Miniaturized bioactivity screening of complex samples

Heus, F.A.H.

2015

document version
Publisher's PDF, also known as Version of record

Chapter 4

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

Reka A. Otvos[1,2], Ferry Heus[1], Freek J. Vonk[3,5,6], Jenny Halff[1], Ben Bruyneel[1], Iryna Paliukhovich[2], August B. Smit[2], Wilfried M. A. Niessen[1,4] and Jeroen Kool[1]

(1) Division of BioMolecular Analysis, Department of Chemistry and Pharmaceutical Sciences, Faculty of Sciences, VU University Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands
(2) Department of Molecular and Cellular Neurobiology, Center for Neurogenomics and Cognitive Research, Neuroscience Campus, VU University Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands
(3) Institute of Biology, Leiden University, Sylvius Laboratory, Sylviusweg 72, 2333 BE Leiden, The Netherlands
(4) Hyphen MassSpec Consultancy, de Wetstraat 8, 2332 XT Leiden, The Netherlands
(5) School of Biological Sciences, University of Wales, Bangor L57 2UW, UK
(6) Naturalis Biodiversity Center, Darwinweg 2, 2333 CR Leiden, The Netherlands

Toxicon, 2013. 76:270

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.

Introduction

Great advances have been made in protein-based pharmaceuticals during the last ten years [33, 35–37]. 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 (2, 13, 19, 31,39, 41), protein engineering and production techniques (8, 12, 26, 45), as well as current knowledge and available technologies dealing with immunogenicity and immunotoxicity (3, 5, 18, 24, 30) 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 (25). 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 (38). 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 (44). 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 (16). 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 (28). 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 (9, 42). 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 (44). 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* (34)). 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 (7, 43). 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 (6, 14, 22, 40). During the last ten years, drug discovery directed

at the α7 nAChR experienced a leap forward by using the acetylcholine binding protein (40) 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 (4) and has been used as nAChR model since then [11, 15]. We recently developed an HRS approach using AChBP as target (27). Subsequently, we developed a miniaturized screening variant allowing low sample amounts to be screened (20) and used it in a follow up study to profile snake venoms for AChBP bioaffinity with parallel nano-LC–MS based determination of the accurate mass of the bioactive peptides (21). Although with help of the accurate masses of the bioactives found in combination with proteome database searches (i.e. Swissprot/Uniprot) known peptides could be identified, unknown peptides could not be identified further than via their accurate mass. Also, the screening process alone does not allow for further pharmacological studies and databases have to be available for the species screened.

This study introduces a new analytical workflow for the screening of bioactive peptides from venoms using miniaturized HRS followed by rapid purification of the identified bioactives by LC–MS guided purification. This is done in an effective and low sample amount consuming way. The workflow starts with the miniaturized HRS approach as reported before (21). With this miniaturized HRS system, bioaffinity profiling of a venom takes only 1–2 h and requires less than a mg of venom sample. The resulting accurate masses of the bioactive peptides found (by correlation with the parallel bioactivity signal) are used for LC–MS purification guided by these accurate masses. This allows for straightforward purification of the bioactive peptides, not guided by traditional bioactivity anymore, which renders the current workflow very rapid and straightforward. The collected bioactive peptides are then freeze-dried for conservation until further use. With the initial miniaturized HRS approach, the purified peptides are efficiently re-screened in order to validate that the correct bioactive peptide was collected and that it is still bioactive and pure. The purified and thus validated peptides can then be used for further characterization in pharmacological studies and for sequencing towards full structure elucidation (e.g., using proteomics approaches or Edman degradation). The analytical workflow is demonstrated with the venom of *Naja mossambica mossambica* (Mozambique spitting cobra) of the family of the Elapidae snakes.

Materials, methods and experimental

Chemical and biological reagents

The ULC–MS grade 99.97% acetonitrile (ACN), 99.95% trifluoroacetic acid (TFA) and 99.95% formic acid (FA) were obtained from Biosolve (Valkenswaard, the Netherlands). HPLC grade water was produced by a Milli-Q purification system from Millipore (Amsterdam, the Netherlands). Guanidine HCl, dithiothreitol (DTT), iodoacetic acid (IAA), α-cyano-4-hydroxycinnamic acid hippuryl-Arg, [Met^5]-enkephalin, NaCl, trizma base and human angiotensin I were supplied by Sigma–Aldrich (Zwijndrecht, The Netherlands). Mca-

Lys-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg-NH_2 was from Bachem (Bubendorf, Switzerland). Nicotine was obtained from Janssen Chimica (Beerse, Belgium). ELISA Blocking reagent and trypsin were purchased from Hoffmann-La-Roche (Mannheim, Germany). KH_2PO_4, Na_2HPO_4 and NH_4HCO_3 were obtained from Riedel-de-Haën (Seelze, Germany). NAP-5 columns were obtained from GE Healthcare (Buckinghamshire, United Kingdom). *L. stagnalis* acetylcholine binding protein (Ls-AChBP) was expressed from Baculovirus using the pFastbac I vector in Sf9 insect cells and purified as described by Celie et al. (6). The fluorescent tracer ligand DAHBA was synthesized in-house as described by [27].

Biochemical assays and samples

On-line AChBP assay
The on-line fluorescent enhancement assay for Ls-AChBP bioactivity profiling was performed as described by [20].

Snake venom samples
Cardiotoxin (#C9759) from *N. mossambica mossambica* was purchased from Sigma–Aldrich. Lyophilized venom from *N. mossambica mossambica* was acquired as described by [44]. Venom samples contained 10 mg/ml protein after preparation for analysis by dissolving the crude venoms in Milli-Q water/ACN/TFA 95:5:0.1%. This stock solution was stored at −20 °C.

Tryptic digestion
The purified and freeze-dried venom peptides were dissolved in 750 µl of 2.0 M Guanidine HCl (GHCl, pH = 8.5) denaturation buffer. After addition of 6 µl 0.05 mM DTT (50-fold molar excess), the solutions were incubated at 50 °C for 30 min. After cooling to room temperature, a 75-fold molar excess of IAA was added to the solutions, which were then incubated in the dark at room temperature for 30 min. After this incubation step, the samples were desalted with NAP-5-columns. The samples were subsequently freeze-dried with a Speed-Vac freeze dryer and the freeze-dried samples were re-dissolved in 50 µl of 50 mM ammonium bicarbonate buffer (pH = 8.4) and incubated with trypsin, added in a ratio of 100:1 w/w (protein:trypsin), for 24 h at 24 °C. After tryptic digestion, the trypsin was deactivated with FA using a final volume of 1% compared to the total volume. [Met^5]-Enkephalin was finally added as internal standard at a concentration of 2 µM. The samples were stored at −20 °C until use.

Radioligand displacement assay
Competition binding assays were performed with α7 nAChR expressing SH-SY5Y cells in buffer (PBS, 20 mM Tris, pH 7.4/0.05% Tween) with a filtration assay in a final volume of 100 µl. The cells were homogenized and sonicated prior use. The final concentration of the radioligand ^{3}H-methyllycaconitine (^{3}H-MLA) K_D = 1.81 nM, specific activity ~100 Ci/mmol) used in the assay was 2.0 nM. For the 3FTXs the average molecular weight of the peptides was assumed to be about 6000 Da. Based on this, the final concentration of the venom ligands in the assay was approximately 10^{-7}–10^{-14} M for the serial dilutions. Bound

radioligand was harvested on 0.3% polyethyleneimine-pretreated Unifilter-96 GF/C filters (Perkin–Elmer, Waltham, USA) and washed with ice cold 50 mM Tris–HCl buffer (pH 7.4). Next, 25 μl/well scintillation liquid (MicroScint, Perkin Elmer) was added to the dried filters and radioactivity was measured with a 300 min delay time using a Wallac 1450 MicroBeta liquid scintillation counter (Perkin—Elmer). To determine non-specific binding, radioligand saturation experiments were performed with 10 mM nicotine. Binding assay data was analysed using Prism 5.0 (Graphpad Software, Inc. California, USA).

2.3. Instrumentation

On-line screening
The analytical system used for on-line screening was recently described by our group [21]. In brief, this system consists of an Ultimate nano-LC system with Famos autosampler (LC Packings, Amsterdam, the Netherlands) with a post-column 1:1 split to a Shimadzu ('s Hertogenbosch, The Netherlands) ion-trap–time-of-flight mass spectrometer equipped with a Picoview nano-Electrospray ionization source and to a bioaffinity detection system consisting of a microfluidic chip connected to a microfluidic LED Induced Fluorescence detector.

MS guided LC purification
Purification of the bioactive peptides was performed with MS guided LC fractionation (Fig. 1B). 50 μl sample was injected with a Gilson 234 autosampler. The eluent flow of 0.5 mL/min was delivered by a binary Shimadzu LC-10a pump system. For the separation of the peptides, a normal-bore LC-column (4.6 mm × 100 mm, particle size 3.5 μm, Symmetry Shield; Waters, Etten-Leur, the Netherlands) was used. After the analytical column, a 1:9 split was inserted resulting in 10% of the flow being directed to the MS.

After the split and prior to fraction collection, a Shimadzu SPD-20A UV detector at 220 nm was installed. The collected fractions were subsequently lyophilized with a SpeedVac freeze-dryer at room temperature. The freeze-dried fractions were stored at −20 C before further use.

To the 10% eluent split to MS, a 250 μl/min make-up flow of 50%/50% H_2O/MeOH with 0.1% FA was mixed in using a Shimadzu LC-10a pump to obtain favourable ESI flow rates and solvent composition. The MS detection was performed in positive-ion mode with a Q-TOF-2™ instrument (Micromass, Manchester, UK). Operating conditions were: 398 K source temperature, 573 K desolvation temperature, 250 L/h desolvation gas flow, 50 L/h cone gas flow, 17 psi gas cell pressure, 20 V collision voltage for optimum transfer through collision cell, 3500 V capillary voltage, and 30 V cone voltage. The mass range was *m*/*z* 50 to 2000. The data acquisition parameters were 1.0 s $scan^{-1}$, 0.1 s dwell time, full TOF MS continuous scan mode using the option ‘MS profile’. Nitrogen (purity 5.0; Praxair, Oevel, Belgium) and argon (purity 5.0; Praxair) were used as desolvation/cone gas and collision gas, respectively.

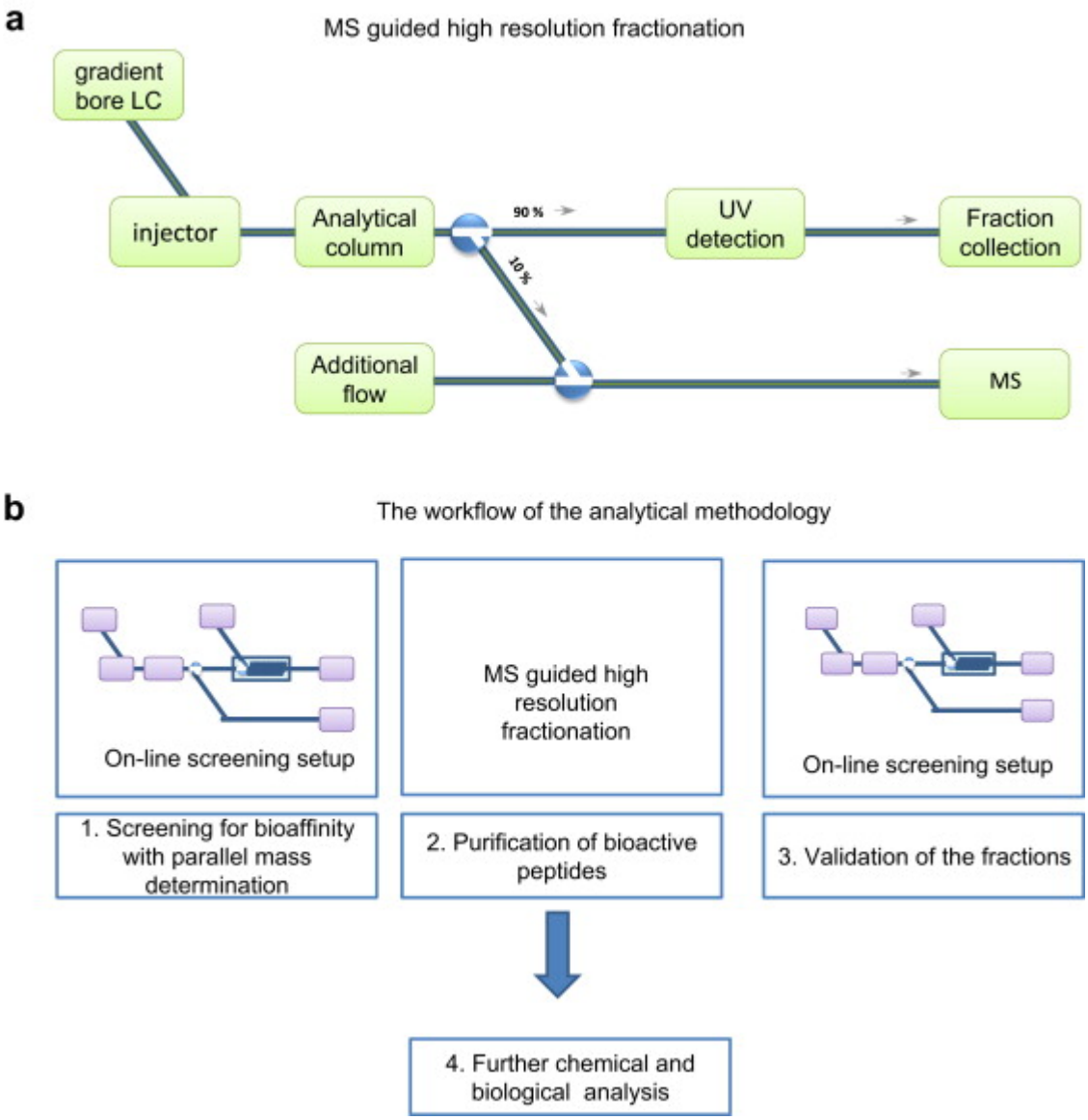


Figure 1. (A) Schematic view of the MS guided LC fractionation. The venom is injected (1′) and separated (2′) with a normal-bore HPLC system. The flow was split post-column in 9:1 ratio, where 10% of the flow was directed to the MS (3′) and 90% of the flow was directed to a UV detector (4′) followed by fraction collection (5′). (B) The complete workflow of the analytical methodology for screening and purifying of bioactives from complex mixtures. Venoms first are screened with the on-line setup for identifying new bioactives. With support of the on-line screening data the bioactive peptides are subsequently purified with MS guided LC fractionation in order to collect the bioactive peptides of interest only. The purified peptides are rescreened with the on-line screening setup to validate that the peptides maintained their original bioaffinity.

Preparative LC-UV guided purification

For further upscaling towards purification of larger amounts of bioactive peptides, UV data was also measured at 220 nm prior to MS analysis. The LC-UV data obtained can directly be transferred to semi-preparative LC-UV peptide purification using UV detection only. The preparative LC system consisted of two Shimadzu LC-10 Ai pumps, a Li ChoChart preparative LC column (250 mm × 10 mm i.d.) packed with 5 µm size Purospher Star RP particles (Merck KGaA, Darmstadt, Germany), operated at 2.5 mL/min with the same gradient program as for the on-line screening system, and a 759A Absorbance Detector

(Applied Biosystems) operated at 220 nm. For these ‘large bore’ LC purifications, 2.5 mg venom was injected. Sample collection after semi-preparative LC was done in 50 mM NH_4HCO_3 buffer (volume ratio of at least 1:3 as compared to collected eluent) in order to prevent peptide denaturation resulting from the composition of the eluent. By subsequent freeze-drying, this volatile buffer was also efficiently removed from the collected fractions. Part of the lyophilized fractions were re-dissolved in water/ACN/TFA 95:5:0.1% and injected in the on-line screening system to check for purity and affinity.

MS peptide sequencing
Peptide sequence analyses were performed with an AB Sciex MALDI TOF/TOF 5800 mass spectrometer (Nieuwerkerk aan den IJssel, The Netherlands.). The 1 µl matrix spot consisted of α-Cyano-4-hydroxycinnamic acid (6 mg/ml) dissolved in ACN:H_2O:TFA (70:30:0.1), diluted 1:1 with the sample before analysis. The MALDI MS instrument was operated in positive-ion mode in the mass range between *m*/*z* 200 and 3100. In MS1 mode, the total number of shots/spectrum was 1500 (250 shots/sub spectrum). MS/MS data were then collected utilizing the positive ion mode for the predominant peaks observed in the MS spectrum. Typically at MS/MS analysis 3000 laser shots (or more if needed for adequate signal to noise) were collected and averaged for each spectrum.

Results and discussion

This paper describes an analytical workflow for bioaffinity screening followed by targeted purification of bioactive venom peptides binding to the Ls-AChBP (Fig. 1C). The workflow is demonstrated on the venom of the snake *N. mossambica mossambica*. We recently published this on-line high-resolution screening (HRS) of neurotoxic snake venoms [21]. After screening the *N. mossambica mossambica* venom for bioactives, the bioactive peptides are purified with MS guided LC purification. The collected fractions are freeze-dried in order to protect the bioactive peptides from degradation in the liquid phase before further use. The lyophilized fractions are then rescreened with the on-line screening setup in order to validate that the correct fractions were collected and the bioaffinity of the peptides is retained after fractionation and lyophilization. We demonstrate that with our workflow bioactive peptides can be rapidly purified from complex venom mixtures. These purified peptides are finally used for further chemical and biological studies. Amino-acid sequencing of the peptides was performed by tryptic digestion and MALDI-MS and MS/MS detection. The crude venom and the purified peptides were finally tested for affinity towards the α7 nicotinic acetylcholine receptor with a radioligand binding assay.

Evaluation of the on-line screening system with commercially available toxins

The data analysis of the mass spectra and the on-line screening results was performed as described previously [21]. The selectivity of the on-line screening system was first demonstrated with commercially available snake toxins of which some were known and some were previously not known ligands of the α7 nAChR. The bioaffinity profiling of α-bungarotoxin, α-cobratoxin, erabutoxin, α-conotoxin, muscarinic toxin 2 and PLA_2 towards Ls-AChBP was recently demonstrated [21]. For further evaluation of the on-line screening system, one additional commercially available toxin, related to the venom analysed in this work, was screened with the on-line screening system, namely a 'cardiotoxin' purified from *Naja mossambica* venom (Sigma #C9759). The bioactivity profile of 500 ng 'cardiotoxin' injected is depicted in Figure 2. This figure shows that the sample consists of multiple peptides of which the identified nominal masses correlated with the masses of peptides P01452, P01469, P01470, P25517 and P01467 (deduced from Swissprot/Uniprot), respectively. These peptides are known as cytotoxins or cardiotoxins. Figure 2 shows that two of these toxins, P01469 (Cytotoxin 2) and P01467 (Cytotoxin 1) display affinity towards Ls-AChBP.

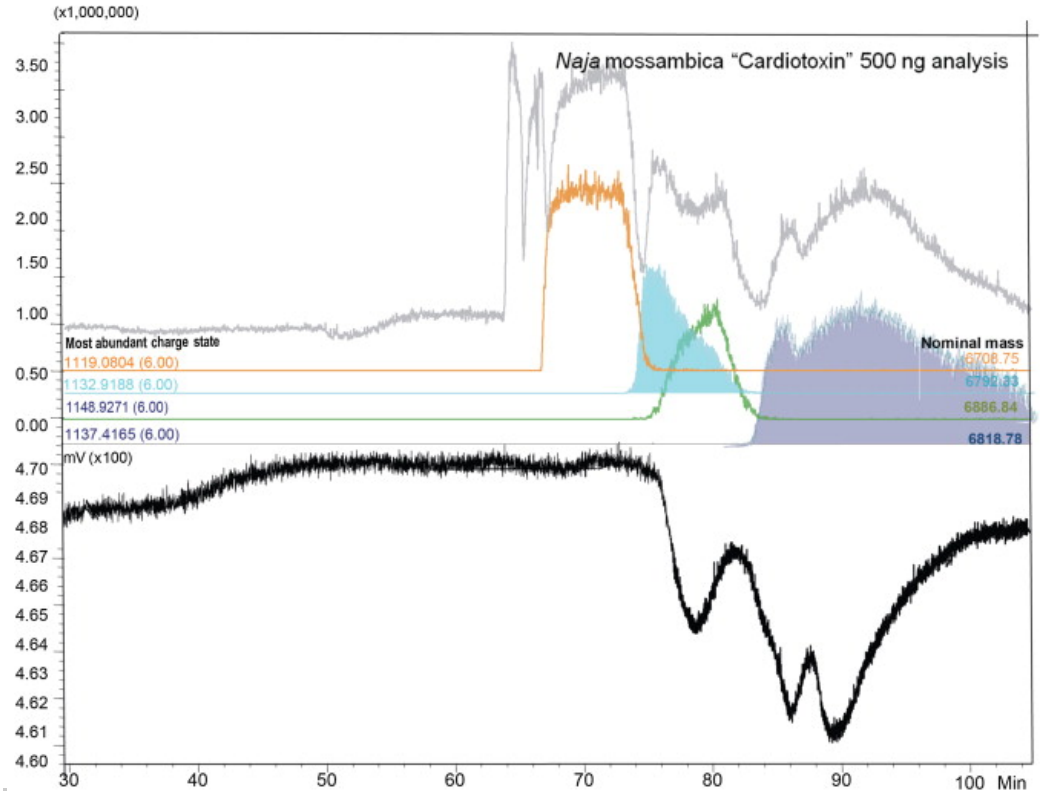


Figure 2. The selectivity of the on-line screening setup was evaluated by us recently [21]. To extend this evaluation, commercially available cardiotoxin ('cardiotoxin' purified from the venom of *Naja mossambica mossambica* by Sigma) was screened by the on-line screening. Figure 2 shows the bioaffinity signal and corresponding MS data (EICs and TIC) of 500 ng 'cardiotoxin' from Sigma. This toxin appeared to consist of a mixture of at least 4 different cardiotoxins present. Two of the cardiotoxins, P01469 (Cytotoxin 2) and P01467 (Cytotoxin 1), showed affinity towards the Ls-AChBP. This result correlates well with the on-line screening result of the crude *Naja mossambica mossambica* venom, where the same cardiotoxins were binding to the Ls-AChBP (see Figure 3 and main text).

Screening for bioactives in the venom of *N. mossambica*

The analysis of *N. mossambica* venom was performed for assessment of neurotoxic ligands with the on-line screening system, effectively narrowing down the number of probable bioactives. The venom of *N. mossambica mossambica* is known to contain phospholipases (Uniprot #P00602, P00603, P00604), cytotoxins (Uniprot #P01467, P01469, P01470, P01452, P25517) and short type neurotoxins (Uniprot #P01431, P01432). These known peptides of the *N. mossambica mossambica* venom were not known before to affect the nAChR.

When correlating the extracted ion chromatograms (EICs) of eluting peptides of a 5 µg *N. mossambica* venom analysis with the on-line screening system, two high affinity, and a low affinity ligands of the Ls-AChBP were detected (see Figure 3A at 55, 58 and 65 min). Their calculated masses correlate with the masses of peptides P01469 (Cytotoxin 2), P01470 (Cytotoxin 3), and P01467 (Cytotoxin 1) (deduced from Swissprot/Uniprot), and are indicated in Figure 3A together with the on-line bioaffinity data. These toxins have been classified by Fry et al. by homology to the group Type IA cytotoxins, a group of cardiotoxic and cytotoxic toxins. Interestingly, these particular toxins have also been classified by Fry et al. as forming a clade separated from the Type IA cytotoxins, and also separated from other African cobra species because of their amino-acid sequence divergence level. Furthermore, Fry et al. suggested comparative assaying to determine differences in potency or specificity between these toxins and the Type IA group of cytotoxins. Indeed, this proved to be a valid suggestion as these toxins showed relatively high affinities to the model Ls-AChBP target and might thus also be neurotoxic. This, to our knowledge, has not been reported before. Potentially, these toxins, together with highly homologous toxins, such as P01468 from *Naja pallida*, could form a new group of so-called Type IV α-neurotoxins because of their affinity/functionality, but being distinctly different in their sequence than, for instance, the Type I α-neurotoxins. So far, predominantly 3Ftx-type cardiotoxins have been reported in comprehensive venomics studies on the *N. mossambica* venom [32]. In addition, two Type I α1 nAChR ligands have been reported (NMM I and NMM III), one of which was also observed in our analyses. According to Ackermann and Taylor [1, 29], this peptide (‘NMM I’ or ‘P01431’; 62 amino acids; (reduced) molar mass 7081 Da; most abundant charge state *m*/*z* 1415.635) binds exclusively to muscle type α1 nAChRs. In our set-up, this peptide did not show bioaffinity towards the Ls-AChBP (Fig. 3A).

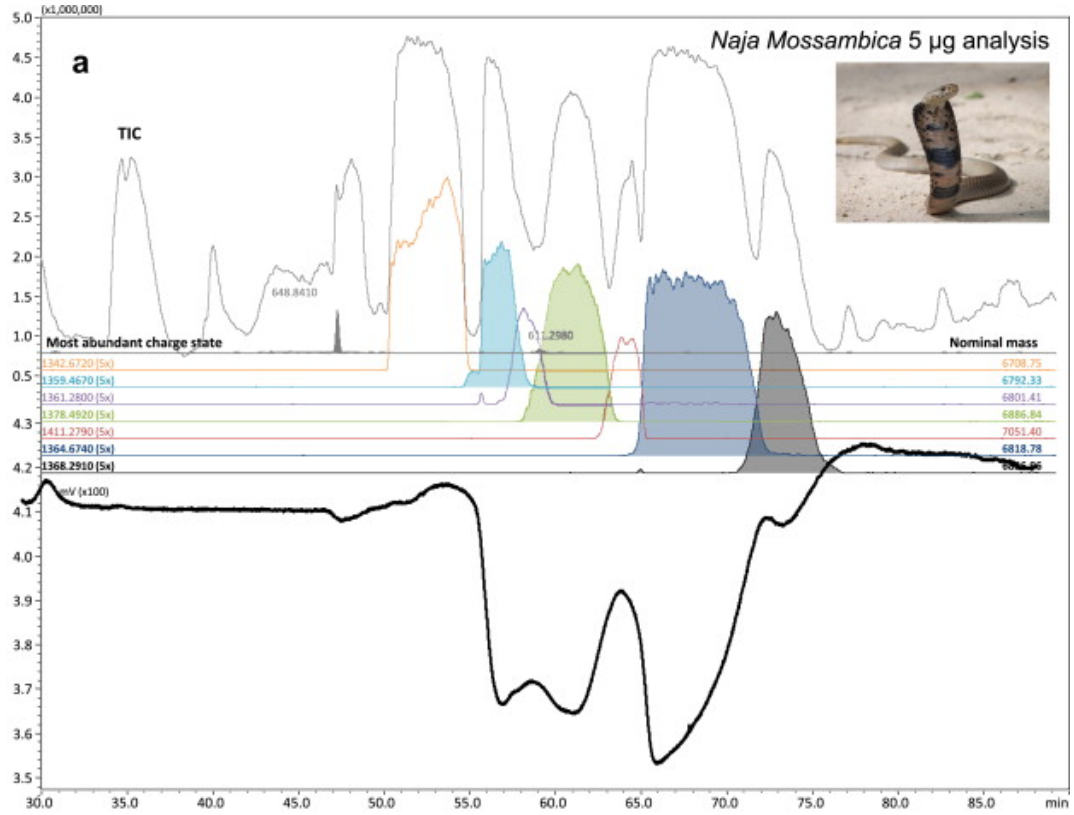
Initially, 5 µg of venom was injected for analysis to observe low-affinity and/or low-abundant binders as well as to provide sufficient sensitivity in MS. This, however, resulted in severe peak broadening for the high-affinity and/or high-abundant bioactive peptides (Fig. 3A). Therefore, subsequently, lower venom concentrations were injected and analysed. In this case, sharper peaks were observed and correlations were more straightforward, which assisted in the elucidation process (Fig. 3B). In principle, co-elution of very low-abundant and highly bioactive peptides could in some cases cause one of the signals observed in the on-line screening assay. In order to rule out this possibility as much as possible, the selectivity of the reversed-phase LC separation was modified by changing the eluent pH from pH 2.5 to pH 7.5.

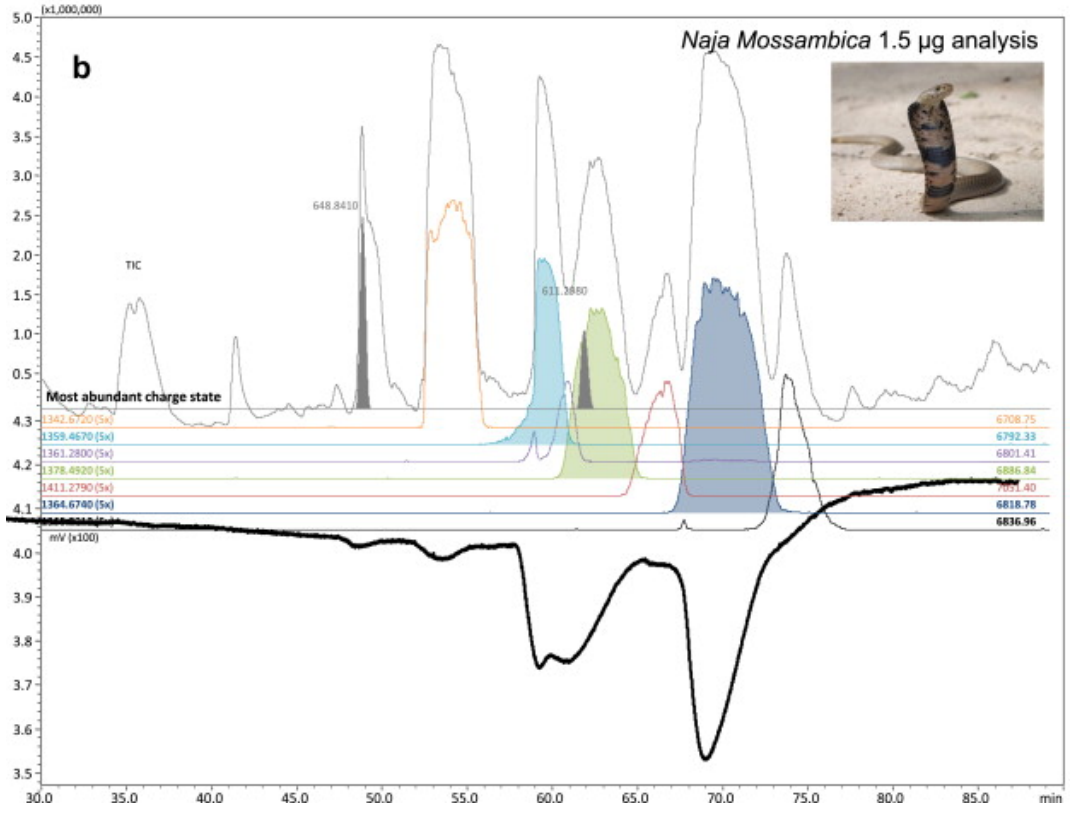
Differences in iso-electric points, the degree of protonation, and therefore the polarity of the toxins will have a significant effect on the selectivity and the separation efficiency in terms of tailing and peak broadening. As an example, Figure 3C shows that the elution order of Cytotoxin 2, Cytotoxin 3, Cytotoxin 1 (at pH 2.5) shifted to Cytotoxin 2, Cytotoxin 1, Cytotoxin 3. As the elution order change simultaneously affects both the MS and biochemical traces, correlation is still possible, but now with other co-eluting peptides. As an additional result, another mildly bioactive peptide could be detected (peptide 1) with a mass of 15,186 Da, which is in the mass range of some reported elapid PLA2s.

Purification of bioactive peptides from the *N. mossambica* venom

As the bioactive peptides are in a complex mixture, purification of the bioactives is of great importance to perform further structural elucidation and/or biological studies. In this study, three bioactive peptides from the venom of *N. mossambica mossambica* were purified with MS guided fractionation. The MS guided fractionation was performed with a conventional bore analytical column LC-UV coupled with mass spectrometry detection in order to collect the bioactive fractions based on their masses identified. The setup built for the MS guided fractionation is depicted in Figure 1B. After the analytical column, the flow was split in a 90:10 ratio, where 90% of the flow was directed to UV detection and fraction collection, and the 10% to the MS. With this analytical setup, Cytotoxin 2, Cytotoxin 3 and Cytotoxin 1 were successfully purified in a straightforward manner as deduced from reinjection in the on-line screening system (see Section “Rescreening for purified peptides” and Figure 4A–C) The collected fractions were freeze-dried in order to protect the peptides from degradation in liquid phase.

For obtaining sufficient amounts of bioactive peptides for further analysis, repeated injections and fraction collections were performed. As this is rather time-consuming, it was decided to subsequently up-scale the fractionation to semi-preparative scale LC (10 mm i.d. column, 2.5 mL/min) based on UV detection. The bioactives identified by LC–MS were easily correlated to the corresponding UV chromatogram and were then translated to the semi-preparative LC-UV fractionation. Using semi-preparative LC fractionation, one injection was sufficient to collect sufficient quantities of venom peptides. During semi-preparative-LC fractionation, the samples were collected in NH_4HCO_3 buffer in order to prevent possible peptide denaturation due to the LC eluents used.


a
Naja Mossambica 5 µg analysis
TIC
648.8410
611.2980
Most abundant charge state
Nominal mass
1342.6720 (5x)
1359.4670 (5x)
1361.2800 (5x)
1378.4920 (5x)
1411.2790 (5x)
1364.6740 (5x)
1368.2910 (5x)
6708.75
6792.33
6801.41
6886.84
7051.40
6818.78
mV (x100)
(x1,000,000)
min


b
Naja Mossambica 1.5 µg analysis
TIC
648.8410
611.2880
Most abundant charge state
1342.6720 (5x)
1359.4670 (5x)
1361.2800 (5x)
1378.4920 (5x)
1411.2790 (5x)
1364.6740 (5x)
6708.75
6792.33
6801.41
6886.84
6818.78
6836.96
mV (x100)
(x1,000,000)
min

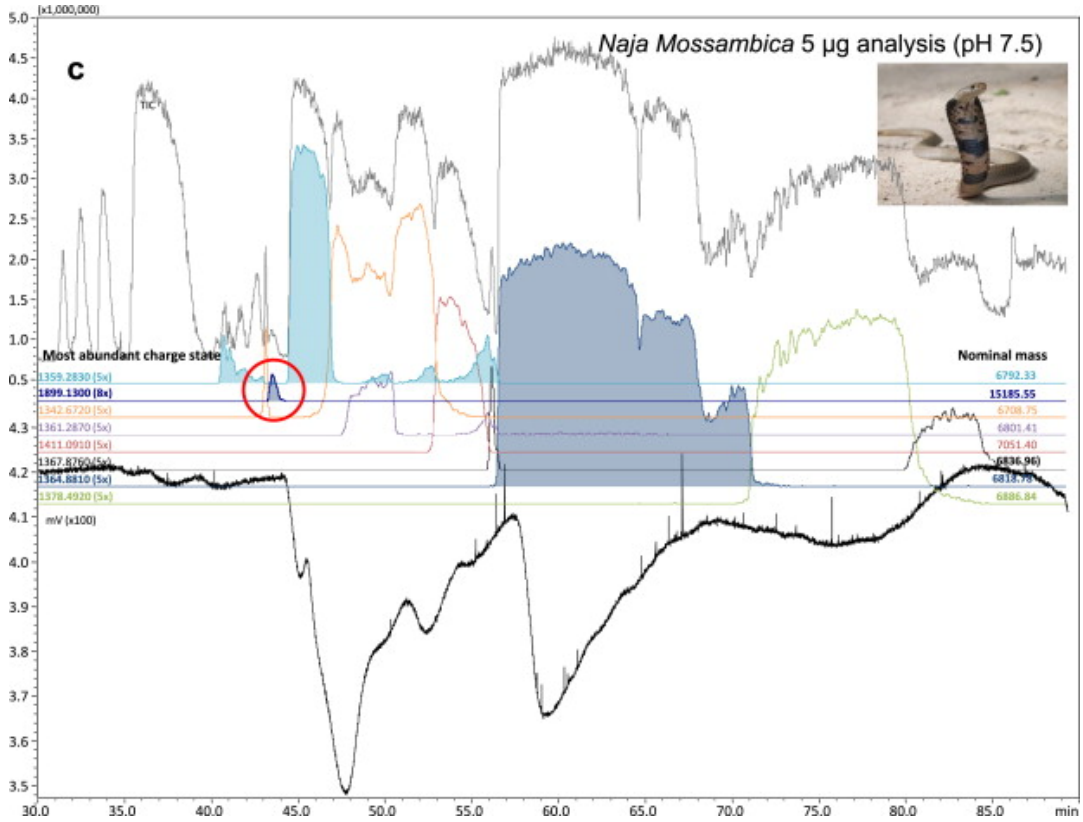


Figure 3. A typical on-line screening result obtained from injection of a 5 µg *Naja mossambica mossambica* venom sample is depicted (a). The bioaffinity and the corresponding MS data show three major bioactive peptides in the venom. The venom analysis was repeated with 1.5 µg venom injected (b) to provide better chromatographic peak shapes enabling a more straightforward correlation with MS and the bioaffinity data. (c) shows the on-line screening result of 5 µg venom measured using eluent at pH 7.5 instead of pH 2.5. Due to differences in iso-electric points, the degree of protonation and the polarity of the toxins, a significant effect on the selectivity and the separation efficiency is to be expected. Indeed, the elution order P01469, P01470, P01467 (at pH 2.5) shifted to P01469, P01467, P01470. As an additional result, another mildly bioactive peptide was detected (*peptide 1*) with a mass of 15,186 Da, which is in the range of some reported elapid PLA_2s.

Rescreening for purified peptides

The purified and freeze-dried peptide fractions were dissolved in the same solvent mixture as the full venom and the fractions were rescreened with the on-line system to validate that the fractions retained their bioaffinity towards Ls-AChBP. The results of the on-line screening of purified Cytotoxins 2, 3 and 1 are shown in Figure 4A–C, respectively. These figures show that the peptides were successfully purified and that they kept their biological activity after separation and freeze-drying. The rescreening of Cytotoxin 3 (Fig. 4B) confirmed that Cytotoxin 3 is a low-affinity binder showing a shoulder peak on the closely eluting high-affinity binder Cytotoxin 2. This result confirms the on-line screening result of the commercial ‘cardiotoxin’ too, where Cytotoxin 2 and Cytotoxin 1 showed affinity to the AChBP, but not Cytotoxin 3 (Fig. 2). Rescreening of the purified bioactive peptides collected in buffer was also compared with purified peptides that were not collected in buffer. In this case, no significant differences were found in the bioaffinity and the amount of peptide purified (data not shown). This indicates that Cytotoxin 1–3 peptides have a rigid structure and do not readily degrade during the purification process. Probably, a high number of

cysteine bridges aids in the stability of these peptides. The sample collection in NH_4HCO_3 buffer however can be a good tool to protect other less stable peptides and proteins that are sensitive to higher organic solvent concentrations (eluent).

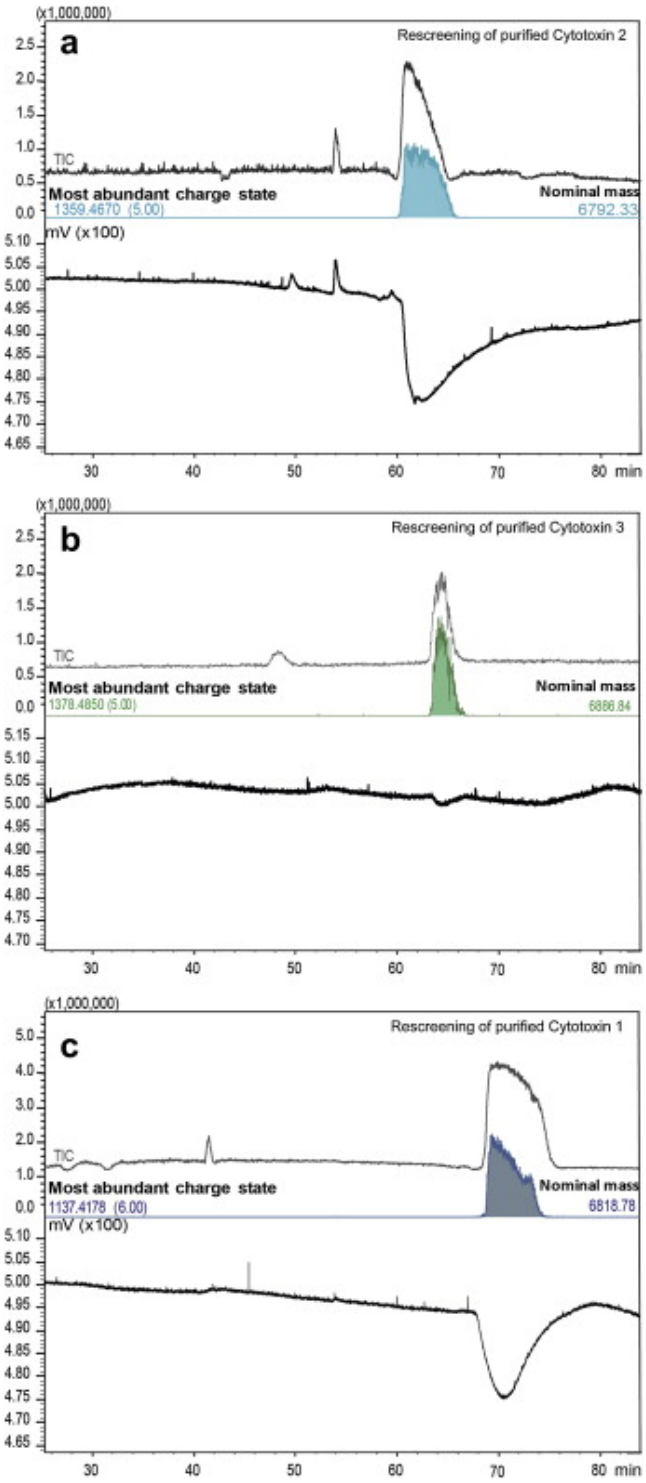


Figure 4. This figure shows the result of the rescreening of the purified peptides using the on-line screening setup. A–C, show the on-line screening result of the purified P01469, P01470 and P01467, respectively. The rescreening validated that the peptides are purified and their bioaffinity remained.

MALDI MS analysis for sequencing of bioactive peptides

Efficient bioaffinity detection followed by fast and straightforward purification of the bioactives found is demonstrated to be possible with our workflow. This enables convenient subsequent biological and chemical experiments to be performed with the purified peptides from venoms. To show this, two high-affinity peptides purified have been sequenced with a MALDI MS based proteomics approach. The purified peptides sequenced correlated by mass in Uniprot to Cytotoxin 2 and Cytotoxin 1. Tryptic digestion was performed on these fractions, followed by MALDI MS analysis of the resulting peptide mixtures. Typical MS spectra obtained from the analyses of Cytotoxin 2 and Cytotoxin 1 are depicted in Figure 5A and 5B. The data analysis was performed by *in-silico* digestion by PeptideMass software (ExPASy Bioinformatics Resource Portal, www.expasy.org) and afterwards by manual identification of the tryptic peptide fragments. The sequence coverage of Cytotoxin 2 was 73% and for Cytotoxin 1, 83%. The *m*/*z*-values of the identified tryptic peptides are highlighted in Figure 5A and 5B; a list of identified *m*/*z*-values and the correlating peptide sequence is shown in Supplementary material Table 1. Since the peptide sequences of Cytotoxin 2 and 1 are very similar, we had to pay special attention to showing the difference between the two purified toxins (Fig. 5C). Cytotoxin 1 and 2 differ only by four amino acids at position 28–31 with AAPM in Cytotoxin 1 and GASK in Cytotoxin 2. The differentiation was achieved by MS/MS sequencing of the tryptic peptide AAPMVPVKR (position 28–35) of Cytotoxin 1, which is not found in Cytotoxin 2. The sequence was confirmed by MS/MS fragmentation as shown in Figure 5C.

Radioligand displacement assay

In the on-line screening setup, the venoms were screened for bioaffinity for AChBP, which is a homologue of the binding site of the α7 nAChR. Although there is a good structural similarity between the AChBP and the α7 nAchR [10], determining the actual binding affinity for the α7 nAChR is the eventual goal. Therefore, we assessed binding affinity for human α7 nAChR by analysis of a concentration–response curves. The [3H]methyllycaconitine radioligand displacement assay was performed with membranes of α7 nAChR expressing SH-SY5 neuroblastoma cells. The radioligand displacement assay was first performed with crude venom of the *N. mossambica mossambica* and showed low affinity binding (Fig. S1A). When the purified Cytotoxin 1 and Cytotoxin 2 were tested, low binding affinity was only observed for Cytotoxin 1, which was also the highest affinity binder observed during the miniaturized screening experiments (Fig. S1B). These radioligand binding experiments showed that Cytotoxin 1 is a low affinity binder to the α7 nAChR receptor, although only a partial displacement curve was measured due to the limited quantity that could be purified in our fast complete workflow. However, the radioligand binding assay did show translational binding from AChBP to the α7 nAchR.

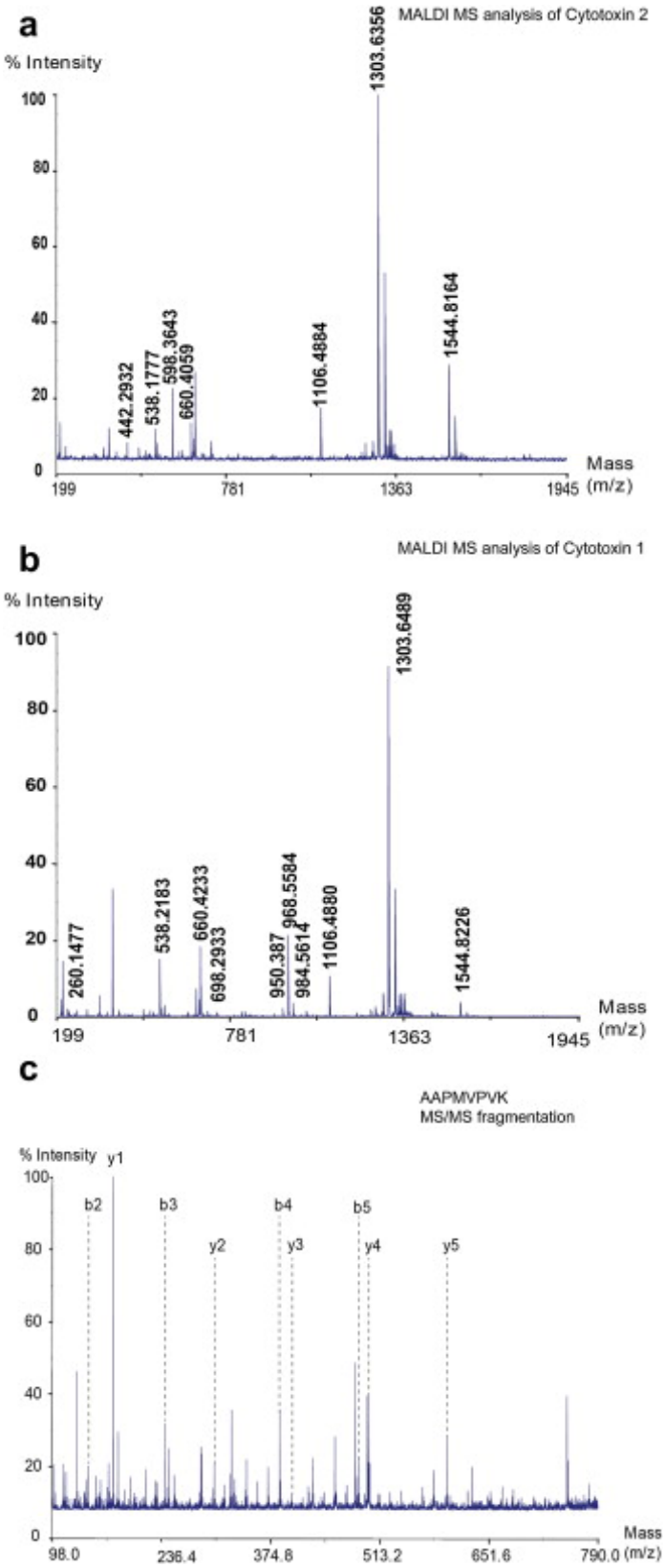


Figure 5. MALDI MS analysis of purified P01469 (A) and P01467 (B) after tryptic digestion. As there is almost complete sequence similarity between P01469 and P01467, most tryptic peptides identified were the same. The only main difference between the two bioactive peptides is that tryptic peptide AAPMVPVKR (*m*/*z* 968,5709) is present in P01467, but not present in P01469, as it is shown in A and B. C shows the sequence analysis of AAPMVPVKR after MS/MS fragmentation.

Conclusion

This study demonstrates that our innovative analytical workflow enables bioactivity assessment of peptides in complex snake venom samples and subsequently allows rapid and effective identification and subsequently purification for further analysis. The workflow was demonstrated with the venom of the Mozambique spitting cobra, *N. mossambica mossambica*. The venom was screened for bioactive peptides with the hyphenated nano-LC on-line coupled post-column to a microfluidic biochemical detection system equipped with a confocal fluorescence detector and to mass spectrometry to correlate bioactivity with identity. With support of the on-line screening data, the bioactive peptides in the venom were subsequently purified with MS guided LC fractionation in order to collect the bioactive peptides of interest only. With this knowledge, also convenient transfer to semi-preparative UV based preparative LC purification was demonstrated and allowed purification of larger peptide amounts, when available. The purified peptides were freeze-dried for keeping them stable for further analysis. These peptides were rescreened with the on-line screening setup in order to validate that the peptides maintained their original bioaffinity. After this validation, the freeze-dried bioactive peptides were digested with trypsin and analysed with MS to confirm their amino acid sequence.

All in all, medium affinity ligands of the Ls-AChBP were identified. These bioactive peptides were correlated to the masses of P01469 (Cytotoxin 2) and P01467 (Cytotoxin 1), deduced from Swissprot/Uniprot. It was found that Cytotoxin 3 was a low-affinity binder co-eluting with Cytotoxin 1. The amino-acid sequence of the two medium affinity ligands were elucidated by MALDI MS analysis, confirming their identity as Cytotoxin 1 and Cytotoxin 2 with 83% and 73% sequence coverage, respectively. Although these toxins were classified as cytotoxins, we showed binding to Ls-AChBP, which was not reported before in literature.

The methodology described can have a broad applicability in drug research from venoms. Numerous non-miniaturized on-line screening platforms exist which can be miniaturized as our platform. The on-line screening system described by [21] and in the current manuscript is in fact the miniaturized version of the screening platform initially described by [27]. Our group developed many on-line screening platforms in the past (many of these are described in a recent review by Kool et al. [28] using various molecular targets, and we are convinced that in most cases transfer to the miniaturized screening platform is feasible.

Acknowledgements

The work of Reka A. Otvos was supported by the AIMMS Bridging PhD project “Identification of novel bioactive substances on brain receptors” (project number 10-001-203). August B. Smit was partially funded by a grant from the European Union Seventh Framework Programme under grant agreement n° Health F2 2007 202088 (Structure, function and disease of Cys loop receptors, “NeuroCypres” project). We would like to thank Dr. Neil Loftus from Shimadzu for the collaboration and use of the Shimadzu nano-ESI source. We

would like to thank René van Elk for the preparation of AChBP. Ka Wan Li is acknowledged for helping with analysis of MALDI MS data.

References

1. Ackermann, E.J., Taylor, P., 1997. Nonidentity of the alpha-neurotoxin binding sites on the nicotinic acetylcholine receptor revealed by modification in alpha-neurotoxin and receptor structures. Biochemistry 36, 12836–12844.
2. Bailon, P., Won, C.Y., 2009. PEG-modified biopharmaceuticals. Expert Opin. Drug Deliv. 6, 1–16.
3. Bhogal, N., 2010. Immunotoxicity and immunogenicity of biopharmaceuticals: design concepts and safety assessment. Curr. Drug Saf. 5, 293–307.
4. Brejc, K., van Dijk, W.J., Klaassen, R.V., Schuurmans, M., van der Oost, J., Smit, A.B., Sixma, T.K., 2001. Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. Nature 411, 269–276.
5. Bussiere, J.L., Martin, P., Horner, M., Couch, J., Flaherty, M., Andrews, L., Beyer, J., Horvath, C., 2009. Alternative strategies for toxicity testing of species-specific biopharmaceuticals. Int. J. Toxicol. 28, 230–253.
6. Celie, P.H., Kasheverov, I.E., Mordvintsev, D.Y., Hogg, R.C., van Nierop, P., van Elk, R., van Rossum-Fikkert, S.E., Zhmak, M.N., Bertrand, D., Tsetlin, V., Sixma, T.K., Smit, A.B., 2005. Crystal structure of nicotinic acetylcholine receptor homolog AChBP in complex with an alphaconotoxin PnIA variant. Nat. Struct. Mol. Biol. 12, 582–588.
7. Chu, N.S., 2005. Contribution of a snake venom toxin to myasthenia gravis: the discovery of alpha-bungarotoxin in Taiwan. J. Hist. Neurosci. 14, 138–148.
8. Daniell, H., Singh, N.D., Mason, H., Streatfield, S.J., 2009. Plant-made vaccine antigens and biopharmaceuticals. Trends Plant Sci. 14, 669– 679.
9. de Jong, C.F., Derks, R.J., Bruyneel, B., Niessen, W., Irth, H., 2006. High-performance liquid chromatography–mass spectrometry-based acetylcholinesterase assay for the screening of inhibitors in natural extracts. J. Chromatogr. A 1112, 303–310.
10. de Kloe, G.E.J., van Elk, R., van Muijlwijk-Koezen, J.E., Smit, A.B., Lingeman, H., Irth, H., Niessen, W.M.A., de Esch, I.J.P., 2011. Online parallel fragment screening and rapid hit exploration for nicotinic acetylcholine receptors. MedChemComm 2, 590–595.
11. de Kloe, G.E., Retra, K., Geitmann, M., Kallblad, P., Nahar, T., van Elk, R., Smit, A.B., van Muijlwijk-Koezen, J.E., Leurs, R., Irth, H., Danielson, U.H., de Esch, I.J., 2010. Surface plasmon resonance biosensor based fragment screening using acetylcholine binding protein identifies ligand efficiency hot spots (LE hot spots) by deconstruction of nicotinic acetylcholine receptor alpha7 ligands. J. Med. Chem. 53, 7192–7201.
12. Decker, E.L., Reski, R., 2008. Current achievements in the production of complex biopharmaceuticals with moss bioreactors. Bioproc. Biosyst. Eng. 31, 3–9.
13. Dinarvand, R., Dorkoosh, F., Hamidi, M., Moghadam, S.H., 2004. Polymeric delivery systems for biopharmaceuticals. Biotechnol. Genet. Eng. Rev. 21, 147–181.
14. Dutertre, S., Ulens, C., Buttner, R., Fish, A., van Elk, R., Kendel, Y., Hopping, G., Alewood, P.F., Schroeder, C., Nicke, A., Smit, A.B., Sixma, T.K., Lewis, R.J., 2007. AChBP-targeted alpha-conotoxin correlates distinct binding orientations with nAChR subtype selectivity. EMBO J. 26, 3858–3867.
15. Edink, E., Rucktooa, P., Retra, K., Akdemir, A., Nahar, T., Zuiderveld, O., van Elk, R., Janssen, E., van Nierop, P., van Muijlwijk-Koezen, J., Smit, A.B., Sixma, T.K., Leurs, R., de Esch, I.J., 2011. Fragment growing induces conformational changes in acetylcholine-binding protein: a structural and thermodynamic analysis. J. Am. Chem. Soc. 133, 5363–5371.
16. Fox, J.W., Serrano, S.M., 2008. Exploring snake venom proteomes: multifaceted analyses for complex toxin mixtures. Proteomics 8, 909– 920.
17. Fry, B.G., Wuster, W., Kini, R.M., Brusic, V., Khan, A., Venkataraman, D., Rooney, A.P., 2003. Molecular evolution and phylogeny of elapid snake venom three-finger toxins. J. Mol. Evol. 57, 110–129.
18. Giezen, T.J., Mantel-Teeuwisse, A.K., Leufkens, H.G., 2009. Pharmacovigilance of biopharmaceuticals: challenges remain. Drug Saf. 32, 811–817.
19. Hamidi, M., Zarrin, A., Foroozesh, M., Mohammadi-Samani, S., 2007. Applications of carrier erythrocytes in delivery of biopharmaceuticals. J. Control. Release 118, 145–160.
20. Heus, F., Giera, M., de Kloe, G.E., van Iperen, D., Buijs, J., Nahar, T.T., Smit, A.B., Lingeman, H., de Esch, I.J., Niessen, W.M., Irth, H., Kool, J., 2010. Development of a microfluidic confocal fluorescence detection system for the hyphenation of nano-LC to on-line biochemical assays. Anal. Bioanal. Chem. 398, 3023–3032.

21. 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 61, 112–124.
22. Hogg, R.C., Bertrand, D., 2004. Nicotinic acetylcholine receptors as drug targets. Curr. Drug Targets CNS Neurol. Disord. 3, 123–130.
23. Hogg, R.C., Raggenbass, M., Bertrand, D., 2003. Nicotinic acetylcholine receptors: from structure to brain function. Rev. Physiol. Biochem. Pharmacol. 147, 1–46.
24. Kessler, M., Goldsmith, D., Schellekens, H., 2006. Immunogenicity of biopharmaceuticals. Nephrol. Dial. Transpl. 21 (Suppl. 5), v9–12.
25. Koehn, F.E., Carter, G.T., 2005. The evolving role of natural products in drug discovery. Nat. Rev. Drug Discov. 4, 206–220.
26. Komarova, T.V., Baschieri, S., Donini, M., Marusic, C., Benvenuto, E., Dorokhov, Y.L., 2010. Transient expression systems for plant-derived biopharmaceuticals. Expert Rev. Vaccines 9, 859–876.
27. Kool, J., de Kloe, G.E., Bruyneel, B., de Vlieger, J.S., Retra, K., Wijtmans, M., van Elk, R., Smit, A.B., Leurs, R., Lingeman, H., de Esch, I.J., Irth, H., 2010. Online fluorescence enhancement assay for the acetylcholine binding protein with parallel mass spectrometric identification. J. Med. Chem. 53, 4720–4730.
28. Kool, J., Giera, M., Irth, H., Niessen, W.M., 2011. Advances in mass spectrometry-based post-column bioaffinity profiling of mixtures. Anal. Bioanal. Chem. 399, 2655–2668.
29. Malany, S., Osaka, H., Sine, S.M., Taylor, P., 2000. Orientation of alphaneurotoxin at the subunit interfaces of the nicotinic acetylcholine receptor. Biochemistry 39, 15388–15398.
30. Martin, P.L., Weinbauer, G.F., 2010. Developmental toxicity testing of biopharmaceuticals in nonhuman primates: previous experience and future directions. Int. J. Toxicol. 29, 552–568.
31. Orive, G., Gascon, A.R., Hernandez, R.M., Dominguez-Gil, A., Pedraz, J.L., 2004. Techniques: new approaches to the delivery of biopharmaceuticals. Trends Pharmacol. Sci. 25, 382–387.
32. Petras, D., Sanz, L., Segura, A., Herrera, M., Villalta, M., Solano, D., Vargas, M., Leon, G., Warrell, D.A., Theakston, R.D., Harrison, R.A., Durfa, N., Nasidi, A., Gutierrez, J.M., Calvete, J.J. Snake venomics of African spitting cobras: toxin composition and assessment of congeneric cross-reactivity of the pan-African EchiTAb-Plus-ICP antivenom by antivenomics and neutralization approaches. J. Proteome Res. 10, 1266–1280.
33. Ponzio, T.A., Feindt, H., Ferguson, S., 2011. License compliance issues for biopharmaceuticals: special challenges for negotiations between companies and non-profit research institutions. LES Nouv. 46, 216–225.
34. Pu, X.C., Wong, P.T., Gopalakrishnakone, P., 1995. A novel analgesic toxin (hannalgesin) from the venom of king cobra (Ophiophagus hannah). Toxicon 33, 1425–1431.
35. Ratner, M., 2010. Pfizer stakes a claim in plant cell-made biopharmaceuticals. Nat. Biotechn. 28, 107–108.
36. Reichert, J.M., 2000. New biopharmaceuticals in the USA: trends in development and marketing approvals 1995–1999. Trends Biotechnol. 18, 364–369.
37. Reichert, J.M., Healy, E.M., 2001. Biopharmaceuticals approved in the EU 1995–1999: a European Union–United States comparison. Eur. J. Pharm. Biopharm. 51, 1–7.
38. Roy, A., Zhou, X., Chong, M.Z., D'Hoedt, D., Foo, C.S., Rajagopalan, N., Nirthanan, S., Bertrand, D., Sivaraman, J., Kini, R.M., 2010. Structural and functional characterization of a novel homodimeric three-finger neurotoxin from the venom of Ophiophagus hannah (king cobra). J. Biol. Chem. 285, 8302–8315.
39. Shoyele, S.A., Cawthorne, S., 2006. Particle engineering techniques for inhaled biopharmaceuticals. Adv. Drug Deliv. Rev. 58, 1009–1029.
40. Smit, A.B., Syed, N.I., Schaap, D., van Minnen, J., Klumperman, J., Kits, K.S., Lodder, H., van der Schors, R.C., van Elk, R., Sorgedrager, B., Brejc, K., Sixma, T.K., Geraerts, W.P., 2001. A glia-derived acetylcholine-binding protein that modulates synaptic transmission. Nature 411, 261–268.
41. Takahashi, H., Letourneur, D., Grainger, D.W., 2007. Delivery of large biopharmaceuticals from cardiovascular stents: a review. Biomacromolecules 8, 3281–3293.
42. van Elswijk, D.A., Schobel, U.P., Lansky, E.P., Irth, H., van der Greef, J., 2004. Rapid dereplication of estrogenic compounds in pomegranate (Punica granatum) using on-line biochemical detection coupled to mass spectrometry. Phytochemistry 65, 233–241.
43. Vincent, A., 2002. Unravelling the pathogenesis of myasthenia gravis. Nat. Rev. Immunol. 2, 797–804.
44. Vonk, F.J., Jackson, K., Doley, R., Madaras, F., Mirtschin, P.J., Vidal, N., 2011. Snake venom: from fieldwork to the clinic: recent insights into snake biology, together with new technology allowing high-throughput screening of venom, bring new hope for drug discovery. Bioessays 33, 269–279.
45. Wurm, F.M., 2007. Manufacturing of biopharmaceuticals and implications for biosimilars. Kidney Blood Press Res. 30 (Suppl. 1), 6–8.

Supplementary material

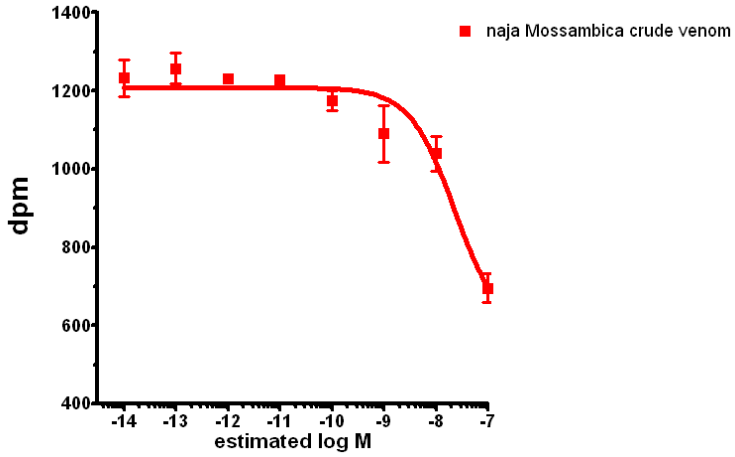


Figure S1A. Displacement of [3H]MLA by the crude *Naja Mossambica mossambica* venom on the human α7 nAChR. The crude venom shows low affinity binding to this receptor.

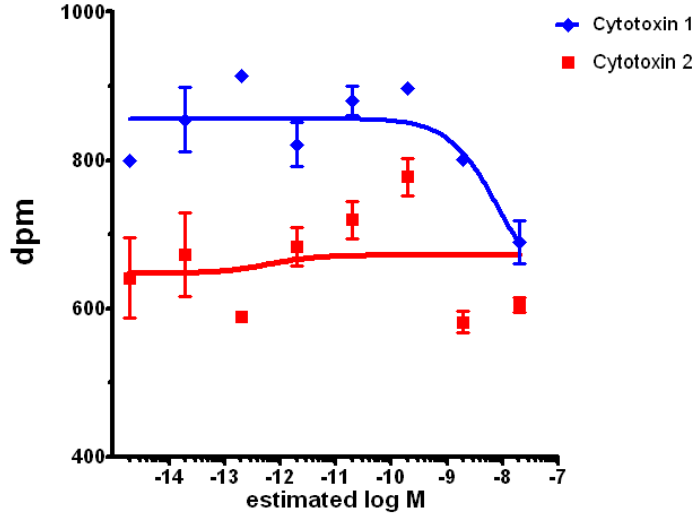


Figure S1B. Displacement of [3H]MLA by the purified Cytotoxin 1 and 2 from the venom of *Naja Mossambica mossambica*. From the two purified toxins low affinity binding was observed only by Cytotoxin 1

Table S1A.
The list of the identified tryptic peptide fragments after the tryptic digestion of Cytotoxin 2

Sequence of Cytotoxin 2: LKCNQLIPPF WKTCPKGKNL CYKMTMRGAS KVPVKRGCID VCPKSSLLIK YMCCNTDKCN					
Mass	position	Number of miscleavages	Artificial modification(s)	Modified mass	Peptide sequence
1486.8	1-12	1	Cys_CM: 3	1544.8	LKCNQLIPPFWK
1245.6	3-12	0	Cys_CM:3	1303.6	CNQLIPPFWK
990.4	36-44	1	Cys_CM: 38,42	1106.4	RGCIDVCPK
834.3	37-44	0	Cys_CM: 38, 42	950.3	GCIDVCPK
660.4	45-50	0			SSLLIK
640.3	19-23	0	Cys_CM: 21	698.3	NLCYK
538.2	24-27	0			MTMR
442.3	32-35	0			VPVK
362.2	28-31	0			GASK

Table S1B.
The list of the identified tryptic peptide fragments after the tryptic digestion of Cytotoxin 1.

Sequence of Cytotoxin 1: LKCNQLIPPF WKTCPKGKNL CYKMTMRAAP MVPVKRGCID VCPKSSLLIK YMCCNTNKCN					
Mass	position	Number of miscleavages	Artificial modification(s)	Modified mass	Peptide sequence
1486.8	1-12	1	Cys_CM: 3	1544.8	LKCNQLIPPFWK
1331.6	24-35	1	MSO: 24, 26, 31	1379.6	MTMRAAPMVPVK
1245.6	3-12	0	Cys_CM:3	1303.6	CNQLIPPFWK
990.4	36-44	1	Cys_CM: 38,42	1106.4	RGCIDVCPK
968.5	28-36	1	MSO: 31	984.5	AAPMVPVKR
834.3	37-44	0	Cys_CM: 38, 42	950.3	GCIDVCPK
660.4	45-50	0			SSLLIK
640.3	19-23	0	Cys_CM: 21	698.3	NLCYK
538.2	24-27	0			MTMR
448.2	13-16	0	Cys_CM: 14	506.2	TCPK
260.1	1-2	0			LK
236.0	59-60	0			CN