptide mixture containing one known AChE binder and several assumed non-binders. After assay development and validation, as a proof of concept, *Naja pallida* (red spitting cobra) venom was spiked with a known AChE binder, fasciculin II, and subsequently analyzed. Following this, we assessed venoms sourced from various elapid and viperid snake genera (*Dendroaspis*, *Aspidelaps*, *Micrurus*, *Vipera* and *Crotalus*), of which some (e.g. *Dendroapsis* spp. – the mambas) were expected to contain AChE inhibitors (i.e. fasciculins).

2. Experimental section

2.1. Materials

The peptides angiotensin II, bradykinin, cardiotoxin and AChE from *Electrophorus electricus* type VI-S (*eel*AChE), together with chemicals used for the enzyme immobilization procedure and tryptic digestion, and snake venoms from *Naja pallida* and *Vipera ammodytes* were all purchased from Sigma-Aldrich. *Crotalus basiliscus* venom was from Ventoxin (USA), *Aspidelaps scutatus scutatus* venom was from African Reptiles & Venoms (Johannesburg, South Africa) and *Micrurus nigrocinctus* venom was from Medtoxin Venom Laboratories (DeLand, Florida, USA). Mamba venoms (*Dendroaspis* spp.) were collected from snakes housed and maintained in the Alistair Reid Venom Research Unit herpetarium at the Liverpool School of Tropical Medicine (UK). Following collection, venoms were lyophilized and stored at 4 °C until reconstitution. Fasciculin II, α-dendrotoxin and δ-dendrotoxin, all isolated from *D. angusticeps* venom, and dendrotoxin I and dendrotoxin-K, both isolated from *D. polylepis* venom, was purchased from Alomone Labs (Israel). α-bungarotoxin was purchased from Tocris (Bio-techne, UK) and mambalgin-1 was donated by Smartox Biotechnology (France). Tacrine hydrochloride was purchased from Sigma-Aldrich. BcMag™ amine-terminated magnetic beads (MB) were from Bioclone (USA). Trypsin gold, MS-grade (Promega) was used in the digestion assays. Methanol and acetonitrile (ACN) were MS grade (Biosolve Chimie, France) and Milli-Q water (Merck Millipore) was used for all experiments.

2.2. Mamba venom proteomics analysis

The tryptic digestion protocol was based on that described in the supplier's instructions (Promega). Briefly, dithiothreitol (DTT) was used as reductant and iodoacetamide (IDA) as alkylation agent. Venom (30 µL of a 140 µg/mL solution prepared in 50 mM ammonium bicarbonate) was added to a 1.5 ml microcentrifuge tube and heated for 10 min at 40 °C. Next, DTT (7.5 µL of 100 mM solution in 50 mM ammonium bicarbonate) was added and the tube heated to 95 °C for 10 min. After cooling to room temperature, IDA (3.7 µL of 100 mM solution in 50 mM ammonium bicarbonate) was added to the mixture and the tube was placed in the dark for 20 min. 1.0 µL of trypsin solution (1.0 µg/mL) was then added and the mixture was left at 37 °C overnight. Formic acid (0.1% in 57.8 µL of Milli-Q water) was finally added to each digested sample, resulting in a final volume of 100 µL. The samples were then transferred to chromatography vials and 1.0 µL analyzed using nanoLC-MS.

2.3. Chromatography system and conditions

An UltiMate™ 3000 UHPLC$^+$ nano system (Thermo Scientific) was used in all chromatography separations with an analytical capillary column (Acclaim® PepMap RSLC C_{18}, 75 µm × 15 cm, 2 µm, 100 Å) and, a nano-trap column Acclaim PepMap100 C_{18} (100 µm × 2 cm, 5 µm, 100 Å). For the analytical capillary column, the flow rate used was 500 nL min^{-1}, with the mobile phase consisting of 0.1% FA in water (solvent A) and ACN:water (80:20, v/v, 0.1% FA) (solvent B). In the nano-trap column, the solvent used was ACN:water (1:99 (v/v), 0.05%

trifluoroacetic acid). The nanoLC system was coupled to an ESI-qTOF mass spectrometer (Impact HD, equipped with an Advance CaptiveSpray Source, Bruker Daltonics). The Captive® spray source conditions were: 1300 V for the capillary voltage, 0.2 bar for the nebulizer gas (N_2) pressure, 3.0 L min^{-1} and 150 °C for the dry gas (N_2) flow rate and temperature. The analyses were performed in full scan mode, positive ionization mode, with a range of 200–2800 m/z.

For validation of the ligand fishing assay, samples with the reference peptide mixture (bradykinin, angiotensin-II, mambalgin-1, fasciculin-II, cardiotoxin and α-bungarotoxin), and snake venom (*Naja pallida*) spiked with fasciculin-II, were analyzed using the following LC conditions: gradient elution at 0.50 µL/min with solvent A and B increasing from 1 to 50% solvent B over 110 min; an increase to 90% solvent B over 1 min, then isocratic elution at 90% solvent B for 8 min. The injection volume was 1.0 µL and, the concentration and clean-up of the sample was performed for 4 min on the nano-trap column at 10 µL/min. Note that the α-bungarotoxin sample (Sigma-Aldrich, B137) was a mixture of two isoforms, while the cardiotoxin sample (Sigma-Aldrich, C9759) comprised at least seven cardiotoxins.

The LC conditions used to analyze the snake venom samples obtained from the ligand-fishing assays were: isocratic elution at 0.50 µL/min with 1% of solvent B from 0 to 10 min; step gradient elution with solvent A and B varying from 1 to 35% solvent B from 10 to 20 min; an increase to 65% solvent B in 60 min and then to 85% solvent B in 10 min; from 90 to 100 min the concentration of solvent B was kept at 85%. Prior to the LC separation, for an injection volume of 1.0 µL, preconcentration and clean-up of the samples was performed on the nano-trap column during 4 min at 10 µL/min.

The LC conditions used for analyzing the trypsin-digested samples were as follows: isocratic elution at 0.50 µL/min with 1% of solvent B from 0 to 10 min; gradient elution with solvent A and B varying from 1 to 55% solvent B from 10 to 50 min; an increase to 85% solvent B in 1 min; from 51 to 55 min the concentration of solvent B was kept at 85% and went then back to 1% in 1 min followed by 10 min at this percentage. The proteomics data generated were analyzed using the Swissprot_2016 database. Searches included charge states of +1, +2 and + 3. MS tolerance was set at 0.05 Da with an MS/MS tolerance of 0.05 Da. The enzyme used for digestion was trypsin and the global modification set was carbamidomethyl.

2.4. Preparation of eelAChE-modified magnetic beads

In accordance with a previous report (Vanzolini et al., 2013), amine-containing- amino-acid residues of *eel*AChE (0.4 mg/mL) were covalently linked to the surface of modified magnetic beads (500 µL of a suspension of 50 mg/mL) by the production of an imine group using glutaraldehyde as linker. After the enzyme immobilization, the produced imine groups were reduced and the non-reacted aldehyde groups were end-capped. Using a magnetic separator (BcMag™ magnetic separator-6, Bioclone), the supernatant was discarded from the magnetic beads and, after which 1.0 mL of a solution containing a mixture of sodium cyanoborohydride (5.0 mg/mL) and hydroxylamine (3.3 mg/mL) prepared in phosphate buffer (100 mM, pH 7.0) was added to the beads. The reaction was stirred (at 9.0 rpm in an orbital shaker) for 24 h at 4 °C. Then, the prepared *eel*AChE-MB was washed three times with 600 µL of Tris-HCl buffer (100 mM, pH 8) and kept in the same buffer (600 µL). Aliquots of 30 µL were transferred to microcentrifuge tubes. 30 µL of Tris-HCl buffer (100 mM, pH 8) was added to each aliquot, then they were stored at 4 °C until use. Using the same procedure, control-MBs were prepared by replacing the enzyme solution with phosphate buffer (100 mM, pH 7.0).

2.5. Ligand-fishing assay: small molecules binder versus peptides binder

Contrary to the ligand fishing method developed for small molecules (like tacrine and galantamine) (Vanzolini et al., 2013, 2015), reusing the *eel*AChE-MBs for the ligand-fishing assay gave non-reproducible results with the venoms. One reason may be a change in the enzyme's conformation or denaturation of a percentage of the enzyme during the extraction step, in which the pH of the modified protocol was decreased to 3.5. Additionally, propidium iodide was used in the extraction solution in order to displace fasciculin-II from the allosteric site. Note that in the extraction step used in the method developed for small molecules, a pH value of 8.0 was used, and this rendered the enzyme fully active after extraction. Hence, in the case of the method for small molecules, the *eel*AChE-MBs can be re-used for multiple ligand-fishing experiments. These conditions did not result in detection of fasciculin-II. Adding methanol to the extraction solution also did not result in detection of fasciculin-II, probably due to precipitation of fasciculin-II. As the *eel*AChE-MBs for venom ligand-fishing could not be re-used, the original assay volume of 500 µL was decreased to 30 µL thereby reducing the number of beads used per ligand-fishing assay by a factor of 16. Additionally, this step significantly reduced the volume needed of small and precious venom samples. As ligand-fished venom extracts were analyzed in the current method using nanoLC (the ligand-fishing method for small molecules uses normal bore LC), this reduction in assay volume is acceptable as only 1 µL of sample is required for nanoLC-MS identification of ligands.

2.6. Ligand fishing assay with reference peptide mixtures

An *eel*AChE ligand-fishing assay for venom screening was performed with a reference peptide mixture containing the AChE peptide binder fasciculin-II (1.4 µg/mL), and the assumed non-binders mambalgin-1 (5.0 µg/mL), angiotensin-II (0.10 µg/mL), cardiotoxin (25 µg/mL) and α-bungarotoxin (25 µg/mL) in ammonium acetate (AE) solution (15 mM, pH 8).

In the assay developed, one microcentrifuge tube containing 30 µL of the prepared *eel*AChE-MBs was used. Briefly, the *eel*AChE-MBs were washed three times with 100 µL of AE solution, and the supernatants discarded. The *eel*AChE-MBs were then suspended in 100 µL of the reference peptide mixture. Each tube was then gently stirred in a Biosan® shaker for 30 min at 4 °C and then placed into a magnetic separator (DiaMag™ magnetic rack, Diagenode) for 120 s. The supernatant ($S_{AChE-MB}$-1) was collected and the *eel*AChE-MBs were washed twice with 100 µL of AE solution by manual shaking for 10 s followed by placing tubes into the magnetic separator for 120 s. The *eel*AChE-MBs were then re-suspended in the extraction solution, 200 µM propidium iodide prepared in AE buffer (15 mM, pH 3.5), and gently stirred with a Biosan® shaker, at room temperature, for 15 min. After placement in the magnetic separator DiaMag™ for 120 s, the supernatant ($S_{AChE-MB}$-3) was collected and the *eel*AChE-MBs were washed twice with 100 µL of ammonium acetate (15 mM, pH 8) by manual shaking for 10 s followed by placing the tube into the magnetic separator for 120 s; the pooled supernatant collected after the wash steps was retained for further analyses. To maintain a similar pH in all samples, 1.0 µL of formic acid 30% (v/v) was added to 90 µL of the supernatants $S_{AChE-MB}$-1 and $S_{AChE-MB}$-3.

Aliquots of 40 µL of $S_{AChE-MB}$-1 and $S_{AChE-MB}$-3 were transferred to LC vials containing inserts of 200 µL. A volume of 1.0 µL was injected in the nanoLC-MS system for analysis.

The protonated molecular ion $[M + xH]^{x+}$ used to identify each of the tested peptides was determined based on the most abundant charge state: $[M+3H]^{3+}$ 354.1974 m/z (bradykinin); $[M+3H]^{3+}$ 349.5216 m/z (angiotensin-II); $[M+11H]^{11+}$ 596.8250 m/z (mambalgin-1); $[M+10H]^{10+}$ 675.9123 m/z (fasciculin-II); two peptides for α-bungarotoxin (which was a mixture of two isoforms): $[M+11H]^{11+}$ 726.7992 m/z and 729.3483 m/z, and seven peptides for cardiotoxin (which was a mixture of at least seven isoforms): $[M+11H]^{11+}$ 660.1296 m/z, 618.4101 m/z, 610.8623 m/z, 620.7744 m/z, 642.0534 m/z, 627.0529 m/z, 622.5068 m/z.

For plotting the ligand-fishing assay results, an area for each peptide

present in $S_{AChE\text{-}MB}$-1 and $S_{AChE\text{-}MB}$-3 was calculated using the software Data Analysis (Bruker Daltonics®). Together with each experiment, a control experiment was carried out using the control-MBs for which the supernatants $S_{control\text{-}MB}$-1 and $S_{control\text{-}MB}$-3 were collected and analyzed using the same conditions.

To evaluate and validate the applicability of the ligand fishing assay for snake venoms, and the suitable concentration range of crude venom to be used, the methodology was applied to *Naja pallida* venom at three concentrations (10, 50, 500 µg/mL) spiked with fasciculin-II at 1, 10 and 100 µg/mL, and un-spiked, in a matrix fashion. The ligand fishing assay was then performed in duplicate for each condition (i.e. for each concentration of venom and fasciculin tested) using the *eel*AChE-MBs and the control-MBs. The results were plotted using the areas found for the fasciculin-II ion ($[M+10H]^{10+}$ 675.9123 m/*z*) in the supernatants $S_{AChE\text{-}MB}$-3 and $S_{control\text{-}MB}$-3.

2.7. Application of the ligand fishing assay for snake venom screening

The validated screening assay was applied to the venom of the following snake species: *Dendroaspis polylepis*, *D. jamesoni*, *D. viridis*, *Vipera ammodytes*, *Crotalus basiliscus*, *Aspidelaps scutatus scutatus* and *Micrurus nigrocinctus*. All venom samples were prepared as 50 µg/mL solutions in AE solution (15 mM, pH 8). The ligand fishing assays were performed six times for the mamba venoms (*Dendroaspis* spp.) and in duplicate for the others.

The areas (measured as peak area of extracted ion chromatograms (EICs) from the highest *m/z* value observed for each peptide) for the peptides present in $S_{AChE\text{-}MB}$-3 and $S_{control\text{-}MB}$-3 were calculated and plotted as an affinity ratio:

$$\text{Affinity ratio} = \text{Compound area present in } S_{AChE-MB} - 3/\text{Compound area present in } S_{control-AChE} - 3.$$

2.8. Ellman's assay for AChE activity

*eel*AChE enzymatic activity based on substrate conversion was measured using an adapted version of Ellman's assay. 10 µL of either buffer (0.1 M potassium phosphate, pH 7.5), or test reagent were added to individual wells of clear polystyrene 384 well plates (Nunc MaxiSorp, Thermo-Fisher, U.K.). Unless stated otherwise, all test reagent concentrations were prepared as 1:3 serial dilutions from 20 µM, (i.e. 6.6 µM, 2.2 µM, 0.7 µM, 0.2 µM, 823 nM, 274 nM or 91 nM). To each well, 25 µl of *eel*AChE (0.03 U/ml) was added and incubated at room temperature for 45 min. After incubation, 50 µl of substrate buffer (2.5 mM [5,5-dithio-bis-(2-nitrobenzoic acid)], 0.4 mM acetylthiocholine iodide) was added using a Multidrop 384 Reagent Dispenser (Thermo-Fisher, the Netherlands). Immediately following substrate addition, absorbance (405 nm) was measured every five minutes for a period of 13 cycles (1 h) at 25 °C using a FLUROstar Omega microplate reader (BMG Biotech, U.K.). All experiments were performed in triplicate. For data analysis, means of replicates were baseline adjusted and normalized against *eel*AChE activity without additive (100% activity). IC50 and EC50 curves were generated in GraphPad Prism 7.

3. Results and discussion

3.1. Ligand fishing assay validation

A ligand-fishing assay for AChE was reported previously with a focus on screening mixtures containing small organic molecules (Vanzolini et al., 2013). The current study describes the development of a new assay format suitable for screening complex peptide and protein mixtures, including venoms. We demonstrate that screening venom mixtures requires a fundamentally different assay approach in which the potential denaturation of peptides and proteins is taken into account, as well as the highly active and stable proteases present in many snake venoms. In short, the principal characteristics of animal venom as matrix requires significant adaptations and modifications compared to the method developed for screening small molecules.

A typical ligand-fishing assay for discovering small molecular ligands is based on four principal steps: matrix load, wash of non/low-binders, extraction of binders, and a final wash/equilibration step to prepare the immobilized MBs for the next assay. Compared to the reported protocol (Vanzolini et al., 2015), some modifications were considered when taking the peptides and proteins present in venoms into account; the main reason is the precipitation and/or denaturation of peptides/proteins in the presence of high concentrations of organic modifiers. Based on this, the extraction step in the previously reported protocol (Vanzolini et al., 2015) was modified from a small percentage of organic solvent to a pH decrease. Also, to avoid the potential action of venom proteases cleaving the immobilized *eel*AChE enzyme, resulting in inactivation, the load step was tested at 10 min and at 30 min at two different temperatures, 4 °C and room temperature. The rationale behind this strategy is that reducing incubation temperature will considerably decrease enzymatic activity, but is likely to influence binding to a lesser extent. Secondly, as binding events (association constants) are usually in the order of seconds (small molecules) to minutes (peptide/protein binders) and enzymatic reactions from venom proteases require time to potentially digest the immobilized *eel*AChE, shorter incubation times favor binding vs. undesired *eel*AChE digestion by venom enzymes.

The reference peptide mixture prepared for the development and validation of the ligand-fishing assay contained peptides with masses in a range of 1–7 kDa. It included the venom peptides mambalgin-1, angiotensin-II, cardiotoxin and α-bungarotoxin, and the known AChE binder fasciculin-II used to verify the assay applicability for peptide/protein mixture screening. The reference AChE binder fasciculin-II is a three-finger toxin (3FTx) and is an example of the family of fasciculin peptides extracted from the venom of *Dendroaspis angusticeps* that exhibit high affinity for AChE (Radic et al., 1994).

The developed ligand-fishing assay as described in the experimental section was first applied to the reference peptide mixture. The results obtained are summarized in the graph presented in Fig. 1. As expected, fasciculin-II was present in a significantly higher amount in $S_{AChE\text{-}MB}$-3 (supernatant of the extraction step) than in $S_{AChE\text{-}MB}$-1 (supernatant of the load step) (measured as the peak areas of the EICs from the highest *m/z* value observed), demonstrating that fasciculin-II had affinity for the immobilized *eel*AChE enzyme. Furthermore, the non-binders (bradykinin, mambalgin-1 and cardiotoxin) were present in similar amounts in both $S_{AChE\text{-}MB}$-3 and in $S_{control\text{-}AChE}$-3, which is indicative of non-specific interactions with the beads and not with the *eel*AChE enzyme. Angiotensin and α-bungarotoxin were found with a higher area in $S_{AChE\text{-}MB}$-1 than in $S_{AChE\text{-}MB}$-3, demonstrating that they remain in solution without significant binding to the enzyme, as expected for these non-binders.

For cardiotoxin and bungarotoxin, multiple ions are plotted as the commercially available products used consisted of a mixture of different cardiotoxins (CardTx-1 to CardTx-7) (Otvos et al., 2013) and bungarotoxins (alpha-BuTx-1 and alpha-BuTx-2) (Heus et al., 2013).

After method optimization and evaluation with the peptide reference mixture, the ligand fishing assay validation was performed using *Naja pallida* venom at 10, 50 and 500 µg/mL spiked with fasciculin-II at 1, 10 and 100 µg/mL, in a matrix fashion. The results are presented in Fig. 2, where the fasciculin-II area ratio $S_{AChE\text{-}MB}$-3/$S_{control\text{-}AChE}$-3 was plotted. The measured ratios were significantly higher than 1.0 in all cases, demonstrating that the *eel*AChE affinity ligand fishing of fasciculin-II was successful for all concentrations of fasciculin-II spiked in all snake venom concentrations tested. The lower fasciculin-II area ratio at 500 µg/mL is probably caused by ion suppression during ESI when analyzing the venom at this high concentration. In general, it is expected that a high affinity ratio from low or medium intensity signals

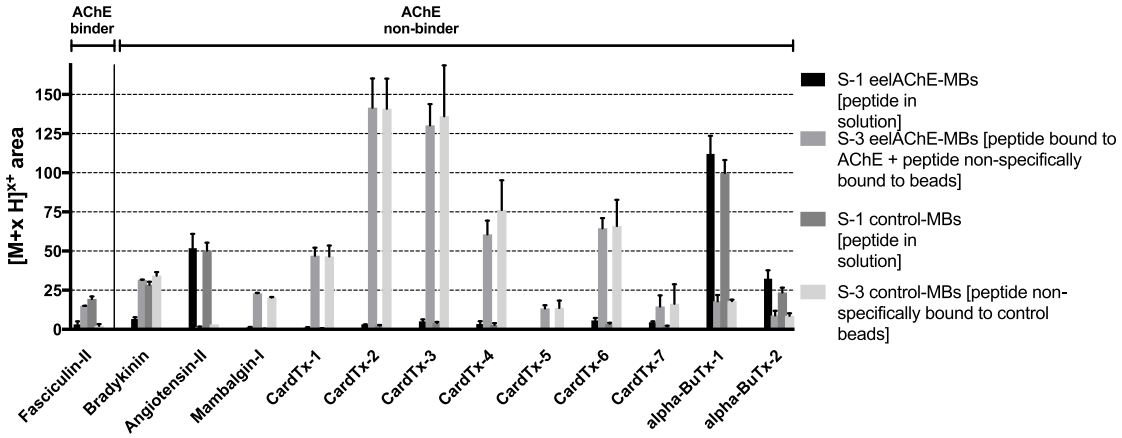

Fig. 1. Results of the ligand-fishing assay performed with the peptide reference mixture. The areas of the following protonated molecular ions were used to plot the graph: $[M+3H]^{3+}$ 354.1974 m/z (bradykinin); $[M+3H]^{3+}$ 349.5216 m/z (angiotensin-II); $[M+11H]^{11+}$ 596.8250 m/z (mambalgin-1); $[M+10H]^{10+}$ 675.9123 m/z (fasciculin-II); α-bungarotoxin (alpha-BuTx)-1 $[M+11H]^{11+}$ 726.7992 m/z, alpha-BuTx-2729.3483 m/z, cardiotoxin (CardTx)-1 $[M+11H]^{11+}$ 660.1296 m/z, CardTx-2618.4101 m/z, CardTx-3610.8623 m/z, CardTx-4620.7744 m/z, CardTx-5642.0534 m/z, CardTx-6627.0529 m/z, CardTx-7622.5068 m/z.

in MS is indicative for a binding interaction. A medium or around the threshold affinity ratio ($S_{AChE\text{-}MB}$-3/$S_{control\text{-}AChE}$-3 = 1.0), in combination with a high signal in MS, indicates a high concentration of a protein and thus can indicate a low affinity binder and/or significant non-specific binding.

3.2. Screening of snake venoms for eelAChE inhibiting peptides and proteins

The assay was then applied to screening several snake venoms including those from the western green mamba (*D. viridis*), Jameson's mamba (*D. jamesoni*) and black mamba (*D. polylepis*). We chose these venoms since the closely related eastern green mamba (*D. angusticeps*) is an abundant source of AChE binding fasciculins (Koh et al., 2006; Laustsen et al., 2015; Rodriguez-Ithurralde et al., 1983; Viljoen and Dawie, 1973). Four other snake venoms from the families Elapidae and Viperidae (*Aspidelaps scutatus scutatus*, *Micrurus nigrocinctus*, *Crotalus basiliscus* and *Vipera ammodytes*) were selected as likely 'negative controls', as no literature reported toxins in these venoms that bind AChE.

Results from the negative control snake venoms are presented in Fig. 3 in which a threshold equal to 2.5 for the compound affinity ratio value ($S_{AChE\text{-}MB}$-3/$S_{control\text{-}AChE}$-3) was selected based on the most intense affinity ratio value. Compounds below this ratio are considered as non-specific or exhibiting very low affinity interactions. Proteins and peptides with ratios above the threshold were deemed to be potential specific binders to the immobilized *eel*AChE target. We applied statistical calculations (using Prism 7.0 for Mac) to validate this approach, by applying multiple *t*-test comparisons (corrected for multiple comparisons using the Holm-Sidak method) to compare the signals from the S-3 fractions of the AChE-beads against their respective data from the S-3 beads without AChE (controls). Significance levels were set to $P < 0.01$ (**) and $P < 0.05$ (*) as indicated in Fig. 3.

Ligand fishing assays with venom from *Micrurus nigrocinctus* revealed one peptide with an affinity ratio close to the threshold (Fig. 3), which we consider to be a low affinity binder, or a binder with low abundance in the venom with good ionization efficiency in the ESI-MS. No peptides with affinity ratios above the threshold were detected in the remaining negative control snake venoms (Fig. 3), although venom from *Crotalus basiliscus* contained several small molecular weight compounds that were detected in the measurements of the supernatant $S_{AChE\text{-}MB}$-3, albeit with affinity ratios below the described affinity threshold (Fig. 3).

In the mamba venoms, four peptides yielded affinity ratios higher than the threshold ratio of 2.5; two had ratios of ~2.8, one of ~5, and one of ~15. The two peptides with affinity ratios of ~2.8 were from *D. viridis* ($[M+11H]^{11+}$, 683.8821 m/z) and *D. jamesoni* ($[M+11H]^{11+}$, 672.4984 m/z). The peptide with a ratio of ~5 was also fished from *D. jamesoni* venom ($[M+11H]^{11+}$, 664.4489 m/z). Finally, the peptide with a ratio of ~15, substantially higher than any other peptide detected in our experiments, was from *D. polylepis* venom ($[M+9H]^{9+}$, 721.5765 m/z) (Fig. 3).

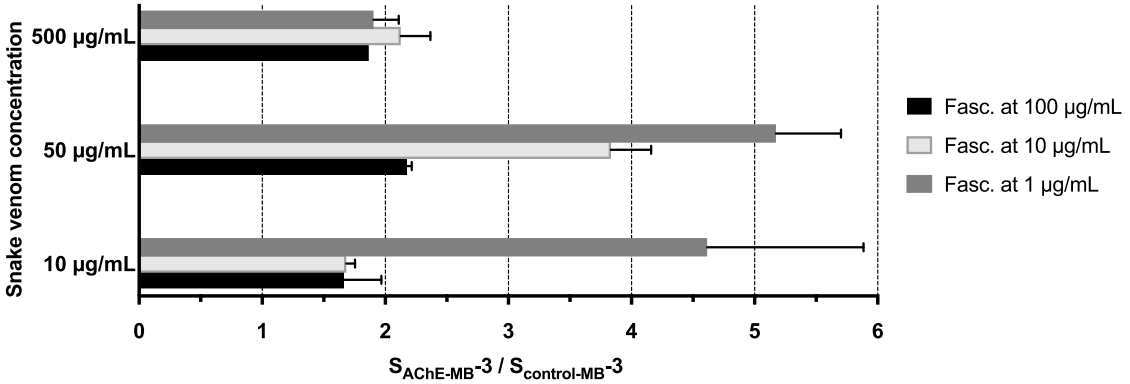

Fig. 2. Results of the ligand-fishing assay performed with Naja pallida venom at 10.0, 50.0 and 500 µg/mL spiked with fasciculin-II at 1.00, 10.0 and 100 µg/mL The area ratio of the fasciculin-II ion ($[M+10H]^{10+}$, 675.9123 m/z) measured in the supernatants $S_{AChE\text{-}MB}$-3 and in $S_{control\text{-}MB}$-3 is depicted. As the ratios of the extracts of the AChE-MB experiments vs. the control-MB experiments are plotted, higher fasciculin-II concentrations give lower ratios due to more non-specific binding at higher concentrations of fasciculin-II.

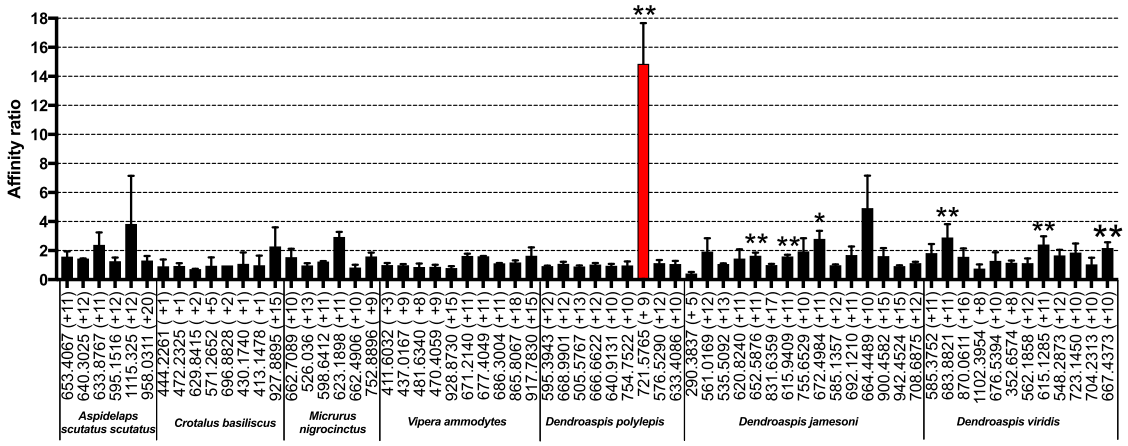

Fig. 3. Affinity ratio values calculated for an accurate mass of each peptide (or small molecular weight compounds in the case of Crotalus basiliscus) identified in $S_{AChE\text{-}MB}$-3 of the ligand-fishing assays performed with the snake venoms. The peptides are indicated with one m/z value per peptide (the most intense charge state for each peptide observed) used to plot extracted ion current chromatograms (XICs) for both supernatant $S_{AChE\text{-}MB}$-3 and $S_{control\text{-}MB}$-3 in order to calculate the compound peak areas from these sets of two EICs. The charge state of each ion used for analysis is indicated (in parentheses) for each compound. ** ($P < 0.01$) and * ($P < 0.05$) indicate significant differences for *t*-test comparisons of the signals from the S-3 fractions of the AChE-beads vs. the respective data from the S-3 beads without AChE (controls).

Average masses determined from deconvoluted mass spectra from the fished peptides were determined to be 6485.15 Da (ratio ~2.8, *D. viridis*), 7386.42 Da (ratio ~2.8, *D, jamesoni*), 6634.45 Da (ratio ~5, *D. jamesoni*) and 7511.48 Da (ratio ~15, *D. polylepis*). The only known AChE binding proteins isolated from mamba venoms have molecular masses of 6817.84 Da (fasciculin-I) and 6768.77 Da (fasciculin-II) (Le Du et al., 1992). Thus, the detected compounds appear to be novel AChE binding peptides. The measured masses of these peptides suggest that they might be three-finger toxins (3FTx; typical mass range 5–10 kDa) or dendrotoxins (typical mass range 6–7 kDa).

Despite being previously isolated from *D. polylepis* (Joubert and Taljaard, 1978) and *D. viridis* (Marchot et al., 1993), no fasciculins were recovered from any mamba venoms subjected to the ligand fishing assay. Previous proteomic analysis of mamba venom (from the same individuals whose venoms were used in this study) (Ainsworth et al., 2017; Petras et al., 2016) detected the presence of only very small amounts of fasciculin in *D. polylepis* venom (< 1% total venom content) and none in *D. viridis* or *D. jamesoni*. Additional experiments were performed to confirm the absence of fasciculin in the *D. viridis* venom used for ligand fishing assays. *D. viridis* venom was spiked with three different concentrations of fasciculin-II (1, 10 and 100 μg/mL) and analyzed in two different ways. Firstly, the spiked samples and a non-spiked venom sample were analyzed for identification of fasciculin-II. In the spiked venoms, XICs of the most abundant *m/z* matched that of fasciculin-II, clearly demonstrating recovery of fasiciulin-II in all concentrations spiked, including that of the lowest concentration tested (1 μg/mL). In the control, non-spiked venom, no fasciculin-II was detected. Secondly, the same venom samples were digested by trypsin followed by nanoLC-MS/MS analysis. Subsequent Mascot database searching identified fasciculin-II in all the spiked venoms. In the non-spiked samples, no fasciculin was identified. From this data, it was concluded that the particular mamba venoms analyzed either did not contain fasciculin, or they were present at a concentration too low to be detected using the analytical equipment and methodology presented in this study.

3.3. Identification of AChE binding peptides

The average mass calculated from the deconvoluted mass spectra (7511.48 Da) of the peptide from *D. polylepis* with very high binding affinity ratio (~15) suggests that it might belong to the 3FTx or dendrotoxin families. To positively identify the unknown AChE binding peptide, the *D. polylepis* $S_{AChE\text{-}MB}$-3 ligand-fished supernatant was digested with trypsin followed by nanoLC-MS/MS analysis. Subsequent Mascot searching using the SwissProt database with reptiles set as taxonomy returned dendrotoxin-I (P00979) from *D. polylepis* as the top hit, with a protein score of 891.71 (digest matches) and a sequence coverage of 63.3%.

The mamba genus-specific dendrotoxins are a family of neurotoxins that enhance the release of ACh into the synaptic cleft, through high affinity blockage of pre-synaptic voltage-gated K^+ channels (Harvey, 2001). To our knowledge, dendrotoxin-I has not been described previously as being able to bind AChE. It is interesting to note that dendrotoxin-I is the major component of *D. polylepis* venom, comprising 24% of total venom protein content (Ainsworth et al., 2017; Petras et al., 2016). The relative abundance of dendrotoxin-I in *D. polylepis* venom may explain the high binding ratio in the ligand fishing experiments (Fig. 3), compared to the other members of the genus tested, whose venom profiles are divergent to that of *D. polylepis* and exhibit 3FTx dominated venom profiles with relatively small quantities (3.4%–16.3% total toxin content) of dendrotoxins (Ainsworth et al., 2017).

3.4. Dendrotoxins stabilize eelAChE activity in vitro

The biological activity of dendrotoxin-I and the *D. angusticeps* homolog, α-dendrotoxin, binding to *eel*AChE was investigated using an adapted Ellman's assay for measuring AChE enzymatic activity based on substrate conversion (Fig. 4). In agreement with previous observations (Gonicnik and Stojan, 2002), analysis of *eel*AChE activity without the addition of additives demonstrates a gradual reduction in the rate of substrate conversion from approximately 15 min onwards throughout the course of the experiment. Experiments in which *eel*AChE was pre-incubated with either dendrotoxin-I or α-dendrotoxin did not demonstrate similar reductions in the rate of substrate conversion, with the rate of conversion appearing constant throughout the experiment. These results suggest that dendrotoxin-I and α-dendrotoxin stabilize *eel*AChE over time (Fig. 4a). At 60 min, stabilization of *eel*AChE with both dendrotoxins resulted in an apparent ~40% increase in *eel*AChE activity (dendrotoxin-I EC50 = 3.97 μM, α-dendrotoxin EC50 = 1.62 μM) (Fig. 4b) as compared to *eel*AChE alone. In order to investigate if potential non-specific binding of other proteins can result in any stabilization effect on *eel*AChE, several proteins were tested in the assay in combination with *eel*AChE, including BSA, HSA,

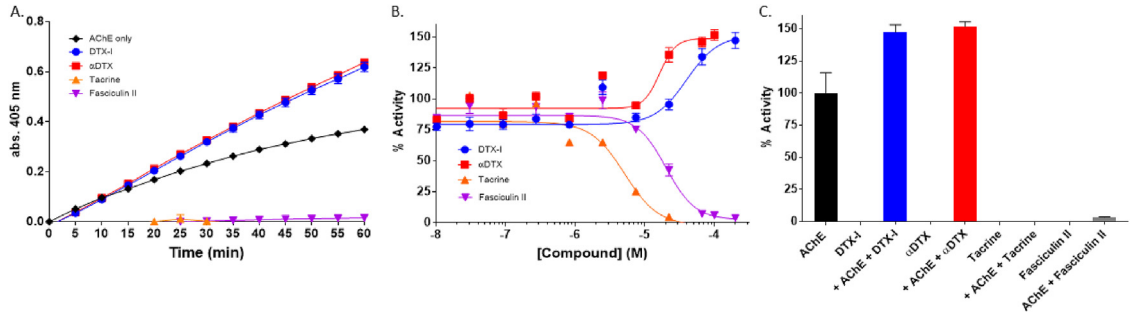

Fig. 4. Investigation of eelAChE enzymatic activity in presence of dendrotoxin-I, α-dendrotoxin, fasciculin and tacrine using an adapted Ellman's assay. A) Time course of substrate conversion by 0.03 U/ml eelAChE with and without additives at 20 μM concentration. B) IC50/EC50 curves of 0.03 U/ml eelAChE incubated with 1:3 serial dilutions of 20 μM additive. Data normalized against 0.03 U/ml eelAChE activity without additive. C) Activity at 60 min post experiment start of compounds with and without 0.03 U/ml eelAChE, demonstrating additives alone do not convert substrate. Data points represent the mean of triplicate experiments. Error bars represent standard deviation of the mean. Error bars are not shown where they are smaller than the data point.

myoglobin, and ELISA Blocking reagent (up to 1 mg/mL). No stabilization effect was observed in any of these experiments.

Repeating the assay in absence of *eel*AChE with dendrotoxins present at the highest concentrations tested did not result in any substrate conversion, demonstrating that neither dendrotoxin-I or α-dendrotoxin have any enzymatic activity towards the substrate (Fig. 4c). Further experiments suggest other dendrotoxin homologs, such as dendrotoxin-K and σ-dendrotoxin have similar *eel*AChE stabilizing properties (Supplemental Fig. 1), indicating this activity may be an inherent attribute of dendrotoxins. Additional experiments in which α-dendrotoxin was incubated at 10 μM tested in combination with AChE inhibitors fasciculin-II or tacrine, at their full inhibitory concentrations, resulted in no detected *eel*AChE activity (Supplemental Fig. 2), suggesting α-dendrotoxin binds to *eel*AChE in an allosteric manner. Further investigations will be required to determine if the observed activities are similar in mammalian AChE and to deduce any biological significance in envenoming. Regardless of any physiological effect, this finding may be useful to stabilize AChE in biotechnological applications.

4. Conclusions

A variety of approaches have previously been described to identify AChE inhibitors in natural or synthetic libraries (Miao et al., 2010). The vast majority of such approaches are based on colorimetric methods using Ellman's reagent or Fast Blue B salt reagent, with solution based enzyme assays (Di Giovanni et al., 2008). The affinity-based screening assay reported herein is a label-free assay, where the ligand-fishing technique was used for the first time to identify AChE ligands in complex peptide/protein mixtures. For analysis of venoms, the assay had to be modified significantly compared to the published method for screening a compound synthetic library (Vanzolini et al., 2015). For example, the paramagnetic beads used for ligand fishing can be used only once and covalent *eel*AChE immobilization was required to prevent release of *eel*AChE from the beads during ligand extraction under low pH conditions. After method development, we demonstrated the applicability of the method to screening venoms. We identified dendrotoxin-I from black mamba venom as a high-affinity *eel*AChE binder, an activity not previously associated with this class of toxins. Further experiments demonstrated that both dendrotoxin-I and α-dendrotoxin stabilize, thereby enhancing, *eel*AChE activity *in vitro*.

Acknowledgments

This work was funded by a research grant 2013/01710-1 and also by a BEPE grant 2015/18504-0 from São Paulo State Research Foundation (FAPESP). Prof. Dr. Glenn King is thanked for critically reviewing the manuscript. The authors also thank the National Council for Scientific and Technological Development (CNPq) and National Institute of Science and Technology (INCT) – Controle Biorracional de InsetosPraga (CBIP).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.toxicon.2018.06.080.

References

Ainsworth, S., Petras, D., Engmark, M., Sussmuth, R.D., Whiteley, G., Albulescu, L.O., Kazandijan, T.D., Wagstaff, S.C., Rowley, P., Wüster, W., Dorrestein, P.C., 2017. The medical threat of mamba envenoming in sub-Saharan Africa revealed by genus-wide analysis of venom composition, toxicity and antivenomics profiling of available antivenoms. J. Proteom. 172, 173–189.

Athauda, D., Maclagan, K., Skene, S.S., Bajwa-Joseph, M., Letchford, D., Chowdhury, K., Hibbert, S., Budnik, N., Zampedri, L., Dickson, J., Li, Y., Aviles-Olmos, I., Warner, T.T., Limousin, P., Lees, A.J., Greig, N.H., Tebbs, S., Foltynie, T., 2017. Exenatide once weekly versus placebo in Parkinson's disease: a randomised, double-blind, placebo-controlled trial. Lancet 390, 1664–1675.

Berkov, S., Mutafova, B., Christen, P., 2014. Molecular biodiversity and recent analytical developments: a marriage of convenience. Biotechnol. Adv. 32, 1102–1110.

Cieśla, Ł., Moaddel, R., 2016. Comparison of analytical techniques for the identification of bioactive compounds from natural products. Nat. Prod. Rep. 33.

Di Giovanni, S., Borlozb, A., Urbainb, A., Marstonb, A., Hostettmannb, K., Carrupta, P.-A., Reista, M., 2008. In vitro screening assays to identify natural or synthetic acetylcholinesterase inhibitors: thin layer chromatography versus microplate methods. Eur. J. Pharm. Sci. 33, 109–119.

Gonicnik, M., Stojan, J., 2002. Multi-step analysis as a tool for kinetic parameter estimation and mechanism discrimination in the reaction between tight-binding fasciculin 2 and electric eel acetylcholinesterase. BBA. Protein Struct. Mol. Enzymol. 1597, 164–172.

Harvey, A.L., 2001. Twenty years of dendrotoxins. Toxicon 39, 15–26.

Harvey, A.L., 2014. Toxins and drug discovery. Toxicon 92, 193–200.

Heus, F., Vonk, F., Otvos, R.A., Bruyneel, B., Smit, A.B., Lingeman, H., Richardson, M., Niessen, W.M., Kool, J., 2013. An efficient analytical platform for on-line microfluidic profiling of neuroactive snake venoms towards nicotinic receptor affinity. Toxicon 112–124.

Hu, F., Zhang, H., Lin, H., Deng, C., Zhang, X., 2008. Enzyme inhibitor screening by electrospray mass spectrometry with immobilized enzyme on magnetic silica microspheres. J. Am. Soc. Mass Spectrom. 19, 865–873.

Iyer, J.K., Otvos, R.A., Kool, J., Kini, R.M., 2016. Microfluidic chip-based online screening coupled to mass spectrometry: identification of inhibitors of Thrombin and Factor Xa. J. Biomol. Screen 21, 212–220.

Jonker, N., K, J., Irth, H., Niessen, W.M., 2011. Recent developments in protein-ligand affinity mass spectrometry. Anal. Bioanal. Chem. 399, 2669–2681.

Joubert, F.J., Taljaard, N., 1978. Complete primary structure of toxin-C from dendroaspis-polylepis (black mamba) venom S. Afr. J. Chem. 31, 107–110.

Jumblatt, J.E., Marquis, J.K., Mautner, H.G., 1981. On the specificity of 125-I-alpha-bungarotoxin binding to axonal membranes. J. Neurochem. 37, 392–400.

Karlsson, E., Mbugua, P.M., Rodriguez-Ithurralde, D., 1984. Fasciculins, anticholinesterase toxins from the venom of the green mamba *Dendroaspis angusticeps*. J. Physiol. 79, 32–240.

King, G.F., 2011. Venom as platform for human drugs: translating toxins into therapeutics. Expert Opin. Biol. Ther. 11, 1469–1484.

King, G.F., 2015. Venoms to Drugs: Venom as a Source for the Development of Human Therapeutics. Royal Society of Chemistry.

King, G.F., Hardy, M.C., 2013. Spider-venom peptides: structure, pharmacology, and

potential for control of insect pests. Annu. Rev. Entomol. 58, 475–496.
Koh, C.Y., Kini, R.M., 2012. From snake venom toxins to therapeutics-cardiovascular examples. Toxicon 59, 497–506.
Koh, D.C.I., Armugam, A., Jeyaseelan, K., 2006. Snake venom components and their applications in biomedicine. Cell. Mol. Life Sci. 63, 3030–3041.
Kool, J., Giera, M., Irth, H., Niessen, W.M.A., 2011a. Advances in mass spectrometry-based post-column bioaffinity profiling of mixtures. Anal. Bioanal. Chem. 399, 2655–2668.
Kool, J., Jonker, N., Irth, H., Niessen, W.M.A., 2011b. Studying protein-protein affinity and immobilized ligand-protein affinity interactions using MS-based methods. Anal. Bioanal. Chem. 401, 1109–1125.
Laustsen, A.H., Lomonte, B., Lohse, B., Fernández, J., Gutiérrez, J.M., 2015. Unveiling the nature of black mamba (*Dendroaspis polylepis*) venom through venomics and anti-venom immunoprofiling: identification of key toxin targets for antivenom development. J. Proteom. 119, 126–142.
Le Du, M.H., Marchot, P., Bougis, P.E., Fontecilla-Camps, J.C., 1992. 1.9-A resolution structure of fasciculin 1, an anti-acetylcholinesterase toxin from green mamba snake venom. J. Biol. Chem. 267, 22122–22130.
Marchot, P., Khélif, A., Ji, Y.H., Mansuelle, P., Bougis, P.E., 1993. Binding of 125I-fasciculin to rat brain acetylcholinesterase. The complex still binds diisopropyl fluorophosphate. J. Biol. Chem. 268, 12458–12467.
Marszałł, M.P., Moaddel, R., Kole, S., Gandhari, M., Bernier, M., Wainer, I.W., 2008. Ligand and protein fishing with heat shock protein 90 coated magnetic beads. Anal. Chem. 80, 7571–7575.
Miao, Y., He, N., Zhu, J.-J., 2010. History and new developments of assays for cholinesterase activity and inhibition. Chem. Rev. 110, 5216–5234.
Miljanich, G.P., 2004. Ziconotide: neuronal calcium channel blocker for treating severe chronic pain. Curr. Med. Chem. 11, 3029–3040.
Moaddel, R., Wainer, I.W., 2009. The preparation and development of cellular membrane affinity chromatography columns. Nat. Protoc. 197–205.
Moaddel, R., Marszałł, M.P., Bighi, F., Yang, Q., Duan, X., Wainer, I.W., 2007. Automated ligand fishing using human serum albumin-coated magnetic beads. Anal. Chem. 79, 5414–5417.
Monge-Fuentes, V., Gomes, F.M., Campos, G.A., Silva, J.d.C., Biolchi, A.M., Dos Anjos, L.C., Gonçalves, J.C., Lopes, K.S., Mortari, M.R., 2012. Neuroactive compounds obtained from arthropod venoms as new therapeutic platforms for the treatment of neurological disorders. J. Venom. Anim. Toxins Incl. Trop. Dis. 21.
Norton, R.S., 2017. Enhancing the therapeutic potential of peptide toxins. Expert Opin. Drug Discov. 12, 6110623.
Otvos, R.A., Heus, F., Vonk, F.J., Halff, J., Bruyneel, B., Paliukhovich, I., Smit, A.B., Niessen, W.M., Kool, J., 2013. Analytical workflow for rapid screening and purification of bioactives from venom proteomes. Toxicon 76, 270–281.
Oujji, N.B., Bakas, I., Istamboulié, G., Ait-Ichou, I., Ait-Addi, E., Rouillon, R., Noguer, T., 2012. Acetylcholinesterase immobilized on magnetic beads for pesticides detection: application to olive oil analysis. Sensors-Basel 12, 7893–7904.
Petras, D., Heiss, P., Harrison, R.A., Süssmuth, R.D., Calvete, J.J., 2016. Top-down venomics of the East African green mamba, *Dendroaspis angusticeps*, and the black mamba, *Dendroaspis polylepis*, highlight the complexity of their toxin arsenals. J. Proteom. 146, 148–164.
Pochet, L., Heus, F., Jonker, N., Lingeman, H., Smit, A.B., Niessen, W.M.A., Kool, J., 2011. Online magnetic bead based dynamic protein affinity selection coupled to LC-MS for the screening of acetylcholine binding protein ligands. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 879, 1781–1788.
Qing, L.S., Xue, Y., Deng, W.L., Liao, X., Xu, X.M., Li, B.G., Liu, Y.M., 2011. Ligand fishing with functionalized magnetic nanoparticles coupled with mass spectrometry for herbal medicine analysis. Anal. Bioanal. Chem. 399, 1223–1231.
Radic, Z., Duran, R., Vellom, D.C., Li, Y., Cervenansky, C., Taylor, P., 1994. Site of fasciculin interaction with acetylcholinesterase. J. Biol. Chem. 269, 11233–11239.
Rodriguez-Ithurralde, D., Silveira, R., Barbeito, L., Dajas, F., 1983. Fasciculin, a powerful anticholinesterase polypeptide from *Dendroaspis angusticeps* venom. Neurochem. Int. 5, 267–274.
Saez, N.J., Senff, S., Jensen, J.E., Er, S.Y., Herzig, V., Rash, L.D., King, G.F., 2010. Spider-venom peptides as therapeutics. Toxins 2, 2851–2871.
Vanzolini, K.L., Jiang, Z.J., Zhang, X.Q., Vieira, L.C.C., Correa, A.G., Cardoso, C.L., Cass, Q.B., Moaddel, R., 2013. Acetylcholinesterase immobilized capillary reactors coupled to protein coated magnetic beads: a new tool for plant extract ligand screening. Talanta 116, 647–652.
Vanzolini, K.L., Vieira, L.C.C., Corrêa, A.G., Moaddel, R., Cass, Q.B., 2015. Acetylcholinesterase immobilized on modified magnetic beads as a tool for screening a compound library. Microchim. Acta 182, 2209–2213.
Vetter, I., Hodgson, W.C., Adams, D.J., McIntyre, P., 2015. Venoms-based drug discovery: bioassays, electrophysiology, high-throughout screens and target identification. In: King, G.F. (Ed.), Venoms to Drugs: Venom as a Source for the Development of Human Therapeutics. Royal Society of Chemistry, pp. 97–128.
Viljoen, C.C., Dawie, P.B., 1973. Snake venom toxins the purification and amino acid sequence of toxin FVII from *Dendroaspis angusticeps* venom. J. Biol. Chem. 248, 4915–4919.
Weller, M.G., 2012. A unifying review of bioassay-guided fractionation, effect-directed analysis and related techniques. Sensors-Basel 12, 9181–9209.
Wubshet, S.G., Brighente, I.M., Moaddel, R., Staerk, D., 2015. Magnetic ligand fishing as a targeting tool for HPLC-HRMS-SPE-NMR: α-glucosidase inhibitory ligands and alkylresorcinol glycosides from *Eugenia catharinae*. J. Nat. Prod. 78, 2657–2665.
Yasuda, M., Wilson, D.R., Fugmann, S.D., Moaddel, R., 2011. Synthesis and characterization of SIRT6 protein coated magnetic beads: identification of a novel inhibitor of SIRT6 deacetylase from medicinal plant extracts. Anal. Chem. 83, 7400–7407.
Zhu, Y.T., Ren, X.Y., Yuan, L., Liu, Y.M., Liang, J., Liao, X., 2015. Fast identification of lipase inhibitors in oolong tea by using lipase functionalised Fe3O4 magnetic nanoparticles coupled with UPLC-MS/MS. Food Chem. 173, 521–526.