High resolution screening analytics for identification of natural bioactives
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High resolution screening analytics for identification of natural bioactives

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CHAPTER 1

Introduction
This thesis describes the development of advanced analytical techniques for identifying bioactive compounds from natural resources. Natural extracts are complex mixtures which may be rich in useful bioactive compounds and, therefore, are attractive sources for new leads in drug discovery. The work presented essentially aims at the identification of novel bioactives targeting brain receptors, with a focus on the alpha-7 nicotinic acetylcholine receptor ($\alpha_7$-nAChR) and the serotonin type 3 receptor (5-HT$_3$R).

The Introduction of the thesis is divided into three main parts: "Targets", "Sources" and "Approaches".

The "Targets" part of the introduction will discuss the relevance of the drug targets studied in this thesis.

The "Sources" part will discuss the relevance for drug discovery of finding bioactive compounds from various natural sources.

The "Approaches" part will give an overview of classical and new analytical approaches which are used for the identification of new bioactive compounds targeting ion channels. In addition, an overview is given of the venom-based drug discovery approach and the diverse hyphenated analytical systems used for complex mixture screening.
1. Targets

Ligand-gated ion channels in the nervous system

The brain consists of hundreds of billions of neurons (nerve cells), which are communicating with each other predominantly via chemical signaling in specialized parts of the neurons, so called synapses. In synapses the presynaptic neurons can release endogenous compounds, neurotransmitters, which can bind to specific neurotransmitter receptors which are present on the post-synaptic neurons, or alternatively pre-synaptic terminals, resulting in modulation of synaptic activity. The neurotransmitters can be categorized in small molecule-type transmitters that are enzymatically synthesized (for example acetylcholine, GABA or serotonin) and peptide-type neurotransmitters encoded by the genome (for example the opioid peptides). The neurotransmitter receptors are categorized in two major groups: G-protein coupled receptors (GPCRs) and ligand-gated ion channels (LGICs). Ion channels can be gated (i.e. opened or closed) by voltage (voltage-gated ion channels) or by a specific ligand binding to the channel (LGICs). The main role of ion channels is to generate ion concentration gradients, that change the membrane potential which may result in the propagation of electrical signals in the neurons. The LGICs can be divided in subcategories based on their structure: the Cys-loop type LGICs consist of 5 subunits, the ionotropic glutamate receptors (iGluR) are formed from 4 subunits, and the ionotopic ATP receptors (P2X-R), which consist of 3 subunits. The categorization of LGICs is shown in Table 1.

Many central nervous system (CNS) related diseases are caused by the altered regulation, function or expression of neurotransmitter receptors. This thesis focuses on two of these receptors that are involved in several CNS diseases and therefore comprise important therapeutic targets: the alpha-7 nicotinic acetylcholine receptor (α7-nAChR) and the serotonin type 3 receptor (5-HT₃R) (respectively, figures 1A and 1B).

<table>
<thead>
<tr>
<th>Type</th>
<th>Subtypes/subunits</th>
<th>Main functions in the CNS</th>
<th>Involvement in CNS diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anionic Cys-loop receptors</td>
<td>α₁-6, β₁-3, γ₁-3, δ ε π θ</td>
<td>Neuronal hyperpolarization, resulting in inhibitory effect on neuronal activity</td>
<td>Anxiety, insomnia, agitation</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;₆&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>α₁-4, β</td>
<td>Inhibitory neurotransmission</td>
<td>Hyperekplexia</td>
</tr>
</tbody>
</table>
### Cationic Cys-loop Receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>Function</th>
<th>Disorders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotonin (5-HT_{3})</td>
<td>5-HT_{3a,k}</td>
<td>Modulation of neurotransmitter release in interneurons, regulating the nausea-vomiting system in the CNS</td>
<td>Schizophrenia, addiction, anxiety and cognitive dysfunctions, emesis^{6-7}.</td>
</tr>
<tr>
<td>Nicotinic Acetylcholine Receptors (nAChR)</td>
<td>Muscle type: α1, β1, γ, δ, ε Neuronal type: α2-10, β2-4</td>
<td>Large diversity in roles, depending on subtype. Mainly regulating presynaptic neurotransmitter release. Excitatory postsynaptic potential (muscle type and ganglionic), post- and presynaptic excitation. Involved in memory and cognitive functions</td>
<td>Alzheimer’s disease, Parkinson’s disease, epilepsy, schizophrenia, dementia, attention deficit, pain, depression, anxiety, and depression^{8-9}.</td>
</tr>
<tr>
<td>Zinc Activated Ion Channel (ZAC)</td>
<td>Function not yet elucidated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ionotropic Glutamate Receptors (iGluR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMPA</td>
<td>GluA1-4</td>
<td>Fast synaptic transmission, synaptic plasticity</td>
<td>Epilepsy^{11}.</td>
</tr>
<tr>
<td>Kainate</td>
<td>GluK1-5</td>
<td>Postsynaptic kainate receptors: excitatory neurotransmission. Presynaptic kainate receptors: modulating GABA release</td>
<td>Epilepsy^{12}.</td>
</tr>
<tr>
<td>NMDA</td>
<td>GluN1, GluN2A-D, GluN3A-B</td>
<td>Synaptic plasticity; learning and memory</td>
<td>Alzheimer’s disease, Parkinson’s disease, depression, and schizophrenia^{13}.</td>
</tr>
<tr>
<td>Orphan</td>
<td>GluD1-2</td>
<td>Synaptogenesis, synaptic plasticity and motor coordination in cerebellum.</td>
<td>Ataxia, dementia, and schizophrenia^{14}.</td>
</tr>
<tr>
<td>ATP-gated Channels</td>
<td>P2X Purinoreceptors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2X1-7</td>
<td>Noception and modulation of synaptic transmission</td>
<td>Chronic pain^{15}.</td>
<td></td>
</tr>
</tbody>
</table>

### 1.1. The nAChRs and the α7-nAChR

The nAChRs belong to the Cys-loop receptor superfamily of the LGICs. The Cys-loop receptor family is named after a 13-amino-acid loop present in these receptors formed by a disulfide bridge. The members of this receptor family are the nAChRs, the GABAA receptors, the 5-HT_{3} receptors and the glycine receptors (GlyR)^{16-19}. The nAChRs can be divided into two groups: the muscle-type nAChRs and the neuronal type nAChRs^{20-21}. The muscle-type nAChRs are found in neuromuscular junctions of the peripheral nervous system, whereas the neuronal types are found in the central nervous system, but are also expressed in non-neuronal tissues and organs, for example in macrophages,
lung or in skin. The nAChR subunits are classified as α subunits when the C loop of the receptor contains two adjacent cysteine residues, whereas in the β subunits these cysteine residues are absent. Up to date there are nine neuronal α subunits (α2-10) and three β subunits (β2-4) identified. Whereas some of the α subunits can form so called homomeric receptors consisting of five homologous α subunits (the α7 and the α9 nAChR), the other neuronal α subunits are forming heteromers consisting of a combination of α and β subunits (for example α4β2, α3β4). Crystal structure studies initially using the acetylcholine binding protein (AChBP) provided detailed information regarding the structure of nAChRs specifically, and LGICs in general (Figures 1C and 1D). AChBPs are soluble proteins expressed in glia cells of Molluscan species, and they are homologous to the extracellular ligand recognition domain of nAChRs. These studies led to breakthrough discoveries in the understanding of the functioning and ligand recognition properties of the nAChRs.

This thesis focuses on the homopentameric α7-nAChR, however, other subtypes of nAChR also have high clinical relevance. For example, the α4β2-nAChR is the predominant nAChR subtype in the brain and it is known to be involved in addiction to tobacco/smoking. For the treatment of tobacco addiction the drug varenicline (Champix) is an approved drug targeting the α4β2-nAChR. Besides tobacco addiction, α4β2-nAChRs are also involved in cognitive disorders and in pain, and there are several compounds targeting α4β2-nAChR in clinical trials for the treatment of these. A reason for studying the α7-nAChR in this thesis is that this receptor is expressed well in SH-SY5Y neuroblastoma cells (therefore making it amenable for cellular screening). Also, the α7-nAChR is a homopentameric receptor, thus making the obtained results more straightforward to interpret. Besides, also the α7-nAChR has been implicated in CNS diseases.

In the brain the α7-nAChR is localized mainly in various brain regions among which those involved in cognitive function, learning and memory. Alpha-7 nACh receptors were found in the cerebral cortex, hypothalamus, ventral tegmental area, substantia nigra, hippocampus, pineal gland, amygdala, medial habenula, olfactory bulb, and cerebellum. The α7-nAChR is also expressed in non-neuronal tissues, such as in macrophages, lymphocytes, skin and kidney.

Typical characteristics of the α7-nAChR are its high desensitization rate, calcium permeability and the relatively low affinity of acetylcholine and nicotine towards the receptor. The most common functions awarded to the α7-nAChR are modulation of the other neurotransmitter systems, e.g., modulation of synaptic plasticity in the brain (glutamate, dopamine, serotonin (5-HT), GABA and norepineprine), and the activation of messenger pathways (for example gene expression or neuronal survival) on postsynaptic neurons by changes in the intracellular Ca2+ concentration. The abnormal functioning or loss of nAChRs has been associated to many CNS diseases, such as to Alzheimer’s disease, Parkinson’s disease, epilepsy, schizophrenia, attention deficit/hyperactive disorder, pain, anxiety, and depression.

In schizophrenia patients the expression level of the α7-nAChR is reduced in many brain regions compared to healthy subjects. There have been several efforts for developing drugs targeting the α7-nAChR, delivering drug candidates, such as the partial α7-nAChR agonist Encenicline (EVP-6124) which is in Phase II clinical studies, and TC-5619, which is a full α7-nAChR agonist in clinical Phase I. These compounds were found to improve the cognitive functions in schizophrenic patients.
The role of the α7-nAChR in Alzheimer’s disease and in its treatment has been extensively studied, as discussed in recent reviews in the literature. In Alzheimer’s disease, the loss of α7-nAChRs in the hippocampus correlates with decreased cognitive functioning. Also, recent studies show that amyloid beta (Aβ) binds with high affinity to the α7-nAChR, and by this disrupts its normal functioning. However, there are still different opinions in the literature regarding the type of interaction, whether Aβ acts as an agonist or an antagonist on the α7-nAChR, and also some publications stating that there is no interaction at all of Aβ and the α7-nAChR. Nevertheless, there are α7-nAChR agonists, which are in Phase I and Phase II studies that are potential new drug candidates for treatment of the cognitive symptoms of Alzheimer’s disease.

Next to the possible involvement in CNS-related diseases, the α7-nAChR might be involved in non-CNS related processes. Recently, the immunodulatory and anti-inflammatory effects of the α7-nAChR were under investigation. Another recently discovered potential of non-CNS pharmaceutical targeting of the α7-nAChR is in cancer treatment. The α7-nAChR was found to modulate the chemosensitivity of gastric cancer cells, and recent studies showed that silencing α7-nAChR levels increased the sensitivity of gastric cancer cells to chemotherapy drugs. In a recent review the role of nAChRs in disease is discussed in more detail, and an overview...
of the different nAChR targeting drugs which are in clinical studies is provided\(^9\). As a conclusion, the \(\alpha7\)-nAChR is potentially an important therapeutic target against several medical conditions. However, there are still many remaining questions that need to be answered regarding the precise functions and roles of this receptor.

1.2. The 5-HT\(_3\)R

The 5-HT\(_3\)R (Figure 1b) is the only serotonin receptor which belongs to the LGIC family, whereas all others are GPCRs\(^63\). The 5HT\(_3\)R, like the \(\alpha7\)-nAChR, is assembled from 5 subunits: either a homomeric receptor composed of five 5-HT\(_{3A}\) subunits or heteromeric composed by 5-HT\(_{3A}\) and 5-HT\(_{3B}\) subunits\(^6,64\)\-65\. The expression of the subunits in the CNS or peripheral nervous system (PNS) has not yet been completely elucidated. Also, the genes of the 5-HT\(_{3C}\), 5-HT\(_{3D}\), and 5-HT\(_{3E}\) subunits have been described, but their function and localization has not been characterized yet\(^66\). The 5-HT\(_3\)R has high similarity to the \(\alpha7\)-nAChR. The two receptors share high sequence (30%) and structural homology and therefore many ligands binding to the \(\alpha7\)-nAChR are also interacting with the 5-HT\(_3\)R\(^67\). A few examples of ligands which show orthosteric binding both to the 5-HT\(_3\)R and \(\alpha7\)-nAChR are varenicline, epibatidine, and tropisetron\(^68\)\-70\. 5-HT\(_3\)Rs are expressed in both the CNS and PNS, and they are expressed in different brain locations, like in the cortex, amygdala, substantia nigra, and brain areas involved in the vomiting reflex, such as the nucleus tractus solitarius and the area postrema.

The 5-HT\(_3\)R has been associated with CNS diseases, such as schizophrenia, addiction, anxiety and cognitive dysfunctions, however there are currently no 5-HT\(_3\)R targeting drugs used clinically for the treatment of these diseases. In the clinic, the two main applications for 5-HT\(_3\)R antagonists are prevention of chemotherapy- and radiotherapy induced nausea and vomiting, and the treatment of irritable bowel syndrome (IBS). 5-HT\(_3\)Rs are expressed both in the PNS and in the CNS at the chemoreceptor trigger zone of the area postrema. 5-HT\(_3\)R antagonists, such as ondansetron and granisetron used clinically are blocking these receptors, however, it is not certain yet if their action is mediated peripherally, centrally, or both. The 5-HT\(_3\)R antagonist alosetron is widely used in the clinic for the treatment of diarrhea-predominant IBS, targeting the 5-HT\(_3\) receptors of the enteric nervous system in the gastrointestinal track.

Since the \(\alpha7\)-nAChR and the 5-HT\(_3\)R are relevant drug targets, there is a high need for identifying new ligands modulating these receptors, which can be developed further as new drug leads.
2. Sources

Natural extracts as sources of new drug leads

Nature has provided a large number of medicinal products for treating diseases over the course of history. The first medications known in many cultures and civilizations were made from plant extracts. The first examples of the use of natural extracts for medication are the “Ebers Papyrus” (Egyptian civilization, BCE 1500, a record of more than 700 natural extracts with medicinal properties), the Chinese “Materia Medica” (China, BCE 1100, 52 prescriptions) and the “Ayurvedic” system (India, BCE 1000, over 300 medicinal natural extracts described). Besides the middle and far Eastern cultures the ancient Greeks and Romans also used natural products as medicines. Hippocrates, who is considered the father of modern medicine, building the foundation of the western medication system, also applied phytotherapy, or healing with herbs, in his treatments. In the last century many important medicines have been derived from natural sources. Well-known examples of medicines discovered from natural sources are aspirin from the *Salix alba* tree, digoxin from *Digitalis purpurea* and penicillin from *Penicillium notatum*. Evidently, nature is a very important source for finding new drugs and drug leads for the treatment of disease. In the following subchapters examples of bioactive compounds derived from natural extracts targeting ion-channels, with a focus on the α7-nAChR and the 5-HT3R, will be overviewed.

2.1. Active compounds identified from microbes, plants and mushrooms

Today’s pharmacology would not be the same without the discovery of nicotine from the *Nicotiana tabacum* plant (Figure 2A) and muscarine from *Amanita muscaria* mushroom. These two specific agonists allowed researchers to discover and investigate the main two subclasses of AChRs (nicotinic and muscarinic AChRs). In nature many examples can be found of plants that produce small molecular compounds that are targeting nAChRs. Known examples of active compounds acting on nAChRs isolated from plants are: cytisine from the golden chain tree (*Laburnum anagyroides*), the potent and selective α7-nAChR antagonist methyllylcaconitine (MLA) isolated from *Delphinium brownii* plant seeds (Figure 2B), and the positive allosteric modulator galanthamine isolated from *Galanthus woronowii* (green snowdrop). Thus, natural sources are a great source for the discovery of bioactive compounds. These bioactives are often used as initial templates for structural drug lead optimization in which the original molecules are chemically modified by medicinal/synthetic chemical approaches to become final drug leads. Many new nAChR targeting drugs now being tested in preclinical and clinical phases for the treatment of Alzheimer’s disease were initially derived from compounds of natural extracts. Hallucinogenic mushrooms, such as the Psilocybe
mushrooms, contain many tryptamine-like small molecules which are agonists on the serotonin receptors. Next to plants and mushrooms, microbes and bacteria are also rich sources for discovering new bioactive compounds. Over the course of history many life saving medicines were derived from microbial sources, of which penicillin and other antibacterial medicines are examples. Examples of active compounds from bacteria are the anatoxin-a, which is a potent α7-nAChR agonist derived from a cyanobacterium, and ivermectin, which is an α7-nAChR positive allosteric modulator produced by Streptomyces bacteria. A comprehensive overview of nAChR ligands discovered from natural sources can be found in Daly.

2.2. Active compounds discovered from animal venoms

Animal venoms are complex mixtures of biologically active compounds, which can cause various physiological effects, such as neurotoxicity, effects on the blood coagulation system, and are tissue necrotizing, when injected into a prey during the process of envenomation. The composition of venoms can range from small organic molecules to peptides and proteins, such as enzymes. Venoms can also contain metal-ions, lipids, carbohydrates and nucleosides. Diverse animal species, such as the invertebrates (e.g., sea anemones, corals, mollusks, arthropods), and vertebrates (e.g., reptiles, fish and even mammals) produce venoms. These venoms contain a large variety of bioactive compounds, of which some are of interest as pharmacological tools and/or as new lead molecules in drug discovery.

The diversity of toxins in venoms often target a large variety of different types of ion channels, among which are the nAChRs. Venoms from marine animals in this regard are a rich source for discovering new bioactive compounds. For example, the cone snails, which are predatory sea snails, produce very potent neurotoxic venoms, each of which is a cocktail of tenths of different bioactive compounds. For instance, the α-conotoxin IMI (Figure 2D) isolated from Conus imperalis cone snail venom is a potent and highly selective antagonist of the α7-nAChR. ω-conotoxin isolated from the venom of Conus magus, is a novel pain killer used in the clinic under the name Prialt (ziconotide). Neuroactive compounds isolated from different marine species besides cone snails are overviewed in a recent review from Sakai et al.

The venoms of various types of arthropods, such as centipedes, spiders and scorpions, contain a large variety of toxins acting mainly on different voltage-gated ion channels, but also on LGICs. Argiotoxin is an AMPA receptor blocker from the venom of the Argiope lobata. An example of an α7-nAChR ligand from arthropods is the alkaloid anabaseine, which is a potent agonist isolated from the venom of Aphaenogaster ants.

Frogs are not typically known for injecting venoms into their prey and, therefore, they are considered as poisonous (the toxin acts when it is eaten, inhaled or digested). The high affinity nAChR agonist epibatidine was discovered from the poisonous Dendrobatid frog Epipedobates tricolor (Figure 2C). Examples of noncompetitive nAChR antagonists (targeting mainly the α3β4-nAChR) are the isodihydrohistrionicotoxin, isolated from the skin extract of Dendobatid frogs, and pseudophrynaminol discovered from the Australian Pseudophryne frogs.

Snake venoms are one of the richest sources of toxins targeting the α7-nAChR. Based on their structure and function, the snake venom proteins can be divided into the following protein families: three-finger toxins (3FTxs); phospholipase A2s (PLA2s), proteinase inhibitors, serine proteinases, lectines, and metalloproteinases.
Most of the snake venom toxins targeting the α7-nAChR belong to the family of the 3FTxs. α-bungarotoxin was isolated from Bungarus multicinctus (Taiwanese banded krait) venom (Figure 2E), and is an irreversible antagonist of the α-nAChR. This toxin used as pharmacological tool allowed the identification the α7-nAChR. Successful examples can be mentioned for the therapeutic use of snake venom toxins. One of the best known examples of a successful drug on the market is the anti-hypertensive drug captopril, which is an angiotensin-converting-enzyme (ACE) inhibitor derived from a peptide discovered in the venom of the lancehead viper (Bothrops jararaca). Another example of an approved medicine from snake venom is the antiplatelet drug epifibatide (Integrillin), which was derived from a peptide toxin found in the southeastern pygmy rattlesnake (Sistrurus miliarius barbouri). Next to compounds from snake venoms targeting the cardiovascular and neuromuscular system, compounds with antibacterial, antitumor, immunomodulator, and analgesic effects have also been discovered and are extensively studied as drug leads91.

Many novel bioactive compounds discovered in venoms do not pass clinical trials and never make it to a new medicine. This may be due to the fact that compounds identified from natural sources often have high affinity towards target receptors and are often are antagonists/inhibitors, a combination which is in most cases not an advantage. Moreover, venom based peptides and proteins often cannot pass the blood brain barrier (BBB) of mammals, so that they cannot reach their target in the CNS80. In addition, peptide and protein drugs commonly have to be administered parenterally, encompassing an increased risk on adverse immunogenic responses. Although newly discovered bioactive peptides and proteins from venoms may not reach the market as medicines, they still can provide new relevant information for the understanding of receptor expression (binding sites), receptor functions (physiology and structure) and find their way into diagnostics.

Figure 2. Examples of compounds targeting the α7-nAChR from natural sources. (A) nicotine from the Nicotiana tabacum plant, (B) MLA from Delphinium brownie plant, (C) epibatidine from Epipedobates tricolor frog, (D) α-conotoxin ImI from Conus imperalis cone snail venom, (E) α-bungarotoxin from Bungarus multicinctus snake venom. (F) From the estimated 2 million species of the biodiversity (including plants, animals, fungi, micro-organisms and alike) more than 95 % has not been evaluated before for biological activity92.
3. Approaches to find new compounds targeting ion channels

Ion channels are important targets for the development of new drug lead molecules. Several ‘classical’ approaches are used in the drug discovery process for ion-channel targets. More advanced and faster screening techniques have emerged over the last years. Classical approaches in the initial screening process and lead optimization phase include binding assays and assays measuring ion fluxes across the cell membrane (e.g. monitoring the membrane potential). More recently developed screening technologies involve automation and increase in the speed of screening. The newest assay formats can be considered high throughput, such as the fluorescence imaging plate reader (FLIPR) calcium flux assay. Besides these high-throughput screening (HTS) assay formats, nowadays medium/high throughput can be achieved with automated patch-clamp technologies and with the newest HTS instrumentation for automated electrophysiology ion-flux assays. The following subchapter will discuss in detail the different assay formats used in ion-channel drug discovery with a focus on the α7-nAChR and the 5-HT₃R.

3.1. Binding assays

Binding assays are used to detect binding of a ligand to a receptor, enzyme, macromolecule or antibody. In order to measure the interaction between a ligand and a receptor, in most of the assay formats labeling of the ligand or the receptor is required. Depending on the type of labeling, the receptor-ligand binding assays can be classified into radioactive and non-radioactive labeling formats. Assays measuring the displacement of a radioactively labeled ligand are widely used screening techniques due to their sensitivity, general use, straight-forward synthesis of radioactive ligands and the easy automation in HTS. The radioactively labeled ligand assays can be performed in heterogeneous and homogeneous assay formats. In heterogeneous assays the bound ligand needs to be separated from the non-bound ligand by filtration, dialysis or centrifugation. Due to the labor intensiveness of this separation step, homogeneous scintillation proximity assays (SPA) have also been developed. In SPA assays the receptor is immobilized on a scintillation bead or scintillation well, which emits light via energy transfer when a beta-radiating radioactive ligand molecule is bound to the immobilized receptor. This mechanism is based on close proximity. The beta radiation travels short distance in water and decay is thus fast. Close to the scintillation material on a bead or coated well, the radiation is transferred to the scintillation material which converts it into light. Radioligand binding assays for screening ligands for binding to the α7-nAChR and the 5-HT₃R were extensively used in academia and industry, and allowed the identification of many nAChR ligands in the past. The disadvantages
of radioligand binding assays are the high costs (especially the SPA assays) and aspects related to safety, health and disposal of radioactive waste. The filtration assays are also lower in throughput and more labor intensive. Therefore, many efforts have been made to develop non-radioactive methods.

Non-radioactive binding assays are using various types of assay readout strategies in most cases based on fluorescence, chemo-/bio-luminescence or colorimetric detection. More specifically, fluorescence/bioluminescence resonance energy transfer (FRET, BRET)\textsuperscript{109-111}, fluorescence polarization (FP)\textsuperscript{112-113}, total internal reflection fluorescence (TIRF)\textsuperscript{114-115}, and fluorescence enhancement assay principles can be named\textsuperscript{116}. Non-radioactive binding assays can be performed both in homogeneous and heterogeneous assay formats, based on the different types of assay component interactions. There are various types of instrumentation for measuring binding assays, such as plate reader formats, flow cytometry\textsuperscript{117-118}, chromatographic separation formats (frontal chromatography, quantitative affinity chromatography)\textsuperscript{119-121}, and surface plasmon resonance (SPR) analysis\textsuperscript{122-123}. Recently developed non-radioactive binding assays applied to screening for α7-nAChR and 5-HT\textsubscript{3}R ligands are fluorescence enhancement assays using AChBP as a soluble binding protein, which mimics the extracellular domain of the α7-nAChR\textsuperscript{116, 124}, SPR-based AChBP ligand binding assays\textsuperscript{123} and TIRF assays for studying the 5-HT\textsubscript{3}R\textsuperscript{114-115}.

Ligand binding assays in general have the limitation that only information on the binding affinity towards a receptor or ion channel is measured, and the mechanism of action (e.g., if a ligand is an agonist, antagonist or a modulator) is not known. Ligand binding assays measure the interaction with a specific binding site of the receptor and therefore ligands interacting with other sites of the receptor (allosteric modulators) are not detected. Also, testing frequency- and voltage-dependency of ligands towards their target receptors cannot be done using ligand binding assays. Therefore, functional cell-based assays, such as ion-flux assays and electrophysiology are widely used for screening purposes\textsuperscript{93, 125}.

### 3.2. Ion-flux and membrane potential assays

Cell-based ion-flux assays measure the functioning, activation and blocking of ion channels by monitoring the flux of ions passing through the channel. There are several approaches described in the literature for HTS ion-flux assays, such as flux assays based on radioactive isotopes using for example \(^{86}\text{Rb}^+\) for studying of potassium channels and non-selective cation channels\textsuperscript{126-127}, and non-radioactive \(^{86}\text{Rb}^+\) flux assays using atomic absorption spectroscopy\textsuperscript{128}. Since only an endpoint can be measured, the kinetics of the ion-channel activation/inactivation cannot be assessed. More extensively used ion-flux assays are based on using voltage sensitive FRET dyes\textsuperscript{129} or ion-selective fluorescence dyes. From the ion-selective fluorescence dyes the calcium-sensitive fluorescence dyes, such as the fluo-4 and FLIPR calcium dyes, are most widely used\textsuperscript{130-131}. These dyes, which can monitor the changes in intracellular Ca\textsuperscript{2+}-ion concentrations caused by ion influxes, are highly sensitive and provide a high dynamic range. Furthermore, they provide a real-time readout (i.e., they measure calcium fluxes in time upon ligand stimulation). The disadvantage of these assays is that they can only be used for measuring calcium fluxes. After introduction of these calcium-flux assays in high throughput plate readers, such as the FLIPR\textsuperscript{98}, calcium-flux assays became one of the most widely used screening techniques in pharmaceutical industry and academic research for this type of assaying,
also for the initial identification of new α7-nAChR ligands\textsuperscript{132-136}.

A more traditional, but high-content-information-providing technique to measure ion fluxes (ion currents) is electrophysiology. The most commonly used approaches for this type of measurement are the two-electrode voltage clamp (TEVC)\textsuperscript{137} in Xenopus oocytes and the patch clamp techniques\textsuperscript{138} using mammalian cellular systems with the ion channel of interest overexpressed. Using electrophysiology in ion channel drug discovery, the mechanism of action of a ligand can be fully characterized, including the amplitude of a current or voltage change evoked by the ligand on the ion channel and the duration of the signal. Using electrophysiology techniques, millisecond-range signals can be recorded, which is advantageous when an ion channel is desensitized in milliseconds, as is the case for the α7-nAChR. In contrast, techniques such as FLIPR and other ion-flux assays cannot measure these fast current changes. Moreover, electrophysiology does not depend on an ion-selective fluorescence dye and therefore each type of ion-channel can be measured by electrophysiology. A general disadvantage of electrophysiology techniques is the low throughput, labor-intensiveness and the requirement of highly skilled staff. In recent years, however, several efforts have been made towards automation to improve the throughput and complexity of these electrophysiology technologies\textsuperscript{93, 99-100}.

The approaches discussed in section 3.1. and 3.2 are summarized and compared in table 2.

Table 2. Drug discovery approaches used for ion channel targets.

<table>
<thead>
<tr>
<th>Approach</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radioactive binding assays</td>
<td>Sensitivity, General use, Straight-forward synthesis of radioactive ligands, Easy automation in HTS</td>
<td>High costs, Aspects related to safety, health and disposal of radioactive waste, Low throughput and labor intensive (filtration assays), Only binding affinity information obtained</td>
</tr>
<tr>
<td>(homogenous (e.g. SPA) or heterogeneous (filtration, dialysis, centrifugation))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-radioactive binding assays</td>
<td>Fluorescent/bioluminescent: health, safety and waste aspects, Easy automation in HTS, Possible multiplexing (e.g. with flow cytometer)</td>
<td>Information only on binding affinity</td>
</tr>
<tr>
<td>(homogenous and heterogeneous. Different mechanisms: e.g. FRET, BRET, FP, TIRF. Different instrumentation: e.g. plate reader, flow cytometry, chromatographic separation formats)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-radioactive binding assays using SPR</td>
<td>no labeling is required, no interference with light scattering, Often binding kinetics can be measured</td>
<td>Receptor or ligand immobilization is required</td>
</tr>
<tr>
<td>Radioactive ion flux assays</td>
<td>Functional cell-based assay, information on activation/inactivation of receptor</td>
<td>Endpoint can be measured (kinetics of the ion-channel activation/inactivation cannot be assessed), Aspects related to safety, health and disposal of radioactive waste</td>
</tr>
</tbody>
</table>
### Non-radioactive ion flux assays (voltage sensitive FRET dyes, ion-selective fluorescence dyes)

<table>
<thead>
<tr>
<th>Non-radioactive ion flux assays (voltage sensitive FRET dyes, ion-selective fluorescence dyes)</th>
<th>Functional cell-based assay, information on the function of the ligand (agonist, antagonist or modulator)</th>
<th>Ion-selective dyes: e.g. using calcium dependent dyes only calcium dependent ion channel function can be assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity, high dynamic range Kinetic measurement possible (e.g. using FLIPR instrument)</td>
<td>Fluorescent: health, safety benefits compared to radioactive ion flux assays Easy automation in HTS</td>
<td>Low throughput (however, in recent years several efforts were made for increasing the throughput and automation of electrophysiology technologies), labor-intensive requirement for highly skilled staff.</td>
</tr>
<tr>
<td>Electrophysiology (e.g. patch-clamp, TEVC)</td>
<td>Full functional characterization is possible (amplitude of a current or voltage change evoked by the ligand on the ion channel, duration of the signal) Millisecond range signals can also be recorded Does not depend on an ion-selective fluorescence dye and (every type of ion-channel can be measured by electrophysiology</td>
<td>Low throughput (however, in recent years several efforts were made for increasing the throughput and automation of electrophysiology technologies), labor-intensive requirement for highly skilled staff.</td>
</tr>
</tbody>
</table>

### 3.3. Classical approaches for screening of natural extract based mixtures: Bioassay guided fractionation

A limitation of standard HTS approaches, which were discussed in section 3.1 and 3.2, is that they can be used only for the screening of libraries of pure compounds. In the pharmaceutical industry often complex mixtures, such as natural extracts, metabolic mixtures or aptamer mixtures have to be screened for bioactive compounds. In this subchapter, the approaches for screening mixtures are described with a focus on natural extracts. Metabolic mixtures, aptamer mixtures and other mixtures used in drug discovery are not further discussed in this thesis as they are out of scope.

When natural extracts need to be screened for bioactivity, fractionation of the sample usually has to be performed. Commonly, multiple orthogonal separation steps are required in order to obtain sufficiently pure fractions to unambiguously assign the activity monitored by the applied screening assay to an individual bioactive. This traditional approach of natural extract screening is called bioassay-guided fractionation (BGF) and might also be used in environmental and food analysis, where it is called effect-directed analysis (EDA). BGF screens for medicinally relevant bioactives, whereas screening for toxicants (such as endocrine disruptors) is the focus of EDA. These approaches traditionally use low-resolution fractionation (fraction sizes of minutes or milliliters) after liquid-chromatographic (LC) separation of a mixture, which showed bioactivity in an initial crude mixture screening. After bioassaying and chemical analysis of the bioactive fractions, usually it turns out that these fractions are not pure. Therefore, subsequent orthogonal separation steps of the bioactive fractions are required followed by further assaying. This process continues until eventually pure bioactive fractions (i.e. containing only one compound) are obtained for compound identification. During these procedures bioactive compounds may get lost in the process due to various reasons, such as adsorption to LC tubing or to the walls of the collection...
vials, precipitation, degradation/oxidation and/or denaturation in case of peptides/proteins. Moreover, BGF approaches are very time consuming and labor intensive. In addition, BGF approaches require large quantities of initial sample, but for example animal venoms typically are available in very limited amounts only. Still, there are many successful examples in which bioactive compounds were discovered using BGF.

Prior to BGF, for many sample types, such as plants and mushrooms, a pretreatment is needed in order to remove interfering matrix constituents and transfer the bioactive compounds to a solution that can be analyzed. This sample pretreatment may involve steps of pre-washing, drying or freeze-drying of the material, grinding the material to obtain a homogeneous sample, and extracting the active compounds with an adequate extraction technique (for example pressurized-liquid extraction, solid-phase extraction, solid-phase micro-extraction, supercritical-fluid extraction or a surfactant-mediated technique). The isolation of bioactive compounds during the BGF process is usually performed using different separation techniques, including LC on columns with different stationary phases (exploiting different separation mechanisms, such as reversed phase LC and size-exclusion chromatography), thin layer chromatography (TLC), and flash chromatography. When a bioactive compound is successfully isolated after repeated separation and bioassay steps, their chemical structures are identified or confirmed by MS/MS and/or NMR analysis. Plant-derived bioactives, which mostly are low-molecular-weight compounds, may be a starting point for medicinal chemistry approaches to yield optimized leads with desired pharmacodynamic and pharmacokinetic profiles. Bioactives from venoms often are peptides or (small) proteins and usually proteomics and biochemical approaches are needed for their structural and biological characterization. Structure optimization steps might include peptide synthesis of derivatives in case of bioactive peptides and over-expression in suitable expression systems in case of protein bioactives.

3.4. Drug discovery workflows for venom peptide derived leads

Comprehensively identifying and studying complete venom proteomes (i.e. all proteins and peptides in a venom) by mass spectrometry is called venomics. Venomics can be used to better understand venom functioning and evolution, and also for the identification of new lead compounds in drug discovery. When applying venomics for drug discovery, subsequent BGF purification and characterization of toxins of interest are performed. For this, often a two-step purification involving successive SEC and RPLC is used. Next, the amino acid sequence of the peptide/protein toxin is determined by enzymatic digestion followed by MS/MS, or by N-terminal Edman sequencing. Production of the active peptide or protein in large quantities is subsequently performed by solid-phase peptide synthesis or by recombinant expression using an expression system, often Escherichia coli (E. coli). E. coli expression systems are a robust and cheap biological means for producing high yields of the protein of interest with the help of a genetically modified organism. In general, peptides larger than 60 amino acid residues cannot be synthesized by solid-phase peptide synthesis making over-expression in these cases necessary. The synthetic toxin expressed and purified is then analyzed by crystallography and/or NMR studies for determining its three dimensional structure, followed by docking studies and structure-activity relation (SAR) studies to determine the amino acids involved in its interaction with the drug target. The biological activity of the synthetic peptide or protein is usually at a later stage also characterized for
binding interaction with different receptors to assess selectivity and characterized with other off-target bioassays to assess toxicity. The biological activity of the peptide or protein is often improved by site-specific mutagenesis or by synthesis of different peptide derivates (i.e. rational changes or deletions of amino acids) compared to the original toxin. Often, this results in smaller derivates of the original toxin with enhanced pharmacodynamic and pharmacokinetic properties. A successful example in this regard is the peptide prohanin, which was derived from an analgesic, 72-amino-acid alpha-neurotoxin type protein, hannalgesin, from *Ophiophagus hannah* (king cobra) venom. Kini et al. determined that the amino acids on the carboxy terminal end of hannalgesin are involved in the analgesic effect of the toxin. For this they used the proline bracket method, which is a prediction model for the active sites based on the presence of proline residues. Based on the information on the active site they eventually synthesized the short, water soluble, 11 aminoacid peptide prohanin, which showed selective analgesic effect in vivo, and which did not cause neurotoxicity.

The above-mentioned approach was used for peptide and protein bioactives from animal venoms. However examples of extracting small molecular bioactive compounds can also be found from other animal sources, for example, epibatidine from the *Epipedobates tricolor* frog skin extract, or tryptamine-like compounds from the skin extract of *Bufo toads*.

Examples of the traditional approach of isolation and characterization of toxins from animal venom sources will next be given treated. The first example deals with the isolation and characterization of an anticoagulant protein, fasxiator, from *Bungarus fasciatus* snake venom. In this endeavor, bioactive proteins were isolated from the crude venom (100 mg) using a two-step fractionation. First size SEC was used, which was followed by RPLC of the fractions that were tested to be bioactive. One-ml fractions were collected and pooled for bioactivity testing (effect on prothrombin time and activated partial thromboplastin time). The amino acid sequences of the purified bioactives were determined by MS/MS after pyridylethylation and Lys-C/Arg-C digestion, and by Edman degradation. Next, the protein identified as best candidate was over-expressed in an *E. coli* system. The obtained recombinant protein showed good anticoagulant activity and protease specificity towards factor XIa (FXIa). Finally, after elucidating the structure-function relationship and the amino acid residues involved in the interaction with the target FXIa, the potency of the protein was improved by site-directed point-mutagenesis.

The isolation and pharmacological characterization of a neurotoxic three-finger toxin targeting the α7-nAChR from black mamba (*Dendroapsis polylepis polylepis*) snake venom, is described by Wang et al. The aim of this work was to isolate and characterize three-finger toxins with unusual posttranslational modifications. The isolated toxin α-elapitoxin-Dpp2d had an amidated C-terminal arginine. The α-elapitoxin-Dpp2d was first purified from crude venom (30 mg) by ion-exchange chromatography (IEC) followed by RPLC of the fraction containing three-finger toxins including elapitoxin-Dpp2d. The amino acid sequence of this toxin was then determined by de novo peptide sequencing using reduction/alkylation procedures, parallel enzymatic digestion by trypsin and by Glu-C, and MS/MS analysis. The crystal structure of the toxin was determined using the hanging drop vapor diffusion crystallization technique. Using docking studies the interaction of the toxin and the pharmacophore regions were modeled with Ls-AChBP. After expressing the toxin in
a larger quantity using the *E. coli* heterologous expression system and subsequent purification, the pharmacological activity of the toxin was characterized using radioligand binding assays and functional calcium flux assays on the human α7, α3β2, α3β4 and α1β1γδ nAChRs. From the functional cell-based assays α-EPTX-Dpp2d was found to selectively inhibit α7 nAChR, indicating its potential as a pharmacological tool for studying the α7 nAChR.

Cardoso et al\(^{154}\) isolated an inhibiting peptide of the voltage-gated sodium channel hNa\(_{1.7}\), which is a potential drug target for the treatment of pain disorders, from a spider venom. Crude venom (1 mg) of *Tarantula pruriens* was centrifuged and fractionated by RPLC. Obtained fractions were screened for hNa\(_{1.7}\) inhibition. The masses of the bioactive peptides were then determined using matrix-assisted laser desorption ionization time of flight (MALDI-TOF) MS and the amino acid sequence was determined by Edman N-terminal sequencing. The peptide was produced using an *E. coli* expression system. Next to recombinant expression, the toxin was also synthetized using Fmoc solid-phase peptide synthesis. The biological activity of the toxin was tested using membrane potential assays and patch-clamp electrophysiology. The biological activity of the toxin was subsequently characterized on different receptors, such as voltage gated calcium channels and nAChRs, in order to assess selectivity. This was followed by in vivo pain behavior assessment using a mouse model. Finally, the three-dimensional structure of the toxin was determined by 2D-NMR studies.

The ω-conotoxin MVIIA, which was the template of the approved painkiller drug ziconotide (Prialt), was discovered from the venom of the *Conus magus* cone snail\(^{155-156}\). The purification of ω-conotoxin MVIIA was performed by three chromatographic steps: first the crude venom (87 mg) was fractionated on a Sephadex gel filtration column. The activity of the fractions was tested using an intracerebral bioassay in mice. In this assay positive fractions elicited a characteristic "shaker" activity in mice. One active fraction was further fractionated on a semi-preparative C18 column, followed by an analytical C18 separation in order to obtain the pure peptide. In between each fractionation step the activity was monitored with the mouse intracerebral bioassay. Amino acid composition analysis was performed after hydrolysis with an automated analyzer. The amino acid sequence was determined by Edman sequencing. The peptide was synthetized using solid-phase peptide synthesis. Identification and biological characterization of this ω-conotoxin led to the discrimination between calcium channel subtypes and to ziconotide, the synthetic version of the ω-conotoxin. Ziconotide eventually became the pain killer drug Prialt used in the clinic. To summarize, venom-based drug discovery can be a successful endeavor to find new bioactive peptide and protein based drug leads.

A very recent approach in the venom-based drug discovery field is the integration of traditional BGF with proteomics, transcriptomics (all mRNA present in the venom gland) and bioinformatics approaches\(^{157}\). For this approach, proteome and transcriptome databases of the specific animal venom need to be available, providing fast assignment of masses of bioactives, and fast elucidation of sequences of bioactive peptides and proteins. In addition, post-translational modifications can be identified faster using database searches for typical modifications of the identified peptides. However, post-translational modifications can also pose difficulties in identification of peptides, in case they are not known in literature and/or in the databases available.
3.5. Hyphenated approaches for complex mixture screening

In order to overcome the limitations of BGF, new analytical techniques have been developed for the screening and identification of bioactive compounds in complex mixtures. High-Resolution Screening (HRS)\(^{158-159}\) is based on the coupling of an LC separation with a continuous-flow bioassay for direct assessment of the biological activity of eluting compounds. The assay reagent mixture is continuously mixed with the LC effluent and directed to a coil providing incubation and reaction time. HRS commonly encompasses parallel MS detection using a post-column split to yield accurate mass information on eluting compounds, including the bioactive ones. A typical HRS system is depicted in Figure 3A. HRS is fast and allows screening of multiple samples per day. Various types of biochemical assays have been successfully applied in HRS systems, although mostly enzymatic and binding assays\(^{116, 160-161}\). A disadvantage of the on-line HRS approaches is that assays have to be relatively fast (incubation times of seconds to maximal a few minutes) in order to obtain a measurable response. Assays with long incubation or assay preparation time, such as radioligand binding assays, are out of scope. Cell-based assays also cannot be used in HRS systems, primarily due to the technical difficulty of combining living cells with chromatographic eluents. In addition, the traditional HRS approaches require relatively large quantities of sample and biological reagents. The latter could be circumvented by developing miniaturized HRS systems, which consume lower reagent amounts while analyzing small sample volumes. So-called at-line nanofractionation approaches can be used allow time-consuming or cell-based assays. In this setup, the separation column effluent is split to provide parallel MS detection and high-resolution fraction collection. A typical at-line nanofractionation setup is shown in Figure 3B. The small fractions (in the range of seconds) are continuously collected onto 96, 384 or 1536 well plates. Usually, the plates are then freeze-dried, followed by direct on-plate pipetting of the bioassay reagents using robotics followed by incubation and plate-reader readout. Potential drawbacks of at-line nanofractionation compared to HRS approaches are that the screening is more time consuming, that it usually requires a freeze-drying step in which active compounds from the mixture could degrade or evaporate, and that the obtained bioassay readout has a lower time resolution.

Next to the post-column techniques HRS and at-line nanofractionation, which apply bioassays after the chromatographic separation, other bioactivity screening approaches of mixtures are available. These can be divided in pre-column and on-column bioactivity profiling methods. These methods in general perform either an affinity extraction of ligands from a complex mixture using a selective pre-column, or a chromatographic separation using an affinity column\(^{162}\). Using these pre-column and on-column approaches the bound and unbound protein before detection of bioactives can be separated. Techniques measuring protein–protein affinity and immobilized ligand–protein affinity interactions\(^{163}\), MS binding-assay approaches\(^{164}\), and coupling ultrafiltration\(^{165}\) or size-exclusion separations\(^{166}\) to LC-MS can be named in this regard. Pre-column and on-column bioactivity profiling methods are comprehensively described in recent reviews\(^{158, 163, 167}\). An example of yet another pre-column bioactivity profiling methodology used in nAChR drug discovery is a magnetic bead based affinity-selection methodology for identification of AChBP ligands in mixtures\(^{119}\).
### Table 3. Summary and comparison of complex-mixture screening techniques used in drug discovery approaches discussed in this thesis.

<table>
<thead>
<tr>
<th>Name of approach</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioassay guided fractionation (BGF)</td>
<td>Applicable approach for identification of bioactives from complex mixtures</td>
<td>Bioactive compounds often get lost in the process</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time consuming and labor intensive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Require large quantities of initial sample</td>
</tr>
<tr>
<td>On-line high resolution screening (HRS)</td>
<td>Biological activity assessment is directly combined after chromatographic separation.</td>
<td>Only assays with short incubation times (i.e. seconds to few minutes range) are applicable.</td>
</tr>
<tr>
<td>At-line nanofractionation approach</td>
<td>Various types of bioassay can be applied in it (also with longer incubation and preparation time) Higher resolution fractions are collected than in BGF</td>
<td>Time consuming, lower resolution compared to on-line HRS Requires a freeze-drying step (in which active compounds can be lost)</td>
</tr>
<tr>
<td>Pre-column and On-column hyphenated screening approaches</td>
<td>Rapid screening technique Wide applicability</td>
<td>Extended controls must be performed to validate the functionality of immobilized targets Limited stability and memory effects from high-affinity ligands</td>
</tr>
</tbody>
</table>

#### 3.6. Assay validation

When new assays are developed, the quality of the developed assay is evaluated with different statistical parameters. The most commonly used parameter to describe the quality of an assay is the Z\textsuperscript{-}-factor, which is calculated with the dynamic range of the assay and the variability of the data\textsuperscript{168}. Other commonly used statistical parameters to describe assay quality are only the dynamic range, the signal-to-noise ratio (S/N) and the signal-to-background ratio (S/B)\textsuperscript{168}. A detailed description of assay quality parameters and assay validation can be found elsewhere\textsuperscript{168-169}.

For statistical evaluation of on-line HRS assays, next to the common statistical parameters, a modified version of the Z\textsuperscript{-}-factor, the Z\textsuperscript{-}-chrom, can be used for some assay formats\textsuperscript{160}. While the Z\textsuperscript{-}-factor is used most often for micro-plate based high throughput assays, the Z\textsuperscript{-}-chrom is suited for the chromatography-based on-line assays\textsuperscript{160}. 
Figure 3. Examples of hyphenated screening systems. (A) Principle of an on-line post-column HRS screening system. After LC (or nano-LC) separation (1) the eluent flow is split into two. Part of the flow is directed to MS (2) for identification of the compounds, and the rest of the flow is directed to a continuous flow incubation coil (3), in which eluting compounds are incubated with bioassay reagents in a reaction coil followed by a fluorescence detector (4) measuring the biological activity (in case of fluorescence assays). (B) Principle of the at-line nanofractionation approach. After LC separation (1) of a complex mixture the flow is split in two. One part of the flow is directed to a UV detector followed by MS (2), while the other (usually the larger part, like 90%) of the flow is collected as nanofractions into well-plates by a nanofraction collector (3). The well-plates are usually freeze-dried after nanofractionation and followed by a bioassay which is performed off-line with the well-plates containing the high resolution fractions.
Scope of this thesis

As discussed in the "Targets" part of the Introduction, there is a need for novel compounds targeting the α7-nAChR and the 5-HT3R. These compounds can be pharmacological and diagnostic tools, or new drug lead molecules for CNS diseases. Nature is a rich source of compounds targeting these receptors. There are several ligands of these receptors that were identified from natural sources, as overviewed in the "Sources" part of the Introduction. However, natural extracts are complex mixtures which make the finding and identification of unknown bioactive components very challenging. There is a an essential need for the development of new analytical approaches for faster and more efficient screening of natural extracts for bioactives.

This thesis aims at development and application of advanced analytical techniques for the identification of bioactives from complex mixtures (such as natural extracts) acting on brain receptors, with focus on the α7-nAChR and the 5-HT3R. These new analytical techniques aim to overcome limitations of the traditionally used bioassay guided fractionation (BGF) methodologies in natural extract screening. These limitations involve the labor intensiveness and time consuming character of BGF, and the large sample amounts required for BGF. The newly developed analytical technologies are based on the parallel hyphenation of liquid chromatography (LC) with mass spectrometry (MS) and a bioassay, allowing mixture component separation by LC followed by both detection of a certain specific bioactivity (i.e. using a bioassay) and chemical identification (i.e. by MS). The bioassays hyphenated with LC and MS described in this thesis, advance from bioassays using soluble binding proteins (AChBP and 5-HTBP) to a combination of much more complex functional cell-based assays. The analytical techniques described were developed in such a manner that similar drug targets, for example other types of receptors, could be implemented in the technology.

Chapter 1 introduces the thesis by starting with an overview of the significance of the different drug targets under study. After introducing these target receptors, the relevance of compounds from different natural sources for drug discovery are overviewed. Finally, classical analytical approaches, which are used for discovery and identification of bioactive compounds from mixtures, new analytical developments in the endeavor of natural extract screening for bioactive compounds, and characteristics and applications of these analytical techniques are discussed.

In Chapter 2 a microfluidic on-line high-resolution screening (HRS) platform is applied in an analytical workflow for identification and direct purification of bioactive compounds. Using a post-column split, nano-LC is coupled in parallel to MS, via nano-electrospray ionization (nano-ESI), and to a microchip bioassay which utilizes a capillary bubble-cell LED-induced fluorescence detector for readout. The platform is operated in the range of nL/min flow rates and with nL range sample injection. This system is highly suited for the analysis of animal venoms, which are often available only in limited amounts. The application of the platform is demonstrated by screening of several snake venoms for AChBP-binding compounds. The homogeneous bioassay is based on fluorescence enhancement caused by a tracer molecule bound to the AChBP. A decrease in fluorescence when the tracer is displaced by an eluting ligand binding to AChBP is used as bioassay readout. The analytical workflow allows the direct MS-guided isolation of bioactives that are detected by the on-line microfluidic assay. Subsequent
bottom-up proteomics approaches can be used for chemical characterization of the bioactives.

In Chapter 3 the microfluidic on-line HRS platform described above is used for the screening and identification of AChBP-binding small molecules and peptides in toad skin and cone snail extracts, respectively. When the *Conus textile* cone snail extract was screened in the microfluidic on-line HRS platform, a bioactive peptide was pinpointed amongst >1,000 other peptides. The analytical workflow to screen and rapidly purify small molecule bioactives was demonstrated with *Bufo alvarius* and *Bufo marinus* toad skin extracts. Tryptamine-like and steroidal-like binders of the AChBP were found in the crude skin extracts, and purified with a straightforward MS guided purification. The full structural identity of the compounds was assessed by NMR and MS/MS, and their biological activity was tested and confirmed in conventional radioligand binding assays. In this manner, after rapid initial screening and pinpointing of bioactives, NMR and/or MS/MS were used for full chemical/structural identification of the purified bioactive compounds followed by confirmation of the biological activity in conventional radioligand binding assays.

Chapter 4 describes the development of a fluorescence enhancement assay for the serotonine binding protein (5HTBP) in plate reader format. Subsequently, the bioassay is implemented in the microfluidic online HRS platform. The 5HTBP is an engineered binding protein which has the protein scaffold of the AChBP with the ligand recognition properties of the 5-HT₃R. This fluorescence enhancement assay is a good initial screening technique for finding novel bioactives targeting the 5-HT₃R. The applicability of the new technique is demonstrated by the screening of different snake venoms for compounds binding to 5HTBP.

The aim of Chapters 5 and 6 is to implement functional cell-based assays in HRS-type screening approaches involving LC and MS in order to obtain biological cellular responses instead of binding affinity information only.

Chapter 5 describes the development of an at-line cell-based screening methodology, which combines LC followed by at-line nanofractionation and parallel MS with a functional fluorescence-based calcium-flux assay. This calcium-flux assay was performed with the human neuroblastoma cell line SH-SY5Y stably over-expressing the α7-nAChR. This at-line nanofractionation system was developed and optimized in assay modes for screening agonists and for allosteric modulators of the α7-nAChR. The application of this methodology was demonstrated by the screening of a hallucinogenic mushroom extract (from *Psilocybe mckennaii*). In this study the new approach of two orthogonal separations of the crude extract performed after each other using the same crude extract for each separation was used for precise bioactive compound identification from multiple co-eluting non-bioactive compounds.

Chapter 6 describes the analytical advancement to a new technique in which the Ca²⁺-flux assay is performed in post-column continuous flow format using a flow cytometer (FC) as readout device. The screening system consisted of an LC system coupled online to FC and to MS using PEEK-tubing and superloops for the infusion and mixing of the bioassay components. The bioassay applied in this system was based on the same functional calcium-flux assay as described in Chapter 5 using human α7-nAChR expressing SH-SY5Y neuroblastoma cells. The advantage of using on-line screening compared to the at-line nanofractionation approach described in Chapter 5 is that the on-line assay in continuous flow format is much faster and that the bioassay readout
obtained yields a higher bioassay-chromatographic resolution compared to the at-line approach described in Chapter 5. This LC-FC-MS screening system was developed in agonist and in mixed antagonist-agonist assay modes. The latter assay mode allows the simultaneous detection of agonists and antagonists. In proof-of-principle experiments the application of the screening system was demonstrated by the screening of tobacco plant leaf extract in agonist mode, and snake venoms in mixed antagonist-agonist assay mode. This analytical technology for the first time demonstrates the successful on-line coupling of chromatographic separations to FC using a continuous flow mammalian cellular bioassay as biological readout.
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CHAPTER 2

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

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.
1. Introduction

Great advances have been made in protein-based pharmaceuticals during the last ten years. 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, protein engineering and production techniques, as well as current knowledge and available technologies dealing with immunogenicity and immunotoxicity 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. 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 to 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. 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. 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. 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\textsuperscript{24}. Analyzing natural extracts with a 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\textsuperscript{25–26}. 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\textsuperscript{22}. Examples of medicinal products derived from snake venom compounds are the antiplatelet drug Eptifibatide (Integrilin) derived from southeastern pygmy rattlesnake (\textit{Sistrurus miliarius barbouri}) venom and the analgesic toxin Hannalgesin from the venom of the King cobra (\textit{Ophiophagus Hannah})\textsuperscript{27}. 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\textsuperscript{28–29}. The latter is based on the affinity of α-bungarotoxin (neurotoxin from the venom of the Taiwan krait \textit{Bungarus multicinctus}) to the acetylcholine receptor.

Nicotinic Acetylcholine receptors (nAChRs) are associated with many CNS diseases like migraine, epilepsy and pain\textsuperscript{30–34}. During the last ten years, drug discovery directed at the α7-nAChR experienced a leap forward by using the acetylcholine binding protein\textsuperscript{34} as drug model. \textit{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\textsuperscript{35} and has been used as nAChR model since then\textsuperscript{36–37}. We recently developed a HRS approach using AChBP as target\textsuperscript{38}. Subsequently, we developed a miniaturized screening variant allowing low sample amounts to be screened\textsuperscript{39} 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\textsuperscript{40}. 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\textsuperscript{40}. With this miniaturized HRS system, bioaffinity profiling of a venom takes only 1-2 hours 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 toward 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.

2. Materials and methods

2.1 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, [Met⁵]-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-NH₂ 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). KH₂PO₄, Na₂HPO₄ and NH₄HCO₃ were obtained from Riedel-de-Haën (Seelze, Germany). NAP-5 columns were obtained from GE Healthcare (Buckinghamshire, United Kingdom). *Lymnaea 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.* 30. The fluorescent tracer ligand DAHBA was synthesized in-house as described by Kool *et al.* 38.

2.2 Biochemical assays and samples

2.2.1 On-line AChBP assay

The on-line fluorescent enhancement assay for Ls-AChBP bioactivity profiling was performed as described by Heus *et al.* 39.

2.2.2 Snake venom samples

Cardiotoxin (# C9759) from *Naja mossambica mossambica* and Phospholipase A₂ (PLA₂; # P7778) from *Naja mossambica mossambica* were purchased from Sigma Aldrich. Lyophilized venom from *Naja mossambica mossambica* was acquired as described by Vonk *et al.* 2011 22. 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.

2.2.3 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 hr at 24 °C. After tryptic digestion, the trypsin was deactivated with FA using a final volume of 1% compared to the total volume. [Met³]-Enkephalin was finally added as internal standard at a concentration of 2 µM. The samples were stored at –20 °C until use.

2.2.4 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 ³H-methyllycaconitine ([³H-MLA] Kᵦ = 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 Dalton. 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 minute 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 analyzed using Prism 5.0 (Graphpad Software, inc. California, USA).

2.3. Instrumentation

2.3.1 On-line screening

The analytical system used for on-line screening was recently described by our group⁴⁰. 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.

2.3.2 MS guided LC purification

Purification of the bioactive peptides was performed with MS guided LC fractionation (Figure 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 x 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% H₂O/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.
2.3.3 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 x 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 NH₄HCO₃ 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.

2.3.4 MS peptide sequencing

Peptide sequence analyses were performed with an AB Scien 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:H₂O: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.

3. 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 (Figure 1c). The workflow is demonstrated on the venom of the snake *Naja mossambica mossambica*. We recently published this on-line high-resolution screening (HRS) of neurotoxic snake venoms. After screening the *Naja 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.
3.1 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\textsuperscript{40}. 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 $\alpha 7$-nAChR. The bioaffinity profiling of $\alpha$-bungarotoxin, $\alpha$-cobratoxin, erabutoxin, $\alpha$-conotoxin, muscarinic toxin 2 and PLA\_2 towards Ls-AChBP was recently demonstrated\textsuperscript{40}. For further evaluation of the on-line screening system, one additional commercially available toxin, related to the venom analyzed in this work, was screened with the on-line screening system, namely a 'cardiotoxin' purified from \textit{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 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.

\textbf{Figure 2.} The selectivity of the on-line screening setup was evaluated by us recently\textsuperscript{40}. To extend this evaluation, commercially available cardiotoxin ('cardiotoxin' purified from the venom of \textit{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 \textit{Naja Mossambica Mossambica} venom, where the same cardiotoxins were binding to the Ls-AChBP (see Fig. 3 and main text).
3.2 Screening for bioactives in the venom of *Naja mossambica*

The analysis of *Naja 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 *Naja mossambica mossambica* is known to contain phospholipases (Uniprot # P00602, P00603, P00604), cytotoxins (Uniprot # P01467, P01460, P01470, P01452, P25517) and short type neurotoxins (Uniprot # P01431, P01432). These known peptides of the *Naja 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 *Naja 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 *Naja mossambica* venom. 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, 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. (Figure 3a).
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Naja Mossambica 5 μg analysis

Most abundant charge state

Nominal mass

mV (x100)

TIC

UV (x100)
Figure 3. A typical on-line screening result obtained from injection of a 5 µg *Naja Mossambica* Mossambica venom sample is depicted (Fig. 3a). 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 (Fig. 3b) to provide better chromatographic peak shapes enabling a more straightforward correlation with MS and the bioaffinity data. Figure 3c 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 PLA₂s.

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 (Figure 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 (Figure 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.

3.3 Purification of bioactive peptides from the *Naja 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 *Naja 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 3.4 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 NH₄HCO₃ buffer in order to prevent possible peptide denaturation due to the LC eluents used.

3.4 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 (Figure 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 (Figure 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$_4$HCO$_3$ buffer however can be a good tool to protect other less stable peptides and proteins that are sensitive to higher organic solvent concentrations (eluent).

### 3.5 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, http://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 Information 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 (Figure 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.
Figure 4. This figure shows the result of the rescreening of the purified peptides using the on-line screening setup. Fig. 4a, 4b, and 4c, 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.
Figure 5. MALDI MS analysis of purified P01469 (Fig. 5a) and P01467 (Fig. 5b) 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 the Figure 5a and 5b. Figure 5c shows the sequence analysis of AAPMVPVKR after MS/MS fragmentation.
3.6. Radioligand displacement assay

In the on-line screening setup, the venoms were screened for bioaffinity for AChBP, which is a homolog of the binding site of the α7-nAChR. Although there is a good structural similarity between the AChBP and the α7-nAChR\textsuperscript{45}, 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 \textsuperscript{[3]H}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 *Naja Mossambica mossambica* and showed low affinity binding (Figure S1.a.). 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 (Figure S1.b.). 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 work flow. However, the radioligand binding assay did show translational binding from AChBP to the α7-nAChR.

4. 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, *Naja 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 analyzed 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 Heus et al. 2013 and in the current manuscript is in fact the miniaturized version of the screening platform initially described by Kool et al. 2010. Another example of a miniaturized platform using acetylcholine esterase as target has been published by us (de Boer et al., 2005). Our group developed many on-line screening platforms in the past (many of these are described in a recent review by Kool et al., 2011) 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 nº 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.
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Supporting information

Figure S1.a. Displacement of \([3H]\)MLA by the crude *Naja Mossambica mossambica* venom on the human \(\alpha-7\) nAChR. The crude venom shows low affinity binding to this receptor.

Figure S1.b. 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

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<th>Number of miscleavages</th>
<th>Artificial modification(s)</th>
<th>Modified mass</th>
<th>Peptide sequence</th>
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<tr>
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<td>1-12</td>
<td>1</td>
<td>Cys_CM: 3</td>
<td>1544.8</td>
<td>LKCNQLIPPFWK</td>
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<tr>
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<td>3-12</td>
<td>0</td>
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<td>1303.6</td>
<td>CNQLIPPFWK</td>
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<tr>
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<td>1</td>
<td>Cys_CM: 38, 42</td>
<td>1106.4</td>
<td>RGIDVCPK</td>
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<tr>
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### Table S1b. The list of the identified tryptic peptide fragments after the tryptic digestion of Cytotoxin 1

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CHAPTER 3

Miniaturized bioaffinity assessment coupled to mass spectrometry for guided purification of bioactives from toad and cone snail

(* Equal contribution)
Abstract

A nano-flow high-resolution screening platform, featuring a parallel chip-based microfluidic bioassay and mass spectrometry coupled to nano-liquid chromatography, was applied to screen animal venoms for nicotinic acetylcholine receptor like (nAChR) affinity by using the acetylcholine binding protein, a mimic of the nAChR. The potential of this microfluidic platform is demonstrated by profiling the Conus textile venom proteome, consisting of over 1,000 peptides. Within one analysis (<90 min, 500 ng venom injected), ligands are detected and identified. To show applicability for non-peptides, small molecular ligands such as steroidal ligands were identified in skin secretions from two toad species (Bufo alvarius and Bufo marinus). Bioactives from the toad samples were subsequently isolated by MS-guided fractionation. The fractions analyzed by NMR and a radioligand binding assay with α7-nAChR confirmed the identity and bioactivity of several new ligands.
1. Introduction

Besides being promising pharmacological tools, bioactive compounds from “natural sources” are considered of interest to the pharmaceutical industry as potential medicines\(^1\). Compounds from natural sources comprise an array of molecules, which have evolved to target specific biological tasks and aim at, e.g., avoidance of predation, immobilizing a prey, or preventing a prey’s reproduction. A challenge of modern drug discovery is to identify these compounds for affinity towards a biochemical process or a specific protein target, to isolate them and to develop such compounds into innovative drugs. The first step is the selection of a natural source, which could be an extract used for (traditional) medicinal purposes. Other sources rich in different biologically active compounds are animal venoms, which contain toxins, either to catch prey or to evade predation.

Cone snails, such as *Conus textile*, are predatory sea snails with venom containing more than a thousand conopeptides, which are small peptides with 12 to 35 amino acids and some disulfide bridges\(^2\). Many of these conopeptides target specific ion channels in the peripheral nervous system and involved in the transmission of pain stimuli\(^3\)\(^-\)\(^4\) with often high specificity and affinity, such as the nicotinic acetylcholine receptor nAChR\(^5\)\(^-\)\(^7\), which therefore may be potential biopharmaceuticals. This is reflected by the recent FDA approval of the conopeptide under the name Ziconotide (Prialt), which blocks a neuronal N-type voltage-gated calcium channel in the treatment of neuropathic pain\(^7\)\(^-\)\(^8\). The a9\(\alpha\)10-type nAChR bioactive conopeptide ACV1 (from Metabolic Pharmaceuticals Limited, Melbourne, Australia), involved in pain relief, reached clinical trials, but was abandoned due to efficacy lack in humans\(^9\). Another conopeptide-based drug candidate, xen2174 (from Xenome Ltd, Brisbane, Australia), increases norepinephrine levels in the spinal cord. An inherent advantage of conopeptide analysis is that identification is relatively straightforward as it can be accomplished with MS\(^2\) peptide sequencing\(^10\)\(^-\)\(^13\) (after reduction).

Toad skin excretions comprise a complex cocktail of small peptides, tryptamine derivatives (with masses between 100 and 400 Da) and steroidal compounds (with masses between 400 and 800 Da), and might be a natural source for bioactive compounds\(^14\). In contrast to conopeptides, they are expected to pass the blood brain barrier more easily. During evolution, the toads produced the tryptamine derivatives from serotonin, which has been the archetype ‘scaffold’\(^15\). These compounds, being excreted from glands in the skin of these toads, serve to deter animals, function as anti-predation toxins, and are known to cause convulsion, hallucinogenic effects and (even) death\(^16\). Toad skin excretions from the Cane toad skin (*Bufo marinus*) and the Colorado River toad skin (*Bufo alvarius*) may also exhibit AChBP affinity.

As the compounds in natural extracts are present in complex mixtures, the screening of these extracts for bioaffinity and identification of the bioactives needs to be optimally combined. Commonly, separation is the first step in natural extract screening after which biochemical assays on collected fractions are applied to screen for bioaffinity. Typically, “hits” are validated biochemically and their structure is elucidated by mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR), after iterative purification rounds with orthogonal separations. This traditional approach is laborious.
and requires relatively large amounts of sample\textsuperscript{5,17-19}. A specific challenge of natural extract screening is the accurate matching of bioactives with their chemical structure\textsuperscript{20-24}. Traditionally, bioassays are performed after low-resolution fractionation of the extract. Besides being laborious, this process is often only capable of identifying a small portion of the bioactives present\textsuperscript{25}, and closely eluting bioactives are not screened individually.

New screening approaches for natural extracts, e.g., affinity-selection MS (AS-MS)\textsuperscript{26}, ultrafiltration MS\textsuperscript{27} and high-resolution screening (HRS)\textsuperscript{28-32}, implement integrated liquid chromatographic (LC) separation and LC-MS. AS-MS techniques measure binding between a protein and its potential ligands by separating the protein-ligand complexes from the non-binding mixture components. Identification of the ligands is done subsequently with MS or MS-MS, whereas HRS techniques apply biochemical assays coupled on-line to LC-MS.

To screen for bioactive compounds that act on the membrane-bound nAChRs, the soluble homolog acetylcholine binding protein (AChBP) can be used\textsuperscript{33-36}. Recently, a HRS system, featuring an on-line fluorescence enhancement biochemical assay, for AChBP screening was developed\textsuperscript{37}. The assay format was subsequently miniaturized in order to enable screening of venoms of which only limited amounts are available for analysis\textsuperscript{38}. The miniaturized format was successfully applied for profiling of neurotoxic snake venoms\textsuperscript{39}. An advantage of this ‘on-line’ HRS technique lies in the ability to directly pinpoint ligands, even when the compounds in the mixture are poorly separated, which is often the case when screening complex venom samples.

Here, we combine HRS, featuring nano-LC separation, on-line miniaturized biochemical assaying and parallel MS analysis for rapid ligand identification, to MS-guided purification to enable full structure elucidation by, e.g., NMR or advanced MS technologies, and biological assaying of the purified ligands towards the human α7-nAChR using a radioligand binding assay (RBA). Using this screening approach, we readily identified AChBP ligands in cone snail venom (\textit{Conus textile}) and in natural extracts from Cane toad skin (\textit{Bufo marinus}) and the Colorado River toad (\textit{Bufo alvarius}).

2. Materials and methods

2.1. Chemical and biological reagents

\textit{Ls-AChBP} (from the snail species \textit{Lymnaea stagnalis}) was expressed from \textit{Baculovirus} using the pFastbac I vector in Sf9 insect cells and purified from the medium, as described by Celie et al.\textsuperscript{35}. Human neuroblastoma cells (SH-SY5Y) expressing human α7 nAChRs were obtained from Christian Fuhrer (Department of Neurochemistry, Brain Research Institute, University of Zurich, Zurich, Switzerland). The fluorescent tracer ligand DAHBA, (E)-3-(3-(4-diethylamino-2-hydroxybenzylidene)-3,4,5,6-tetrahydropyridin-2-yl)pyridine, was synthesized in house\textsuperscript{37}. Human neuroblastoma cells (SH-SY5Y) expressing human α7-nAChR were washed with PBS, collected as a pellet and stored at \textasciitilde80 °C until further use. \textsuperscript{3}H-Methyllycaconitine (\textsuperscript{3}H-MLA) was from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). KCl, DL-dithiothreitol (DTT), guanidine-HCl, NaCl, trizma base, HCl, Tween 20, [Met\textsuperscript{5}]-enkephalin and human angiotensin I, and \textit{Bufo marinus} and \textit{Bufo alvarius} extracts were purchased from Sigma-Aldrich (Zwijndrecht,
The Netherlands). KH₂PO₄, Na₂HPO₄ and NH₄HCO₃ were obtained from Riedel-de-Haën (Seelze, Germany). I-Nicotine (99.0%) was purchased from Janssen Chimica (Beerse, Belgium). Enzyme linked immunosorbent assay (ELISA) blocking reagent (ELISA-BR) was purchased from Hoffmann-La-Roche (Mannheim, Germany). Mca-Lys-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg-NH₂ was from Bachem (Bubendorf, Switzerland). Methanol-d₄ was purchased from Cambridge Isotopes Laboratories, Inc. (Buchem B.V., Apeldoorn, The Netherlands). ULC/MS grade trifluoroacetic acid (TFA; 99.95%), formic acid (FA; 99.95%) methanol (MeOH; 99.98%) and acetonitrile (ACN; 99.97%) were purchased from Biosolve (Valkenswaard, The Netherlands). HPLC grade water was produced using a Milli-Q purification system from Millipore (Amsterdam, The Netherlands).

2.2. Instrumentation.

2.2.1. Microfluidic confocal fluorescence detection system

The screening platform consisted of a nano-LC unit with a post-column split to an MS and a microfluidic chip acting as biochemical reactor, where the LC flow and a bioassay solution were mixed and incubated, and a microfluidic LED based laser-induced fluorescence (LIF) detector as described in detail in Heus et al.39.

2.2.2. Nano-LC and solvent delivery system

The Ultimate nano-LC system with a Famos autosampler was from LC Packings (Amsterdam, The Netherlands). The gradient system was operated at 400 nL/min. Mobile phase solvent A consisted of water/ACN 95:5 and 0.05% FA and solvent B of water/ACN 5:95 and 0.05% FA for Conus textile venom analysis. The toad extracts were analyzed with solvent A consisting of water and 0.1% TFA and solvent B of ACN and 0.1% TFA. Sample volumes of <500 nL were injected into the analytical capillary column (150 mm × 75 μm internal diameter (i.d.) ) packed in-house with Aqua C18 particles (particle size 3 μm, 200 Å pore diameter; Phenomenex, Torrance, CA, USA). For Conus textile venom analysis, a 70 min gradient elution was applied running five min isocratic at 5% solvent B, then rising to 60% solvent B in 65 minutes. For Bufo alvarius and Bufo marinus analysis, a 70 min gradient elution was applied running five min isocratic at 1% solvent B, then rising to 60% solvent B in 65 min.

2.2.3. Microfluidic chip

The microfluidic chip and chip holder (type 4515), produced by Micronit Microfluidics, (Enschede, The Netherlands), was described in detail elsewhere38. One inlet was used to connect the nano-LC carrier flow; the other inlet to a Model 980532 syringe pump (Harvard Apparatus, Holliston, MA, USA) to infuse the AChBP and tracer ligand DAHBA at a flow rate of 5 μL/min, as described previously37-38.

2.2.4. Microfluidic LED based LIF detector

The flow cell of the detector consisted of a 150 μm i.d. extended light path “bubble cell” with 50 μm i.d. connecting capillaries (G1600 64232, Agilent Technologies, Amstelveen, The Netherlands). This bubble cell served as the actual flow-through detector cell. Light from a high-intensity LED passed a 465 nm single band pass filter, was collimated by a lens, reflected by a dichroic mirror under 90° and focused into the centre of the bubble cell by a 20× quartz microscope objective. Emitted light passed the same dichroic mirror, a focusing lens, and a 520 nm single band pass filter, after which it was detected
by photomultiplier tube. A detailed description of this detector can be found in\textsuperscript{18}.

2.2.5. Nano-LC-MS

A Shimadzu (‘s-Hertogenbosch, The Netherlands) ion-trap–time-of-flight (IT–TOF) hybrid mass spectrometer equipped with a Picoview nano-electrospray ionization (ESI) source from New Objective (Woburn, MA, USA) was operated in positive-ion mode. A 40 mm × 180 µm outer diameter × 30 µm i.d. stainless-steel emitter was used as the spray needle (ESS22, Proxeon/Thermo Scientific, Waltham, MA, USA). The spray needle was connected to the nano-LC system via a 1,000 mm × 10 µm i.d. bare fused-silica capillary by a low void volume connector (type P 720, Upchurch Scientific, Oak Harbor, WA, USA) which was integrated in the nano-ESI source. The temperature of the heating block and curved desolvation line were set to 200 °C. The interface voltage was set at 1.7 kV, resulting in a current of ~32 µA.

2.2.6. MS-guided fractionation

For purification, normal bore LC was performed using a 100 mm × 4.6 mm Symmetry Shield column at a flow rate of 0.5 ml/min. A Gilson 234 was used for sample injection (50 µl). The mobile phases used were the same as in nano-LC with the following gradient: 25 min at 2% B; 25 min linear increase to 95% B; 5 min at 95% B. By means of a post-column Y-split, 90% of the eluent was directed through a Shimadzu LC-10a UV detector (220 nm) to a Gilson 234 fraction collector. Collected fractions were freeze-dried utilizing a centrifugal evaporator at room temperature. To the remaining 10% of the eluent, an additional flow of 50%/50% H\textsubscript{2}O/MeOH with 0.1% FA at 250 µl/min was mixed via a Shimadzu LC-10a pump to obtain favorable ESI flow rates and solvent composition. MS detection was done using ESI in the positive mode with a Q-TOF II mass spectrometer (Micromass, Manchester, UK). A 398 K source temperature, 573 K desolvation temperature, 250 L/h desolvation gas flow, 60 L/h cone gas flow, 17 psi gas cell pressure (i.e. 20 V collision voltage for optimum transfer through collision cell), 3,500 V capillary voltage, and a cone voltage of 30 V were used. The mass range was m/z 50 to 2,000. The data acquisition parameters were 1.0 s spectrum\textsuperscript{-1} in MS profile mode and 0.1 s delay time. Nitrogen (purity 5.0; Praxair, Oevel, Belgium) and argon (purity 5.0; Praxair, Oevel, Belgium) were used as desolvation/cone gas and collision gas, respectively.

2.2.7. Off-line NMR

NMR spectra were recorded on a Bruker Avance III spectrometer equipped with a 5 mm Cryo Probe CPTXI with z-axis gradients and automatic tuning and matching accessory (Bruker Biospin, Rheinstetten, Germany). The spectrometer was operated via Bruker TopSpin 2.1 software, running under CentOS. Data was processed using TopSpin 2.1 running under Linux. The resonance frequency for \textsuperscript{1}H NMR was 599.76 MHz and for \textsuperscript{13}C 150.82 MHz. All spectra were recorded in methanol-d\textsubscript{4} at 298 K. Standard Bruker parameter settings – with the pulse sequences indicated in brackets according to the Bruker nomenclature – were used for recording of \textsuperscript{1}H (zgpr), \textsuperscript{1}H–\textsuperscript{1}H COSY (cosygpprf), \textsuperscript{1}H–\textsuperscript{13}C TOCSY (mlevphpfr), \textsuperscript{1}H–\textsuperscript{13}C HSQC (hsqctgpsi) and \textsuperscript{1}H–\textsuperscript{13}C HMBC (hmbcpgndqf) NMR spectra. Presaturation of the residual water resonance was performed prior to the \textsuperscript{1}H-1D and \textsuperscript{1}H-2D NMR experiments. All 2D NMR experiments were recorded with 2K data points in the direct dimension and 128 data points in the indirect dimension.
The spectra were processed using zero filling such that data matrices of 2K x 1K were obtained. The spectra were calibrated against the residual solvent signal of methanol-d$_4$; δ $^1$H 3.31 ppm, δ $^{13}$C 49.0 ppm.

2.3. **Biochemical assays and samples**

2.3.1. **Biochemical assay**

Fresh solutions of 5 nM Ls-AChBP and 15 nM DAHBA were made every day by dissolving in a bioassay solution containing 1 mM KH$_2$PO$_4$, 3 mM Na$_2$HPO$_4$, 0.16 mM NaCl, 20 mM trizma base/HCl at pH 7.5 and 400 µg/mL ELISA BR. The bioassay solution was kept in a 2.5 mL syringe (type 1002LTN, Hamilton, Bonaduz, Switzerland) at 4 °C. The on-line bioassay itself was performed at 22 °C.

2.3.2. **Radioligand binding assay**

RBAs for human α7-nAChR were performed as reported previously. α7-Receptor expressing SH-SY5Y cells cells were harvested, washed with PBS and washed 3 times with ice-cold PBS by centrifugation at 4 °C. Aliquotted cell pellets were stored at -80°C. Frozen cell pellets were dissolved in ice-cold PBS, homogenized and sonicated immediately before use and added to the assay. The final volume of binding buffer suspension with nAChR-rich membranes from SH-SY5Y cells was 100 µL. The buffer was a 1.4 mM KH$_2$PO$_4$, 4.3 mM Na$_2$HPO$_4$, 137 mM NaCl, 2.7 mM KCl, 20 mM Trizma base, 0.05% Tween 20 buffer at pH 7.4. As radioligand, 3H-MLA ($K_d = 1.81$ nM) was used at a concentration of 2.5 nM. The SH-SY5Y homogenate was incubated with $10^{-3}$–$10^{-10}$ M of ligand. Crude *Bufo alvarius* skin extracts were dissolved at 300 µg of crude mixture per 100 µL assay buffer; for an (arbitrary) compound concentration of $10^{-3}$M, assuming skin extract compounds average at 300 Dalton. After an incubation period of 1.5 hours under continuous shaking, bound radioligand was collected on 0.3% polyethyleneimine-pretreated Unifilter-96 GF/C filters (Perkin Elmer, Waltham, USA) and washed using ice-cold washing buffer (4 °C) consisting of 50 mM Tris-HCl buffer at pH 7.5. After the filters were dried, scintillation fluid (MicroScint, Perkin Elmer) was added after which radioactivity was measured in a Wallac 1450 MicroBeta liquid scintillation counter (Perkin Elmer). To determine non-specific binding, radioligand saturation experiments were performed with nicotine. The concentration of nicotine used was 1 mM. Binding assay data was analyzed using Prism 5.0 (Graphpad Software, Inc., San Diego, California, USA).

2.3.3. **Conus textile venom**

Lyophilized venom sample from *Conus textile* was acquired as described by Dutertre et al. Lyophilized venom was dissolved in water/MeOH 95:5% at a concentration of 5 mg/mL and subsequently centrifuged at 13,400 rpm for 10 min to remove particulate matter. Aliquots of these samples were stored at -20 °C until use. Before analysis, nicotine was added as internal standard at an end concentration of 40 µM.

Venom peptide cysteine bridges were reduced by adding 2 µL of the 5 mg/mL venom mixture to 18 µL reduction solution, which was then allowed to react at 50 °C for 45 min. The reduction solution contained 1 M DTT and 2 M guanidine-HCl, buffered at pH 8.5 by 50 mM NH$_4$HCO$_3$. The reduced venom was then analyzed by nano-LC-MS. During the LC-MS analysis of the reduced venom mixture, the nano-LC unit remained
hyphenated to the bioaffinity screening setup. This served as an indication whether the reduced peptide(s) remained bioactive. Full MS spectra were acquired between \( m/z \) 150 and 3,000. Data-dependent MS\(^2 \) data were obtained with a precursor ion isolation width of 3.0 atomic mass units and product ions between \( m/z \) 150 and 2,000. In a subsequent analytical run, MS\(^2 \) data was targeted for the reduced \( \alpha \)-TxIA peptide at \( m/z \) 831.360 (from ions obtained between \( m/z \) 150 and 1,800 in the ion trap). MS\(^2 \) product-ion \( m/z \) values were compared with theoretical \( \alpha \)-TxIA peptide fragments as calculated by the University of California, San Francisco’s (UCSF’s) protein prospector. Theoretical fragment calculation was based on the \( \alpha \)-TxIA sequence as published by Dutertre et al.\(^42\).

2.3.4. Toad skin excretion extracts
Lyophilized skin secretion samples from *Bufo alvarius* and *Bufo marinus* were dissolved in water/ACN 99:1 and 0.1% TFA at a concentration of 10 mg/mL and subsequently centrifuged at 13,400 rpm for 10 min. Aliquots of these samples were stored at –20 °C until further use. Before analysis, three reference peptides at an end concentration of 2 \( \mu \)M each were added. The resulting solutions were directly injected in duplicate onto the nano-LC system for parallel bioaffinity screening and MS identification. Samples were re-analyzed at lower or higher concentrations whenever necessary. The three reference peptides were used to align MS data between different runs, whenever necessary. The three reference peptides were [Met\(^5\)] enkephalin, human angiotensin I, and Mca-Lys-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg-NH\(_2\), which were detected at \( m/z \) 627.146, 648.848, and 611.307, respectively.

3. Results and Discussion
This work describes the application of an efficient analytical HRS platform for the bioaffinity profiling and subsequent MS-guided purification of compounds from natural sources. Previously, we showed its applicability to the bioaffinity profiling of neurotoxic snake peptides (between 60 and 90 amino acids) for Ls-AChBP binding.\(^39\) Here, profiling is demonstrated for small peptides and small bioactive molecules in neurotoxic cone snail venoms and in extracts of toad skin excretions, respectively. In this extended workflow, the toxins identified with the miniaturized HRS platform were subsequently isolated by ‘normal-bore’ LC using MS-guided fractionation. Thereafter, isolated small molecular ligands were structurally elucidated by NMR and biologically assessed using an RBA for assessing human \( \alpha 7 \)-nAChR binding.

The miniaturized analytical screening setup was used as described by Heus et al.\(^39\). Half of the 400 nL/min effluent from the nano-LC is split to the nano- ESI interface. The other half is hyphenated to the microfluidic chip where it is continuously mixed with the bioassay solution, which is infused at 5 \( \mu \)L/min by a syringe pump. The chip outlet is connected to the confocal LED-induced fluorescence detector, which monitors the eluting toxins for bioaffinity after in-flow and in-chip incubation. The biochemical assay is based on fluorescence enhancement in which the tracer ligand DAHBA shows increased fluorescence when bound to the AChBP. Eluting ligands, when competing with DABHA for Ls-AChBP binding, are observed as a negative peak. Identified bioactives may then be subsequently purified by normal-bore LC–MS, guided by the accurate mass of the ligands, for further pharmacological studies on human nAChRs. This subsequent
MS-guided purification setup is shown in Figure 1.

3.1. Method evaluation for cone snail venoms

To evaluate the applicability and performance of the miniaturized methodology for the screening of bioactive peptides in snail venom, a dilution series of the venom was first analyzed in order to assess sensitivity, repeatability, and correlation of peak shapes observed in MS and the biochemical assay, as well as the (post-column) band broadening. This was done by injecting 0.1, 0.5 and 2.5 μg of Conus textile venom in a 500 nL sample (Supporting Information, Figure S1). In Conus textile venom, one AChBP ligand was found. The analysis of 2.5 μg venom showed some peak broadening, but not as extreme as was observed for some high-affinity snake toxins.\(^{39}\) Previously, we demonstrated that a purified cone snail (Conus imperialis) peptide was detected with high sensitivity and with not much peak broadening in the bioassay. Some tailing in the bioassay is observed, which is common for on-line bioassays as the response is sigmoidal (see Heus et al.\(^{39}\)). Here, we found a similar peak shape in the bioassay of the crude Conus textile venom analysis for the bioactive peptide. From parallel MS analysis, the correlated \(m/z\) of the bioactive peptide was rapidly identified, as shown in Figure 2. The peak in the extracted ion chromatogram (EIC) of the ion with \(m/z\) 829.347 correlated to the bioassay peak of the cone snail peptide. The estimated limit of detection of the bioactive peptide was around 0.5 μg venom in 0.5 μL injected.

![Figure 1. Schematic diagram of the MS-guided purification. Venom is injected for analysis (1). After (large bore) LC (2), a split allows eluting toxin constituents to collection vials for NMR analysis (3) with help of a 1:9 post-LC split and to go to ESI-MS for monitoring of toxin mass (4).](image)

3.2. Cone snail toxin screening

Correlation of the MS data with the bioaffinity data (Figure 2) conveniently identified the binding conotoxin peptide amongst the over 1000 peptides present in the cone snail venom\(^2\). The 2\(^+\)-ion with \(m/z\) 829.347 (Figure 2; insert) matches with the known \(\alpha_7\)-nAChr ligand \(\alpha\)-TxIA with (GCCSRPPCIANNPDLC, amidated C-terminal, theoretical \(m/z\) of \([M+2H]^{2+}\) is 829.338, mass error + 9 mDa), found by Dutertre et al.\(^{42}\). For further
confirmation of the peptide identity, the whole venom was reduced with DTT and then analyzed in data-dependent MS² analysis (Figure 3). The ion with \( m/z \) 829.347 was no longer found, but a \( 2^+ \)-ion with \( m/z \) 831.359 (mass error +6 mDa) emerged, i.e., the original peptide \( m/z + 2 \), which corresponds to an accurate peptide mass of 1661.694 Da. Figure 3 shows the MS² spectrum of this peptide in the venom extract and in the inserts the total ion current (TIC) chromatogram (A) and the MS¹ spectrum of the relevant chromatographic peak (B). The \( m/z + 2 \) after reduction in the \( 2^+ \)-ion indicates the reduction of two cysteine bridges, which is in line with the peptide \( \alpha \)-TxIA.

![Figure 2. Typical result of a crude snail venom analysis where 2.5 μg of crude Conus textile venom was injected in a 500 nL sample. High resolution MS¹ analysis and on-line bioassay data were obtained in parallel. This analytical run obtained one ligand, identified as \( \alpha \)-TxIA by the high resolution MS¹ analysis. 40 μM nicotine functioned as an alignment compound between the MS and the bioassay trace (the split nicotine peak is due to the addition of 5% MeOH to the sample; the chromatographic run started at 5% ACN). The binding signal in the bioassay trace aligned with the doubly charged ion with \( m/z \) 829.347 (mass spectrum is shown in the insert), which corresponds to a monoisotopic mass of 1656.678 Da.](image)

The partial sequence of the peptide can be derived from the MS² spectrum, as shown in Figure 3. In fact, this experiment shows a strategy for confirmation of the presence of disulfide bridges of a found bioactive peptide, based on reduction of all disulfide bridges: after reduction by DTT, the bioactive peak should disappear, and another peak should appear somewhere in the chromatogram, featuring an \( m/z \) shift corresponding...
to the number of disulfide bridges present. Sequencing of this reduced peptide can be achieved by MS². As we confidently identified the peptide as being the α-TxIA peptide based on accurate mass, the presence of the two expected disulfide bridges and the (partial) sequence information from the MS² data (see Figure 3), and because the peptide was already characterized by Dutertre et al., we decided not to proceed with subsequent purification in this case.

Figure 3. Data-dependent MS² spectrum analysis of the reduced α-TxIA peptide. Fragments that can be attributed to α-TxIA are marked. The doubly charged peptide with m/z 831.360 results in a peptide mass of 1661.689 Da, which is in good agreement with the previously published mass of 1661.67 Da of α-TxIA. The inserts show the total ion current chromatogram and the extracted ion current of m/z 831.354 (insert A) and the MS¹ spectrum of the corresponding chromatographic fraction (insert B).

3.3. Screening of toad skin excretions

For the screening of toad skin excretion extracts, no nicotine was added to the samples as it could interfere with the detection of small-molecule ligands. In the lyophilized skin excretions (5 μg) of the *Bufo marinus* and *Bufo alvarius*, a total of six AChBP ligands were found. Both species exhibited almost identical binding profiles, as shown in Figure 4 and Figure S2 in the supporting information for *Bufo marinus* and *Bufo alvarius*, respectively. The ligands found in *Bufo marinus* were fractionated by normal-bore LC-MS. The advantage of using MS-guided purification is that with MS analysis we can plot EICs of m/z values seen in the area of fraction collection. Plotting the EICs can show the peak purity of our collected fractions, while UV detection does not give information about the purity of the eluting peaks. Once peak purity is known, one can subsequently revert to UV based fractionation as the data obtained from LC-UV-MS can be translated to LC-UV for further easy, cost effective and convenient LC-UV based purification. The ligands found in *Bufo marinus* were fractionated by normal-bore LC. For the fraction
collection 50 µl 10 mg/ml Bufo marinus skin extract sample was injected to the LC. The isolated compounds were then analyzed in a RBA to assess real α7-nAChR binding. Again, AChBP proved to be a valid homologue of the α7-nAChR binding pocket, as the identified ligands showed binding affinity to the α7-nAChR in radioligand displacement assay. NMR data were acquired for additional structural elucidation/confirmation.

In MS analysis, the first bioactive compound (at a retention time of 8.5 min) showed an ion with m/z 303.168, consistent with a most-likely formula of C$_{12}$H$_{23}$N$_{4}$O$_{5}$ (mass error –1.9 mDa). Although an information-rich MS$^2$ spectrum was obtained, we did not succeed in interpreting the data to retrieve the identity of this compound (Supporting information, Figure S3). Structure proposals of the second (minor) bioactive (10.0 min; m/z 205.098; C$_{11}$H$_{13}$N$_{2}$O$_{2}$; +0.8 mDa) and third bioactive compound (10.5 min; m/z 219.112; C$_{12}$H$_{15}$N$_{2}$O$_{2}$; –0.7 mDa) could only be made upon interpretation of the MS$^2$ spectra (Figure S4 and S5 in supporting information). Both compounds, assuming the structure proposals are correct, have not been described in relation to this toad venom. Unfortunately, for these three minor bioactivity peaks, too many co-eluting compounds were present to obtain sufficiently pure fractions for unambiguous NMR analysis.

The fourth bioactive compound (m/z 203.118; C$_{12}$H$_{15}$N$_{2}$O$^+$; mass error within 1 mDa), co-eluting with compound 3, was identified as dehydrobufotenin (Figure 5), a well-documented bufotoxin$^{43}$. In MS$^2$, the subsequent losses of two methyl radical (CH$_3^\bullet$) confirmed the dimethyl-substituted quaternary ammonium character of this compound (Supporting information Figure S6). NMR analysis confirmed these findings (see Section 3.4. and Supporting Information, Figure S7A–C).

The fifth bioaffinity signal at 58.0 min correlated to a compound that was identified as marinobufagin (m/z 401.234; C$_{24}$H$_{33}$O$_{5}$, mass error +5 mDa; in some cases appearing as a proton-bound dimer [2M+H]$^+$ with m/z 801.460), also reported by Gao et al.$^{44}$ (Figure 5). The MS$^2$ spectrum (Supporting Information, Figure S8) is in agreement with data reported earlier$^{14, 45}$. NMR data supported these findings (see Section 3.4. and Supporting Information, Figure S9A,B).

The sixth bioaffinity signal at 62.0 min correlated to a compound with m/z 713.414 (C$_{38}$H$_{57}$N$_{4}$O$_{9}$, mass error +2 mDa). The molecular formula is consistent with arginine-suberoyl marinobufagenin, reported by Yoshika et al.$^{46}$ (Figure 5). The MS$^2$ spectrum shows the expected loss of the marinobufagenin part of the molecule with charge retention on the arginine-suberoyl part (Supporting Information, Figure S10), consistent with data reported by Yoshika et al.$^{46}$. Subsequent losses involve typical small molecule losses (H$_2$O, CO, NH$_3$, and CH$_2$N$_2$) expected for the arginine-suberoyl part. A definitive confirmation of the structure by NMR was not possible because of the low signal-to-noise ratio of the NMR spectrum (see Section 3.4).
3.4. NMR data on toad fractions

Compounds analyzed by NMR are numbered below according to the description in the results and discussion based on the nano-LC elution order.

**Compound 4 (m/z 203.118)** The proton spectrum of compound 4 exhibited three characteristic dehydrobufotenin signals in the aromatic region at δ 7.33 ppm (1H, d, J = 8.6 Hz), δ 7.15 ppm (1H, s) and δ 6.83 ppm (1H, d, J = 8.6 Hz). In the aliphatic region, two methylene signals at δ 4.06 ppm (2H, t, 5.9 Hz) and δ 3.33 ppm (2H, t, 5.9 Hz) and two methyl signals both at δ 3.73 ppm (6H, s) were observed, confirming the structure to be dehydrobufotenin. Carbon assignments were corroborated by 1H, 13C-HSQC and
\(^1\text{H},\, ^{13}\text{C}-\text{HMBC} \) experiments (Supporting Information, Figure S7A-C).

**Compound 5:** \((m/z \, 801.460; \text{proton-bound dimer})\) Compound 5 has been assigned as marinobufogenin. The essential signals in \(^1\text{H}-1\text{D} \) and \(^1\text{H}-2\text{D} \) spectra are the \(\alpha\)-pyrone signals at \(\delta \, 7.90 \, \text{ppm} \, (1\text{H, dd, } J = 9.7, \, 2.4 \, \text{Hz}), \delta \, 7.45 \, \text{ppm} \, (1\text{H, dd, } J = 2.4, \, 1.0 \, \text{Hz}) \), and \(\delta \, 6.26 \, \text{ppm} \, (1\text{H, dd, } J = 9.7, \, 1.0 \, \text{Hz}) \), the epoxy signal \(15\text{CH} \) at \(\delta \, 1\text{H} \, 3.61 \, \text{ppm} \, (1\text{H, d, } J = 8.5 \, \text{Hz}) \) and two methyl signals at \(\delta \, 0.79 \, \text{ppm} \, (3\text{H, s}) \) ppm and \(\delta \, 0.97 \, \text{ppm} \, (3\text{H, s}) \). The specific carbon resonances were observed in \(^1\text{H},\, ^{13}\text{C}-\text{HSQC} \) or \(^1\text{H},\, ^{13}\text{C}-\text{HMBC} \) at \(\delta \, 60.7, \, 75.7 \, \text{ppm} \) for the epoxy and \(\delta \, 124.3, \, 164.6 \, \text{ppm} \) for the \(\alpha\)-pyrone. Almost all of the steroidal signals are doubled as also seen in compound 6. Compound 5 and 6 were most probably purified as two co-eluting fractions as compound 6 was found in compound 5, and vice versa. As this did not interfere with the NMR elucidation and NMR was performed to assist the MS measurements, further purification attempts were not done. Although the resonance of \(18\text{CH} \) were also doubled, most of the extra signals originated from a modification around C3/C5 as could be observed in 2D TOCSY, HSQC and HMBC data (Supporting Information, Figure S9A,B).

**Compound 6:** \((m/z \, 713.414)\) Only the steroidal specific shifts for \(18\text{CH} \) and \(19\text{CH} \) at resp. \(\delta \, 0.79 \, \text{ppm} \) and \(\delta \, 1.00 \, \text{ppm} \) were observed for compound 6. Those chemical shifts are close to those of marinobufagenin (compound 5) but slightly modified, probably in the A or B- ring. The signals of the low content compound 6 are doubled in a 60/40-ratio. The major signals are in the aromatic region equal to the signals of compound 5. In addition, the \(^1\text{H} \) chemical shift at 0.78 ppm corresponding to \(18\text{CH} \) is identical to the one in compound 5. The signal of H15 was not observed at the same shift. The chemical shift of \(19\text{CH} \) at 1.00 ppm is slightly higher than the observed \(19\text{CH} \) shift in marinobufagenin at 0.97 ppm. This might indicate that the suberoyl is added at position 3. Nevertheless, the compound is marinobufagin-like and modified in the A and B-ring indicating di-suberoyl marinobufagin, which is in accordance with the MS data.

### 3.5. *Bufo alvarius* bioaffinity assessment

Our current HRS approach aims at rapid assessment of binders. Therefore, the (usually low) amount of each bioactive compound isolated can only be roughly estimated and a quantitative assessment of relative binding potencies cannot be made. We estimate that between 0.05 and 0.5 mg compound was collected, which is sufficient for NMR analysis and an initial assessment of binding to nAChR in a RBA. First, the crude *Bufo alvarius* skin excretion sample was analyzed by a traditional RBA for \(\alpha7\)-nAChR to assess binding potency of included compounds. This showed 50% binding at a concentration of ~3 mg/mL. Serial dilutions were prepared for the purified fractions 1, 2, 4, 5 and 6. For all fractions, RBA show binding to nAChR, except for fraction 6 (at the current total amount of compound isolated and re-dissolved).

### 4. Conclusions

This work demonstrates that the developed miniaturized on-line HRS system was able to identify a bioactive peptide in the *Conus textile* with AChBP affinity amongst > 1000 (small) peptides. The current methodology was able to identify the bioactive conotoxin within two rapid analytical runs (60 min each). It does so robustly and is able
to use minute amounts of venom (2.5 μg). Therefore, the HRS platform enables efficient screening for bioactives in natural extracts which are available only in low sample amounts, e.g., spider and scorpion venoms. To show that this platform is also applicable to screen non-peptide small compounds (molecular weight of 200 to 1000 Da), skin secretions from the Colorado River toad (*Bufo alvarius*) and Cane toad (*Bufo marinus*) were analyzed. The results demonstrated several tryptamine-like and steroidal ligands, which, to our knowledge, have as of yet never been correlated with AChBP (and/or nAChR) affinity. By extending our workflow with a rapid analytical purification, NMR analysis, and rescreening in either the miniaturized screening system or a conventional RBA, we were able to isolate bioactive toxins from natural extracts in a straightforward and sample conserving way for initial and rapid chemical and biochemical assessment.

**Acknowledgments**

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44. Gao, H.; Zehl, M.; Leitner, A.; et al. Comparison of toad venoms from different *Bufo* species by HPLC and LC-DAD-MS/MS. *J Ethnopharmacol* 2010, 131 (2), 368-76.


Supporting information

Figure S1. On-line bioassay analysis of 3 consecutive 500 nL sample injections containing 0.1, 0.5 and 2.5 μg crude *Conus textile* venom. The 0.1 and 0.5 μg venom injections also contained 40 μM nicotine to align the MS – and bioassay trace.

Figure S2. An analysis of a 500 nL sample containing 5 μg *Bufo alvarius* skin secretion extract obtained identical binding signals and attributed masses as the *Bufo marinus* extract analysis.
**Figure S3.** MS² spectrum of the unknown bioactive compound with \( m/z \) 303.168, eluting at 8.5 minutes. In the table below Figure S3, the proposed molecular formula, and proposed fragmentation of the compound is shown.

**Table S1.** The proposed molecular formula, and proposed fragmentation of the compound is shown for the unknown bioactive compound with \( m/z \) 303.168.

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<th>( m/z )</th>
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<th>Interpretation</th>
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<td>91.361</td>
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Figure S4. (a) MS² spectra of the bioactive compound with \( m/z \) 205.098, eluting at 10.0 min.

Figure S4. (b) The proposed structure, and proposed fragmentation scheme of the bioactive compound with \( m/z \) 205.098, eluting at 10.0 min.
**Figure S5. (a)** MS2 spectrum, proposed structure, and proposed fragmentation scheme of the bioactive compound with m/z 219.112, eluting at 10.5 min.

**Figure S5. (b)** Proposed structure and proposed fragmentation scheme of the bioactive compound with m/z 219.112, eluting at 10.5 min.
**Figure S6. (a)** MS² spectrum, proposed structure, and proposed fragmentation scheme of the bioactive compound with $m/z$ 203.118, eluting at 10.5 min.

**Figure S6. (b)** Proposed structure, and proposed fragmentation scheme of the bioactive compound with $m/z$ 203.118, eluting at 10.5 min.
Figure S7. (A) HMBC-NMR spectrum of the bioactive compound with m/z 203.118, eluting at 10.5 min. (B) HSQC-NMR spectrum of the bioactive compound with m/z 203.118, eluting at 10.5 min. (C) HSQCHMBC-NMR spectrum of the bioactive compound with m/z 203.118, eluting at 10.5 min.
Figure S8. MS² spectrum of the bioactive compound with m/z 401.235, eluting at 58.0 min.

Table S2. Proposed structure, and proposed fragmentation scheme of the bioactive compound with m/z 401.235, eluting at 58.0 min. Most of the peaks in the MS² spectrum can be readily interpreted, e.g., in terms of water and CO losses, although it is generally unclear where exactly these losses occur.

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Figure S9. (a) COSY-NMR spectrum of the bioactive compound with $m/z$ 401.235, eluting at 58.0 min, (b) TOCSY-NMR spectrum of the bioactive compound with $m/z$ 401.235, eluting at 58.0 min.
Figure S10. (a) $MS^2$ spectrum of the bioactive compound with $m/z$ 713.412, eluting at 62.0 min.

Figure S10. (b) Proposed structure and proposed fragmentation scheme of the bioactive compound with $m/z$ 713.412, eluting at 62.0 min. The minor fragments can be considered as secondary fragments of the ion with $m/z$ 331 and are consistent with subsequent losses of H$_2$O, CO and/or NH$_3$, e.g., loss of water to the ion with $m/z$ 313, loss of NH$_3$ to $m/z$ 296, loss of H$_2$O to $m/z$ 278, and either loss of H$_2$O to $m/z$ 260 or CO to $m/z$ 250. The ion with $m/z$ 253 is consistent with the loss of HN=C=CH and 2×H$_2$O from the fragment ion with $m/z$ 331.
CHAPTER 4

Development of plate reader and on-line microfluidic screening to identify ligands of the 5-hydroxytryptamine binding protein in venoms

Abstract

The 5-HT₃ receptor is a ligand-gated ion channel, which is expressed in the nervous system. Its antagonists are used clinically for treatment of postoperative- and radiotherapy-induced emesis and irritable bowel syndrome. In order to better understand the structure and function of the 5-HT₃ receptor, and to allow for compound screening at this receptor, recently a serotonin binding protein (5HTBP) was engineered with the Acetylcholine Binding Protein as template. In this study, a fluorescence enhancement assay for 5HTBP ligands was developed in plate-reader format and subsequently used in an on-line microfluidic format. Both assay types were validated using an existing radioligand binding assay. The on-line microfluidic assay was coupled to HPLC via a post-column split which allowed parallel coupling to a mass spectrometer to collect MS data. This high-resolution screening (HRS) system is well suitable for compound mixture analysis. As a proof of principle, the venoms of Dendroapsis polylepis, Pseudonaja affinis and Pseudonaja inframacula snakes were screened and the accurate masses of the found bioactives were established. To demonstrate the subsequent workflow towards structural identification of bioactive proteins and peptides, the partial amino acid sequence of one of the bioactives from the Pseudonaja affinis venom was determined using a bottom-up proteomics approach.
1. Introduction

5-Hydroxytryptamine (5-HT, serotonin) is a neurotransmitter acting in the peripheral and central nervous systems. In the brain, it is involved in diverse types of functions, such as anxiety responses, learning and memory, sleep, and behavior\(^1\). There are at least 15 types of 5-HT receptors belonging to the G-protein coupled receptors. In contrast, there is only one type of 5-HT\(_3\) receptor (5-HT\(_3\)R), consisting of two types of subunits\(^2\), which belongs to the Cys-loop family of pentameric ligand-gated ion channels (pLGIC)\(^3\)\(^-\)\(^4\). Besides 5-HT\(_3\)R\(_{A/B}\), the Cys-loop receptor family includes \(\gamma\)-aminobutyric acid A (GABA\(_A\)) receptors, glycine receptors (GlyRs) and nicotinic acetylcholine receptors (nAChRs)\(^5\). Antagonists of 5-HT\(_3\)R are in use in the clinic as anti-emetics to control chemotherapy-induced and postoperative nausea and vomiting. This receptor is also validated as a drug target for irritable bowel syndrome and it is has been suggested to play a role in various brain disorders, such as schizophrenia and anxiety\(^6\)\(^-\)\(^7\).

Screening for novel ligands acting on the 5-HT\(_3\)R is usually performed by radioligand binding assays, radioactive ion-flux assays and/or by performing low-throughput electrophysiological patch-clamp studies\(^8\). Since the 5-HT\(_3\)R is an ion channel, cell-based assays which involve measuring the membrane potential using fluorescence dyes have been developed\(^9\). These assays provide useful functional information on potential ligands for the receptor.

Recently, a binding protein was engineered which contains the ligand recognition properties of the 5-HT\(_3\)R. This ligand-binding pocket of the 5-HT\(_3\)R was engineered by mutation in the original scaffold of the \textit{Aplysia californica} acetylcholine-binding protein (AChBP)\(^10\). The AChBP is most similar to the extracellular ligand-binding domain of \(\alpha\)7-nAChR\(^11\). The scaffold of the AChBP was a suitable starting point for engineering the 5-HT\(_3\)-binding protein (5-HTBP) because of the high sequence and structural identity of 5-HT\(_3\)R and \(\alpha\)7-nAChR\(^12\). In this regard, many \(\alpha\)7-nAChR ligands, such as varenicline\(^13\) and epibatidine\(^14\), bind to the 5-HT\(_3\)R as well, and 5-HT\(_3\)R antagonist tropisetron is a selective agonist of \(\alpha\)7-nAChR\(^15\).

In the case of screening complex mixtures, low-throughput bioassay-guided fractionation techniques\(^16\)\(^-\)\(^17\), or newer analytical techniques such as high-resolution screening (HRS) are required\(^18\). High resolution screening (HRS) is a post-column methodology in which a bioassay is coupled directly on-line with a chromatographic separation. Often, via a post-column split, mass spectrometry (MS) is performed in parallel for the identification of compounds. The first HRS systems were developed by the research groups of Przyjazny\(^19\) and Irth\(^20\). One of the recent advances in the field of HRS is the development of miniaturized systems in which nano-LC separation is coupled post-column to an on-line microfluidic assay along with parallel MS detection\(^21\). As microfluidic on-line assays use very low sample volumes, such technologies are most suitable when only small quantities of sample are available for the analysis\(^22\)\(^-\)\(^24\). The major advantage of direct post-column bioaffinity analysis is the capability of analysing individual compounds in mixtures (such as venoms) after chromatographic separation. The parallel MS detection provides mass and MS/MS data for the identification of bioactive compounds observed.

Previously, we developed an assay for AChBP ligands in HRS\(^25\) and miniaturized-HRS format\(^21\). In this study, we took advantage of the homology of AChBP with 5HTBP and developed a fluorescence enhancement based assay for 5HTBP ligands. After optimizing
and validating the assay in a 96-well-plate format, it was transferred to a microfluidic on-line HRS format allowing the analysis of individual bioactives in complex mixtures. The system consisted of nano-LC separation with a post-column split allowing parallel 5HTBP assay and MS detection. This microfluidic on-line assay has the added advantages of needing only small quantities of samples and low consumption of assay materials. The potential of the microfluidic on-line HRS was demonstrated by screening venoms of the snakes *Dendroapsis polylepis*, *Pseudonaja affinis* and *Pseudonaja inframacula* for ligands of the 5HTBP.

2. Materials and methods

2.1. Chemicals and biological reagents

DAHBA ((E)-3-(3-(4-diethylamino-2-hydroxybenzylidene)-3,4,5,6-tetrahydropyridin-2-yl)pyridine), VUF11234 ((E)-1-methyl-5-((2-(pyridin-3-yl)-5,6-dihydropyridin-3(4H)-ylidene)methyl)indoline and VUF10907 ((E)-3-(3-(4-Dimethylaminobenzylidene)-3,4,5,6-tetrahydropyridin-2-yl)pyridine) were synthesized in house as described by Kool et al. VUF10166 was synthesized as described by Thompson et al. ELISA blocking reagent was purchased from Hoffmann-La Roche (Mannheim, Germany). The ULC-MS grade 99.97% acetonitrile (ACN) and 99.95% trifluoroacetic acid (TFA) were obtained from Biosolve (Valkenswaard, the Netherlands). HPLC grade water was produced by a Milli-Q purification system from Millipore (Amsterdam, The Netherlands). NaCl, dithiothreitol (DTT), iodoacetamide (IAM), trypsin from bovine pancreas, Trizma base, KH₂PO₄, Na₂HPO₄, NH₄HCO₃, 5-fluorotryptamine and quipazine were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Granisetron, tropisetron, serotonine-HCl, RS56812, mirtazapine, SR57227, zacopride, iodophenpropit, B-HT920, RS16566 and palonosetron were obtained from Tocris Bioscience - R&D Systems Europe (Abington, Oxon, United Kingdom).

2.2. Expression and purification of 5HTBPs

A1B2D1ᵩ and A1B2D1ᵱ 5HTBP mutants were expressed and purified as described by Kesters et al. In short, the mutagenesis was induced using a QuikChange-based strategy on Ac-AChBP (from snail species *Aplysia californica*). Protein was expressed using a Bac-to-Bac expression system. The protein was purified by affinity chromatography with nickel Sepharose as affinity material (GE Healthcare, Eindhoven, The Netherlands) followed by size exclusion chromatography (Superdex 200 column, GE Healthcare) using a 20 mM Tris buffer of pH 8.0 containing 150 mM NaCl as eluent. Fractions corresponding to pentameric protein were pooled, concentrated to 6 mg/ml and stored at -80°C until use.

2.3. Snake venom samples

Lyophilized venoms from *Dendroapsis polylepis*, *Pseudonaja affinis* and *Pseudonaja inframacula*, *Crotalus adamanteus*, *Crotalus horridus atricaudatus*, *Bitis arietans* and *Bitis nasicornis* were acquired, freeze-dried and sent to us by Ryan Mccleary and Prof. R.M. Kini (National University Singapore). The venom samples (10 mg/ml) were dissolved in...
Milli-Q water/ACN/TFA 95:5:0.1 %. After analysis, the remainder of samples was stored at -20°C for further use, if needed.

2.4. Fluorescence enhancement by anabaseine derivatives with 5HTBP constructs A1B2D1_\text{r} and A1B2D1_\text{w}

The fluorescence enhancement properties of three anabaseine derivatives (Figure S3) bound to the orthosteric binding pocket of two different 5HTBP variants were measured using 1 cm wide glass cuvettes in a Perkin-Elmer LS50B fluorometer (Groningen, The Netherlands). The excitation spectra, $\lambda_{\text{max}}$ (nm), and $\varepsilon$ (L mol$^{-1}$ cm$^{-1}$) values of the anabaseine derivatives are described by Kool et al.$^{25}$. The emission spectra from 500-595 nm were recorded with 0.5 nm intervals with a fixed 488 nm excitation.

2.5. Fluorescence Enhancement Microplate Reader Assay

The buffer used for development of the 5HTBP fluorescence enhancement assay consisted of 1 mM KH$_2$PO$_4$, 3 mM Na$_2$HPO$_4$, 0.16 mM NaCl, 20 mM trizma base/HCl at pH 7.5 with addition of 400 $\mu$g/mL ELISA BR 21. The fluorescence enhancement was measured with a Victor3 Microplate reader from Perkin-Elmer. The excitation and emission wavelengths were set to 485 and 520 nm, respectively. Black-bottomed 96-well microplates were purchased from Greiner Bio-One (Alphen aan den Rijn, The Netherlands). The final assay volume was 100 $\mu$L per well. The 5HTBP was first mixed with the ligands, and then with the tracer ligand. The assay mixture was incubated for 5 min before the measurement. The pharmacological validation was performed by measurement of IC$_{50}$ curves of twelve 5-HT$_3$R ligands and comparing the results obtained with the results obtained from a radioligand binding assay (see below). The $z'$ factors for the determination of assay quality were calculated according to Zhang.$^{29}$

2.6. Radioligand Binding Assay with [3H]-granisetron

The competitive radioligand-binding assay was performed as described by Kesters et al 2013.$^{10}$ His-tagged A1B2D1_\text{r} 5HTBP was diluted in buffer (10 mM HEPES/0.05% Tween) to obtain 50 ng binding protein per well. Serial dilutions of the ligands (10$^{-3}$ to 10$^{-12}$ M from stock concentrations of 10 or 100 mM in DMSO) were used for IC$_{50}$ determinations. The concentration of the radioligand [3H]-granisetron (Perkin-Elmer, specific activity ~ 85 Ci/mmol) was 1.99 nM. After addition of PVT Copper His-Tag SPA beads (final concentration 2 mg/mL), the final well volume was 100 $\mu$L. The plates (white Optiplates, Perkin-Elmer Life Science) were incubated at room temperature under continuous shaking while protected from light for 1.5 h. The SPA beads were then allowed to settle for 3 h in the absence of light before counting in a Wallac 1450 MicroBeta (Perkin-Elmer Life Science, Groningen, The Netherlands). All radioligand binding data were fit by a non-linear, least squares curve analysis procedure using Graphpad Prism (version 5, GraphPad Software).

2.7. Microfluidic on-line assay optimization in nano-LC flow-injection mode

The optimal concentrations of the tracer ligand and the binding protein used in the microfluidic on-line assay were first evaluated in nano-LC flow injection mode (without using an analytical column) in order to measure the pure compounds without
chromatographic retention. The schematic overview of the microfluidic on-line assay in nano-LC flow-injection mode is shown in Supporting information Figure S4A). An Ultimate 3000 nano-LC system (Thermo Scientific, Breda, The Netherlands) was used in combination with the miniaturized bioaffinity detection system. Sample injection volumes were 500 nL. Bioaffinity was measured with a microfluidic fluorescence detection system in which the eluent flow from the nano-LC instrument was first incubated in an on-line 4-µL volume microfluidic chip with the assay mixture consisting of 5HTBP and the fluorescent tracer DAHBA in assay buffer. The assay mixture was continuously fed to the microfluidic system at a flow rate of 5 µL/min using a syringe pump positioned in the dark at 4°C. Infusion was possible for up to 8 h before refilling of the syringe was needed. After on-line incubation, the fluorescence was measured with an LED-induced fluorescence detector. After assay optimization, the pharmacological validation of the on-line assay was performed by measuring IC\textsubscript{50} curves with known 5-HT\textsubscript{3}R ligands. Calculation of the final concentration of ligands in the assay, which is lower than the injection volume due to dilution by the chromatographic separation and in the microfluidic assay, was performed as described elsewhere.

2.8. Microfluidic on-line HRS with snake venoms

The microfluidic on-line HRS system was similar as described (Supporting information Figure S4B). In this system, the nano-LC eluate was split post-column in a 1:1 ratio. One part was directed to the microfluidic fluorescence detection system, and the other part to an ion-trap-time-of-flight (IT-TOF) mass spectrometer equipped with a Picoview nano-Electrospray ionization source (Shimadzu, ’s- Hertogenbosch, The Netherlands). The MS was operated in positive-ion mode with an interface voltage of 1.7 kV, and a heating block and curved desolvation line temperature of 200°C. For chromatographic separation of the snake venom proteins, a 150 mm × 75 µm internal diameter capillary column was packed in-house with Aqua C18 particles (5 µm, 200 Å; Phenomenex, Utrecht, The Netherlands). For gradient elution mobile phase eluent A consisted of water/ACN/TFA 99/1/0.1% and eluent B of water/ACN/TFA 1/99/0.1%. The snake venoms were separated with two nano-LC gradient programs and screened using miniaturized on-line HRS. First, a relatively fast gradient program of 75 min was used: 0-5 min isocratic 5% B; 5-10 min, linear 5-15% B; 10-40 min, linear 15-40% B; 40-50 min, linear 50-70% B; 50-60 min, isocratic 70%; 60-75 min, re-equilibration 5% B. When needed, reanalysis of a venom was performed using a 115-min gradient: 0-5 min, isocratic 5% B; 5-15 min, linear 5-15% B; 15-75 min, linear 15-45%; 75-85 min, linear 45-70% B; 85-100 min, isocratic 70% B; 100-115 min, re-equilibration 5% B. For screening, 500 nL of each snake venom (10 mg/mL) was injected.

2.9. Purification of bioactives from snake venoms

The purification of a bioactive from the Pseudonaja affinis snake venom was performed by using a normal bore LC-MS setup with a 10/90% split to MS analysis and fractionation, respectively. The system details are described elsewhere. In short, the eluents for the LC gradient were pumped with two Shimadzu LC-2AD HPLC pumps at 0.6 mL/min. Mobile phase eluent A consisted of water/ACN/FA 98/2/0.1% and eluent B of water/ACN/FA 2/98/0.1%. For chromatographic separation a C18 column (XBridge, 100 mm x 4.6 mm, 3.5 µm particle size, Waters, Milford, MA) and a linear gradient from 0-90%
B in 20 min was used. The column effluent (90%) was fractionated using a GILSON 235P Autoinjector modified to operate as fraction collector, while 10% of the effluent was directed to a Micromass Q-TOF Ultima mass spectrometer (Waters, Milford, MA). MS measurement was in positive ion-mode using a 150°C source temperature, 200°C desolvation temperature, and 3 kV capillary voltage. Fractions were collected at a rate of 6 s/well into a black 384-well microplate (Greiner Bio One, Alphen aan den Rijn, The Netherlands). The microplate was freeze-dried and stored at -20°C until further use. Only the fractions of interest were re-dissolved for further analysis.

2.10. Tryptic digestion

The freeze-dried fraction of interest was dissolved in 18 µL 25 mM NH₄HCO₃, pH 8 digestion buffer. Present protein was reduced with 2 µL 50 mM dithiothreitol (DTT) at 50°C for 30 min. After reduction, 7 µL digestion buffer was added, and 3 µL 100 mM iodoacetamide (IAM) was added for the alkylation. Finally, 1 µL of 0.1 µg/µL trypsin was added and incubated for 2 h at 37°C. After this incubation, a second aliquot of 1 µL 0.1 µg/µL trypsin was added and the solution was left at 30°C overnight for digestion. The next day, the digestion was stopped by addition of 1% formic acid (final concentration).

The tryptic digests were analyzed by a micrOTOF-Q mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray nano ion source and an Ultimate-3000 nano-LC system (Thermo Fisher Scientific). A 5-mm Pepmap 100 C18 pre-column (300 µm ID, 5 µm particle size, Thermo Fisher Scientific) was used to trap the peptides. The separation was performed on a Acclaim Pepmap RSLC analytical column (75 µm x 15 cm, Thermo Fisher Scientific). Eluent A consisted of water/FA 100/0.1 % and eluent B of water/ACN/FA 20/80/0.1 %. The sample was loaded on the pre-column with 15 µL/min of water/ACN/TFA 98/2/:0.1% for 3 min. After loading, the sample was separated at a flow rate of 600 nL/min applying a 0-30 min linear gradient from 4 to 55% B, and between 30-36 min a linear gradient from 55 to 90% B. Subsequently, 1 min isocratic at 90 % B was followed by column re-equilibration at 4 % B for 10 min. The MS analysis was performed in positive-ion mode (m/z 50-2000) using 1.5 L/min drying gas, 150°C dry heater, 2.5 kV capillary voltage. Data dependent MS2 was performed on the three most abundant ions in the recorded full MS spectra using 25 eV collision energy. The MS data were processed and analyzed with Mascot Distiller (version 2.3.2) and Mascot server (version 2.2) from Matrix Science (London, UK, www.matrixscience.com).

3. Results and Discussion

3.1. Selection of a suitable tracer ligand

We first evaluated three potentially suitable tracer ligands. These benzylidene anabaseines type tracer ligands were shown to have good fluorescence enhancement properties in the AChBP binding pocket. Since the binding pockets of the AChBP and the 5HTBP are similar (as are the binding pockets of the α7-nAChR and the 5-HT₃R), as expected, the benzylidene anabaseines showed significant fluorescent enhancement in the presence of the 5HTBP mutant proteins (Figure 1). Fluorescence enhancement
factors are defined as fluorescence in presence of 5HTBP divided by fluorescence in absence of 5HTBP. VUF11234, DAHBA and VUF10907 showed fluorescence enhancement factors of 6.5, 4.7 and 3.8, respectively, for the A1B2D1R variant. With the A1B2D1w variant, VUF11234 and VUF10907 showed enhancement factors of 3.7 and 2.5, respectively. Although VUF11234 showed the best fluorescent enhancement with the A1B2D1R mutant, it appeared to undergo notable degradation in solution (deduced by MS analysis). DAHBA was therefore selected as most suitable tracer ligand. Because the A1B2D1w 5HTBP mutant showed lower fluorescence enhancement with the benzylidene anabaseines, the A1B2D1R mutant was used for the rest of the study.

![Fluorescence enhancement properties of DAHBA with A1B2D1R 5HTBP.](image)

**Figure 1.** The fluorescence enhancement properties of DAHBA with A1B2D1R 5HTBP. The emission spectrum obtained using excitation at 488 nm is shown between 500 to 595 nm. DAHBA in absence of 5HTBP showed low fluorescence in the 525 nm range (line 2). In presence of D1R 5HTBP the fluorescence was enhanced 4.7 times (line 3). When increasing concentrations (M) of the selective 5-HT3R ligand granisetron was added to the mixture, displacement of DAHBA from the 5HTBP binding pocket occurs, resulting in decreased fluorescence intensity (lines 4-8). The concentrations of granisetron in the figure refer to the addition of 10 µl of the indicated concentration to a 1 ml assay mixture in a 1 cm cuvette.

### 3.2. Fluorescence enhancement assay in microplates

During assay optimization, the high affinity 5-HT3R ligand granisetron was used as competing ligand. In the first experiment, the optimal concentration of 5HTBP was evaluated by comparing no displacement and full tracer displacement at three 5HTBP concentrations (Figure 2A). Increasing concentrations of 5HTBP in the assay evidently enlarged the assay window. The full displacement background signal did not significantly rise upon increasing the 5HTBP concentration. The receptor concentration should be as low as possible in order to allow accurate measurement of high affinity ligands. At the same time, the receptor concentration should be sufficiently high to obtain an adequate assay window. As a compromise, a 5HTBP concentration of 50 nM was chosen, still
providing an acceptable assay window (maximum signal-to-noise ratio, S/N) in plate reader format. When very high affinity ligands ($K_d < 50 \text{ nM}$) have to be measured, a lower binding protein concentration has to be chosen at the expense of a smaller assay window.

The optimal concentration of tracer ligand was determined by generating IC$_{50}$ curves with granisetron using different tracer ligand concentrations (Figure 2B). The optimal concentration of the tracer ligand DAHBA was selected based on an adequate assay window in combination with low background fluorescence. The $z'$-factors were all similar above a tracer concentration of $5.25 \times 10^{-8}$ M, but the best S/N (61.1) and signal-to-background ratio (S/B, 3.36) was obtained with $8.33 \times 10^{-7}$ M tracer concentration (Table 1). This concentration was selected for further plate reader assay optimization and subsequent transfer to the microfluidic on-line assay, providing a $z'$ score of 0.77.

Table 1. Calculated bioassay parameters for different tracer ligand (DAHBA) concentrations.

<table>
<thead>
<tr>
<th>tracer c (M)</th>
<th>$z'$-factor</th>
<th>Dynamic range</th>
<th>S/B</th>
<th>S/N</th>
<th>SW</th>
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<td>$3.33 \times 10^{-6}$</td>
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<td>157302</td>
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<td>4.1</td>
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<tr>
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<td>136610</td>
<td>3.36</td>
<td>61.1</td>
<td>1.5</td>
</tr>
<tr>
<td>$2.08 \times 10^{-7}$</td>
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<td>76749</td>
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<td>38.0</td>
<td>1.4</td>
</tr>
<tr>
<td>$5.25 \times 10^{-8}$</td>
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<td>23738</td>
<td>1.84</td>
<td>13.7</td>
<td>9.9</td>
</tr>
<tr>
<td>$1.3 \times 10^{-8}$</td>
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<td>1.30</td>
<td>1.8</td>
<td>-4.3</td>
</tr>
<tr>
<td>$3.26 \times 10^{-9}$</td>
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<td>1.01</td>
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</tr>
<tr>
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<td>45</td>
<td>1.00</td>
<td>0.01</td>
<td>-5.3</td>
</tr>
</tbody>
</table>

Next, specific and non-specific binding was determined by measuring a tracer saturation curve with and without a displacing ligand. In this experiment, the binding protein concentration was 50 nM while 100 µM granisetron was used as displacing ligand for measurement of the non-specific binding curve. The $K_d$ value was then calculated with the use of Prism software and found to be $2.7 \times 10^{-7} \pm 2.1 \times 10^{-7}$ M. A typical saturation curve was obtained, thus showing specific ligand binding to the binding pocket. The non-specific binding, the total binding curve and the resulting specific binding curve is shown (Figure 2C).

The assay was pharmacologically validated by determining the dissociation constants (Ki) of several 5HTBP ligands with the developed fluorescence enhancement assay and comparing these data with Ki values obtained by a radioligand binding (RLB) assay. Fourteen 5-HT$_3$R ligands, also binding to the 5HTBP 10, were selected for this experiment. The measured Ki values for both the fluorescence enhancement plate reader assay as well as those measured with the radioligand binding assay are listed (Supporting Information Table S1). Figure 2D shows the correlation plot of the Ki’s measured with the two assays. The high correlation ($r^2 = 0.9647$) indicates that the fluorescence enhancement assay is well suited for measuring Ki’s of ligands for the 5HTBP. Furthermore, the affinities measured in both assays are also similar (i.e. they do not all have a same shift to a higher or lower Ki) meaning that similar IC$_{50}$s can be obtained with both assays without the immediate need of calculating Ki’s from IC$_{50}$s.
Figure 2. Development of the fluorescence enhancement assay in microplate reader format. (a) 5HTBP concentration variation (tracer ligand concentration, 10⁻⁷ M). Full tracer displacement was obtained with 100 µM granisetron; (b) Optimization of the tracer ligand concentration by establishing IC₅₀ curves of granisetron using different tracer ligand concentrations. The 5HTBP concentration was 50 nM; (c) Tracer saturation curves. Specific, non-specific and total binding curves are shown; (d) Correlation between the RLB and the microplate reader fluorescence enhancement assay (r² = 0.9647). The measured pKi values are in Supporting Information Table S1.

3.3. Microfluidic on-line assay optimization in nano-LC flow-injection analysis mode

After having optimized the 5HTBP and DAHBA concentrations in microplate reader format and validating and comparing the assay with known ligands, the assay was transferred to the microfluidic on-line assay format. In this format, the non-bound tracer will show a decreased fluorescence when a ligand is displacing the tracer ligand, which will be detected as a negative peak in the assay signal trace. The total signal (height of a negative peak) is measured in flow injection analysis mode, which eliminates any potential interference by the chromatographic column, as the samples directly get into the bioassay allowing testing of the system at different conditions. The concentration of test ligand injected results in full tracer displacement under all conditions to obtain the full bioassay signal under various conditions. The assay window (negative peak height) was increased with higher concentrations of tracer ligand or binding protein in the bioassay. At higher tracer ligand concentrations, there will be increased background fluorescence. In order to minimize the background fluorescence and to avoid using an
unnecessary high concentrations of tracer ligand and binding protein, we optimized their concentrations to keep the background fluorescence at the peak minimum.

Firstly, three different concentrations of 5HTBP were tested (3.0, 7.5 and 15 nM; Figure 3A) with a DAHBA concentration of 240 nM, while 500 nL of 100 µM granisetron was injected as competing ligand. Although the 15 nM 5HTBP showed the largest assay window, the baseline fluorescence was close to the highest measurable intensity for the detector. With 7.5 nM 5HTBP, the assay window was lower, however, the baseline fluorescence decreased significantly and the actual S/N was hardly compromised. A 3.0 nM 5HTBP concentration showed a significantly decreased assay window and S/N. From the three concentrations tested (Figure 3A), the 7.5 nM concentration was selected for use in further experiments.

**Figure 3.** Microfluidic on-line assay optimization using injections of 500 nL 100 µM granisetron. (a) Effect of the 5HTBP concentration (3.0, 7.5 and 15 nM) using a tracer concentration of 240 nM; (b) Effect of the tracer ligand concentration. Duplicate injections are shown for each condition tested. Negative peaks represent displacement of the fluorescent tracer by the ligand. (c), (d), (e) Serial dilutions of granisetron, palonosetron and VUF10166 in the microfluidic on-line assay. The negative peaks represent the bioactivity of the ligands. When the concentration of a ligand reaches binding pocket saturation, the negative peak height will not further increase and the peak becomes broader due to chromatographic tailing and overloading. The concentrations in figure (c), (d) and (e) refer to the injected concentration of the ligand. The bioassay signals on Figure (a) and (b) are non-superimposed, while figure (c) (d) and (e) are showing the signals superimposed.

The tracer ligand concentration in the assay mixture was evaluated in the range of 120-960 nM using a 7.5 nM 5HTBP concentration (Figure 3B). Injections of 500 nL
of a 100 µM granisetron solution were performed in the microfluidic on-line assay. A 120 nM tracer ligand concentration provided a very small assay window. With a tracer concentration of 240 nM, an acceptable assay window was obtained. Larger assay windows were obtained for tracer concentrations of 480 and 960 nM, but the corresponding background fluorescence signals were too high. Therefore, 240 nM was selected for further use in assay evaluation.

After optimization, the assay was pharmacologically validated by measuring semi-quantitative IC50 curves for granisetron, palonosetron, VUF10166, and 5-F-tryptamine (Figure 3C, 3D, 3E). In an on-line format, the concentration actually measured in the assay will be lower than the injected concentration due to addition of the assay mixture and dilution (i.e. band broadening) during the chromatographic process. The dilution due to post-column mixing (DM) follows from the ratio of the ultimate flow through the fluorescence detector and the effluent flow of the nano-LC entering the microfluidic incubation chip. The chromatographic dilution (DC) depends on the observed full width at half maximum (FWHM) of the compound peak, the flow rate in the nano-LC, and the injection volume. The procedure for calculating the ultimate concentration of the tested compound was described previously. For the present on-line 5HTBP assay, the DM was calculated to be 13.5. The DC was calculated for each chromatographic peak separately. Due to the dilution during on-line screening, it was not possible to measure complete IC50 curves. Therefore, the IC50 values were completed by adding a data point for the 100% displacement concentration, which was empirically set at 10-2 M. This way, semi-quantitative IC50s were used to determine the pKi of granisetron, palonosetron, VUF10166, and 5-F-tryptamine. The resulting pKi values correlated well with the pKi values measured with the RLB assay (r² = 0.9598 (Supporting Information Figure S1).

3.4. Microfluidic on-line HRS of snake venoms

After the microfluidic on-line assay was optimized using nano-LC flow injection analysis, a high-resolution mass spectrometer was coupled to the nano-LC system in parallel to the microfluidic on-line assay. With this microfluidic on-line HRS system, several snake venoms were screened for bioactive peptides and proteins. The 5-HT3 ligand palonosetron was analyzed every measurement day to determine the proper functioning of the system and also to establish an accurate time alignment of the bioactivity chromatogram obtained with fluorescence detection and the MS chromatogram. Spiking an internal standard in the venom samples was not preferred because none of the available 5-HT3 ligands was eluting in the dead-volume time and therefore potentially would interfere with the detection of active co-eluting venom components.

With the microfluidic on-line HRS system, we screened the venoms of the following snakes: Pseudonaja inframacula, Pseudonaja affinis, Dendroaspis polylepis, Crotalus Adamanteus, Crotalus horridus atricaudatus, Bitis arietans and Bitis nasicornis. Only the venoms from Pseudonaja inframacula, Pseudonaja affinis and Dendroaspis polylepis snakes were found to contain bioactive peptides that interact with 5HTBP. It had been reported that the Pseudonaja textilis venom contains neurotoxins (pseudonajatoxin a and b, textilotoxin) which are known to have affinity to the neuronal nAChRs. Their affinity to the 5-HT3 receptor, however, has not been established so far, but considering the similarities between the aforementioned receptors it was expected that active binders would be found.
Figure 4. Microfluidic on-line HRS screening of the venom of *Pseudonaja affinis*. The identified ligands: 32.3 min, m/z value of 1244.779 (5+ charges, peptide mass 6218.86 Da); 29.4 min, m/z of 1303.993 (5+ charges, peptide mass 6514.93 Da), and 46.8 min, m/z of 918.745 (4+ charges, peptide mass 3670.95 Da).

Figure 5. Microfluidic on-line HRS screening of *Pseudonaja inframacula*. The identified ligands: 35.1 min, m/z of 1244.779 (5 times charged, 6218.86 Da); 43.4 min, m/z value of 1260.397 (5+ charges, peptide mass 6296.95 Da); 52.0 min, m/z value of 1306.287 (5+ charges, peptide mass 7831.68 Da) or to 1338.006 (5+ charges, peptide mass 6684.99 Da).
Using the new HRS system, a large binding peak eluting at 32.3 min was found in the *Pseudonaja affinis* venom (Figure 4). After correlation with the parallel MS chromatogram, it was found that this bioactive shows an ion with \( m/z \) value of 1244.779 (5+ charges, peptide mass 6218.86 Da). There were two additional ligands found in the venom that showed a low binding peak, eluting at 29.4 and 46.8 min with \( m/z \) values of 1303.993 (5+ charges, peptide mass 6514.93 Da) and 918.745 (4+ charges, peptide mass 3670.95 Da), respectively.

In the venom of *Pseudonaja inframacula*, two large binding and one small binding ligand were found with the on-line HRS screening system (Figure 5). A ligand with a low binding peak eluting at 35.1 min shows an ion with \( m/z \) value of 1244.779 (5+ charges, peptide mass 6218.86 Da). The second bioactive eluting at 43.4 min shows an ion with \( m/z \) value of 1260.397 (5+ charges, peptide mass 6296.95 Da). The third ligand eluting at 52.0 min and could correspond to a venom protein with \( m/z \) values of 1306.287 (5+ charges, peptide mass 7831.68 Da) or to the \( m/z \) value of 1338.006 (5+ charges, peptide mass 6684.99 Da). The mass of the protein with \( m/z \) value of 1306.287 (peptide mass 7831.68 Da) was correlated to the molecular mass of the known toxin pseudonajatoxin-b previously identified from *Pseudonaja textilis*, which is known to bind to the \( \alpha 7 \)-nAChR.

In *Dendroapsis polylepis* venom, we found a bioactive with a small binding peak eluting at 13.0 min, which was correlated to two co-eluting compounds with a \( m/z \) value of 639.271 or 483.255 (1+ charge) (peptide masses 638.26 and 482.25 Da); 29.2 min, \( m/z \) of 1312.864 (5+ charges, peptide mass 6559.28 Da); 33.6 min, \( m/z \) 1202.608 (6+ charges, peptide mass 7209.60 Da) or \( m/z \) 1362.613 (5+ charges, peptide mass 6808.03 Da.).

![Figure 6](image-url)

**Figure 6.** Microfluidic on-line HRS of *Dendroapsis polylepis* venom. The identified ligands: 13.0 min, \( m/z \) value of 639.271 or 483.255 (1+ charge, peptide masses 638.26 and 482.25 Da); 29.2 min, \( m/z \) of 1312.864 (5+ charges, peptide mass 6559.28 Da); 33.6 min, \( m/z \) 1202.608 (6+ charges, peptide mass 7209.60 Da) or \( m/z \) 1362.613 (5+ charges, peptide mass 6808.03 Da.).
peptide mass 6559.28 Da), and at 33.6 min corresponding to two co-eluting proteins with a \( m/z \) of 1202.608 (6+ charges, peptide mass 7209.60 Da) and \( m/z \) of 1362.613 (5+ charges, peptide mass 6808.03 Da).

A list of all identified \( m/z \) values of the bioactives found, with charge state and calculated molecular mass, is shown in Supporting Information Table S2.

### 3.5. Protein identification

To study the feasibility of identifying a bioactive compound detected by the HRS system, we performed in-solution tryptic digestion of the bioactive with an \( m/z \) value of 1244.78 from the *Pseudonaja affinis*. We first refractionated the venom using a conventional bore LC system from which the effluent was post-column split with 10% being analyzed by MS for detecting the compound with \( m/z \) of 1244.78, and 90% being directed to a fraction collector. The fraction with the protein of interest was collected and subjected to tryptic digestion for proteomics analysis by nanoLC-MS (see Experimental section). The MS and MS/MS data obtained were subjected to a Mascot search using the Uniprot.org database which indicated the bioactive to be the Short neurotoxin 3 from *Pseudonaja textilis* (Uniprot number Q9W7K0) with a sequence coverage of 93% and Mascot score of 832 (Supporting Information Figure S2).

### 4. Conclusions

In this work, two new fluorescence-based assays are described, which can be used to find new ligands of the 5-HT\(_3\) receptor and nACh receptor from pure compound libraries and mixture libraries. Both assays use an engineered binding protein, which has the scaffold of the AChBP and the ligand recognition properties of the 5-HT\(_3\) receptor in the binding pocket. The assays were optimized and validated in a micro-plate reader and subsequently transferred to a microfluidic on-line assay format capable of screening mixtures. The assays were found to be robust and well correlating with IC50s measured using a conventional radioligand binding assay.

The main strength of a fluorescence-based bioassay like the assay developed is the cost-effectiveness compared to the radioligand binding assays and the ability to perform in flow-injection mode, which can be coupled post-column to mass spectrometry. These assays are well suitable for mixture analysis. As an application example for the microfluidic on-line assay the screening of several snake venoms for identification of new peptides binding to the 5HTBP was demonstrated. The venoms from *Pseudonaja inframacula*, *Pseudonaja affinis* and *Dendroaspis polylepis* were screened and we successfully pinpointed the accurate masses of bioactives acting on the 5HTBP. The complete workflow of protein identification of bioactive proteins was finally demonstrated with one of bioactives from *Pseudonaja affinis* found during the screening process. Using the accurate mass identified with the microfluidic on-line screening, the venom was subsequently fractionated and the fraction containing the protein of interest was digested with trypsin prior to proteomics based identification of the protein. The Mascot search identified this protein to be Short neurotoxin 3 from *Pseudonaja textilis* (Uniprot number Q9W7K0). In the future these screening assays can be used for screening campaigns to identify novel bioactive compounds from complex mixtures.
Acknowledgments

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). We would like to thank Maikel Wijtmans (Division of Medicinal Chemistry, VU University, Amsterdam) for synthesising and providing the fluorescent tracers used in this work. We would like to thank Marija Mladic for the purification of the bioactive peptide from Pseudonaja affinis venom.
References


Supporting Information

Figure S1. (a) Concentration-response of four 5HT₃ receptor ligands obtained with the microfluidic on-line system. Due to dilution effects in the nano-LC and on-line assay, the actual concentration in the assay is lower than the injected concentration. The dilution factor of ligands injected was calculated as described by Falck et al.²⁶, and then used to estimate the assay concentration of the analyzed ligand; (b) Correlation between the RLB assay and the microfluidic on-line fluorescence enhancement assay ($r^2 = 0.9598$).
**Figure S2.** Mascot results from the tryptic digestion of the bioactive with m/z value of 1244.78 from *Pseudonaja affinis.*
Figure S3. Structure of the three anabaseine derivates tested in the study.

A Microfluidic on-line assay in nano-LC flow-injection mode

B Microfluidic on-line HRS

Figure S4. (a) Schematic view of the microfluidic on-line assay in nano-LC flow-injection mode. With a nano-LC system (1) 500 nL of samples are injected (2). The eluent flow is directed into a microfluidic incubation chip (4) where the sample is mixed with the bioassay mixture, infused in the chip by a syringe pump (3). The fluorescence signal was detected by an in-house built LED-induced fluorescence detector (5). (b) Schematic view of the microfluidic on-line HRS setup. 500 nL of samples are injected (2) and separated with nano-LC (1). After separation by the capillary column (3) the effluent flow was spilt in 1:1 ratio. One part of the flow was directed to a high resolution MS (4), and the other part of the flow was directed into a microfluidic incubation chip (5) where it was mixed with the bioassay mixture infused by a syringe pump (6). After incubation the fluorescence signal was detected by an in-house built LED-induced fluorescence detector (7).
Table S1. Comparison of Ki values measured for 14 5HT₃ ligands using the fluorescence enhancement plate reader assay and the radioligand binding assay.

<table>
<thead>
<tr>
<th></th>
<th>Ki fluorescence enhancement (A1B2D₁,5HTBP mutant) (µM)</th>
<th>Ki RLB (A1B2D₁,5HTBP mutant) (µM)</th>
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<tbody>
<tr>
<td>granisetron</td>
<td>0.24 ± 0.14</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>serotonin</td>
<td>103.03 ± 32.01</td>
<td>218.27 ± 24.98</td>
</tr>
<tr>
<td>tropisetron</td>
<td>0.04 ± 0.72</td>
<td>0.01 ± 0</td>
</tr>
<tr>
<td>quipazine</td>
<td>72.98 ± 32.22</td>
<td>53.84 ± 13.74</td>
</tr>
<tr>
<td>VUF10166</td>
<td>11.54 ± 3.58</td>
<td>2.39 ± 0.58</td>
</tr>
<tr>
<td>RS56812</td>
<td>3.57 ± 0.91</td>
<td>1.6 ± 0.34</td>
</tr>
<tr>
<td>mirtazapine</td>
<td>12.82 ± 3.99</td>
<td>9.29 ± 1.87</td>
</tr>
<tr>
<td>SR57227</td>
<td>85.03 ± 7.62</td>
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<td>zacopride</td>
<td>4.05 ± 1.14</td>
<td>1.62 ± 0.57</td>
</tr>
<tr>
<td>iodophenpropit</td>
<td>1.44 ± 0.2</td>
<td>0.23 ± 0.06</td>
</tr>
<tr>
<td>B-HT920</td>
<td>3.85 ± 0.76</td>
<td>0.93 ± 0.24</td>
</tr>
<tr>
<td>RS 16566</td>
<td>3 ± 0.02</td>
<td>0.38 ± 0.09</td>
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<tr>
<td>5-fluorotryptamine HCL</td>
<td>110.35 ± 14.03</td>
<td>100.95 ± 50.19</td>
</tr>
<tr>
<td>palonosetron</td>
<td>2.2 ± 0.63</td>
<td>0.51 ± 0.11</td>
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Table S2. Mass of bioactives binding to 5THBP found in snake venoms.

<table>
<thead>
<tr>
<th>Species</th>
<th>Most abundant m/z</th>
<th>Charge state</th>
<th>~Nominal mass (Da)</th>
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<tbody>
<tr>
<td>Pseudonaja affinis</td>
<td>1244.779</td>
<td>5</td>
<td>6218.86</td>
</tr>
<tr>
<td>Pseudonaja affinis</td>
<td>1303.993</td>
<td>5</td>
<td>6514.93</td>
</tr>
<tr>
<td>Pseudonaja affinis</td>
<td>918.745</td>
<td>4</td>
<td>3670.95</td>
</tr>
<tr>
<td>Pseudonaja inframacula</td>
<td>1244.779</td>
<td>5</td>
<td>6218.86</td>
</tr>
<tr>
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<td>1260.397</td>
<td>5</td>
<td>6296.95</td>
</tr>
<tr>
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<td>1306.287</td>
<td>6</td>
<td>7831.68</td>
</tr>
<tr>
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<td>1338.006</td>
<td>5</td>
<td>6684.99</td>
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<tr>
<td>Dendroapsis polylepis</td>
<td>639.271</td>
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<td>638.26</td>
</tr>
<tr>
<td>Dendroapsis polylepis</td>
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<td>482.25</td>
</tr>
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<td>1312.864</td>
<td>5</td>
<td>6559.28</td>
</tr>
<tr>
<td>Dendroapsis polylepis</td>
<td>1362.613</td>
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<td>6808.03</td>
</tr>
<tr>
<td>Dendroapsis polylepis</td>
<td>1202.608</td>
<td>6</td>
<td>7209.60</td>
</tr>
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</table>
At-line cellular screening methodology for bioactives in mixtures targeting the α7-nicotinic acetylcholine receptor


(* Equal contribution)
Abstract

The $\alpha_7$-nicotinic acetylcholine receptor ($\alpha_7$-nAChR) is a ligand-gated ion channel expressed in different regions of the central nervous system (CNS). The $\alpha_7$-nAChR has been associated with Alzheimer’s disease, epilepsy and schizophrenia, and therefore, is extensively studied as a drug target for the treatment of these diseases. Important sources for new compounds in drug discovery are natural extracts. Since natural extracts are complex mixtures, identification of the bioactives demands the use of analytical techniques to separate a bioactive from inactive compounds.

This study describes screening methodology for identifying bioactive compounds in mixtures acting on the $\alpha_7$-nAChR. The methodology developed combines liquid chromatography (LC) coupled via a split both to an at-line calcium ($\text{Ca}^{2+}$)-flux assay and to high-resolution mass spectrometry (MS). This allows evaluation of $\alpha_7$-nAChR responses after LC separation, while parallel MS enables compound identification. The methodology was optimized for analysis of agonists and positive allosteric modulators, and was successfully applied to screening of the hallucinogen mushroom *Psilocybe McKennaaii*. The crude mushroom extract was analyzed using both reversed-phase and hydrophilic interaction liquid chromatography. Matching retention times and peak shapes of bioactives found with data from the parallel MS measurements allowed rapid pinpointing of accurate masses corresponding to the bioactives.
Introduction

In drug discovery, screening of natural extracts plays an important role in finding novel bioactive compounds. After screening, hit compounds are identified and then purified for further individual assessment. Traditional screening workflows have been successful for many years, but are often time consuming\(^1\)-\(^2\). In recent years, modern analytical screening methodologies have been developed and used to analyze natural extracts in a faster and more straightforward manner\(^3\)-\(^4\). These advanced approaches combine separation techniques, mass spectrometry (MS) and bioassays to screen complex mixtures for compounds with desired bioactivity\(^5\). In this regard, three main post-column approaches can be discerned: on-line, at-line and off-line screening. The first two approaches are based on a liquid chromatographic (LC) separation of complex mixtures, followed by parallel MS detection and bioassays. In an on-line screening setup, the bioassay is performed in a continuous flow fashion in parallel and simultaneously with MS detection\(^5\). In at-line approaches, MS detection of a part of the LC effluent is carried out, while the other part in parallel is fractionated onto a well plate on which later a bioassay is performed\(^6\). In off-line screening, after chromatographic separation and fraction collection, bioassays are performed on the fractions collected and subsequently (LC-)MS analysis is performed on the bioactive fractions only. This traditional approach often is called Bioassay Guided Fractionation or - in environmental settings - Effect Directed Analysis\(^7\)-\(^8\). On-line screening is the technique with the shortest overall analysis times, but many bioassays cannot be implemented in an on-line format, such as bioassays with long incubation times, cellular bioassays, and radioligand binding assays. At-line screening methodologies were developed allowing application of bioassays separate from the time and place of the LC-MS experiment\(^9\)-\(^10\). Moreover, in at-line screening methodologies, LC eluents are evaporated from the collected fractions and thus their potential interference with the bioassay is avoided. With respect to off-line approaches, at-line screening has the advantage that MS data are directly obtained in parallel to fractionation and that fractionation is performed in a resolution of seconds (so called nanofractionation) instead of minutes (microfractionation). The latter means that chromatographic resolution is virtually maintained, giving the possibility to reconstruct a so-called bioactivity chromatogram from the bioassay results which allows accurate peak shape and retention time matching with the MS chromatogram.

Various types of bioassays have been applied in modern analytical screening methodologies, such as bioassays using enzymes that convert substrates into fluorescent products, radioligand binding assays, and bioassays with receptors for which fluorescent tracers are available showing fluorescence enhancement when bound to the receptor\(^11\)-\(^14\). Most importantly, functional bioassays can also be implemented in these screening methodologies\(^9\). A functional bioassay gives the advantage of not only measuring binding affinity of a ligand, but also measuring functional activity and allowing distinction between for example agonism, antagonism, and allosteric modulation.

The present study describes the development of an at-line screening methodology for discovery of bioactive compounds in complex mixtures (e.g. natural extracts) targeting the α7-nicotinic acetylcholine receptor (α7-nAChR). The α7-nAChR belongs to the family of ligand-gated ion channels and is expressed in different regions of the central nervous system (CNS), mainly in regions that are involved in memory...
Abnormalities of α7-nAChR have been described in various CNS diseases, such as epilepsy, Alzheimer’s disease, pain, addiction, and schizophrenia. Potentiation of α7-nAChR activity is shown to be beneficial in the treatment of these diseases. Therefore, agonists and positive allosteric modulators (PAMs) of α7-nAChR are broadly investigated as potential drug leads against these CNS diseases.

The methodology developed encompasses LC separation of mixtures with parallel MS detection and nanofractionation onto 96-well plates. The 96-well plates are subsequently freeze-dried and then subjected to a functional α7-nAChR activity bioassay. The bioassay used is a fluorescence-readout Ca²⁺-flux assay using human neuroblastoma SH-SY5Y cells stably over-expressing the α7-nAChR. The methodology was developed, optimized and calibrated using mixtures of known α7-nAChR ligands (i.e. agonists, and PAMs). Then, the methodology was applied to the screening of an extract of a Psilocybe mushroom, which is known for its hallucinogenic effects. The effect of known hallucinogenic compounds, like psilocybin, is associated with their affinity for the 5-hydroxitryptamine receptor, but the activity of compounds in hallucinogenic mushrooms on the α7-nAChR has not been investigated. The developed screening technique allows the direct correlation of bioactivity to the m/z values of the corresponding compounds. This provides a straightforward manner to continue into drug discovery using known masses of the bioactives instead of the need to repeatedly performing time-consuming bioassays during traditional bioassay guided fractionation. The isolation and full chemical and biological characterization of bioactive components from natural extracts is out of scope of this method development article.

2. Materials and methods

2.1. Chemicals and biological reagents

Human neuroblastoma SH-SY5Y cells expressing the human α7-nicotinic acetylcholine receptor were obtained from Christian Fuhrer (Department of Neurochemistry Brain Research Institute, University of Zurich, Zurich Switzerland). Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12), G418 (geneticin), Dulbecco’s Phosphate Buffered Saline (PBS), Fluo-4 NW assay kit including Fluo-4 NW dye mix (component A) and probenicid (component B), Hank’s Balanced Salt Solution (HBSS), fetal bovine serum (FBS), penicillin-streptomycin, and 0.25% trypsin-EDTA were purchased from Life Technologies Europe (Bleiswijk, The Netherlands). Matrigel was obtained from Corning B.V. Life Sciences (Amsterdam, The Netherlands). Nicotine, epibatidine, PNU120596, PNU282987, α-bungarotoxin, acetylcholine and TQS were obtained from Tocris Bioscience (R&D Systems Europe, Ltd. Abingdon, UK). HEPES, scopolamine, poly-L-lysine (PLL) and Triton X-100 were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). The ULC-MS grade acetonitrile (ACN) and 99.95% formic acid (FA) were purchased from Biosolve (Valkenswaard, the Netherlands). HPLC grade water (H₂O) was produced by a Milli-Q purification system from Millipore (Amsterdam, The Netherlands).

2.2. Instrumentation

A schematic overview of the analytical system and setup is shown in Figure 1. Mixtures
were separated by reversed-phase LC (RPLC) or hydrophilic interaction LC (HILIC) followed by parallel MS detection and nanofractionation onto 96-well plates. The fractionated 96-well plates were freeze-dried and then measured in a Ca\(^{2+}\)-flux assay.

**Figure 1.** Schematic overview of the at-line cellular screening methodology. (1) Samples are injected and separated using an HPLC system. After separation, the effluent flow is split in a 90/10 ratio. (2) The 10% portion of the flow is directed to a UV detector followed by a mass spectrometer for identification of the eluting compounds. (3) The 90% portion of the effluent flow is directed to a UV detector followed by a nanofraction collector. (4) The well plate containing the collected fractions is then freeze-dried, and processed for the Ca\(^{2+}\) assay. From there, a reconstructed bioactivity chromatogram is plotted and compared with the parallel LC-MS chromatogram, to correlate bioactivity with accurate mass.

2.2.1. *Reversed-phase liquid chromatography-mass spectrometry (RPLC-MS)*

Solvent A contained 98% H\(_2\)O, 2% ACN, and 0.1% FA; solvent B contained 98% ACN, 2% H\(_2\)O, and 0.1% FA. The following gradient program was used for the analysis of known compounds: 10 min linear gradient from 0-90% solvent B followed by 2.5 min isocratic elution at 90% B and subsequent linear decrease from 90% to 0% solvent B in 0.5 min, followed by column equilibration at 0% B for 5 min. The gradient separations were carried out using two Shimadzu LC-20AD HPLC pumps (‘s Hertogenbosch, The Netherlands) at a 0.6 ml/min flow rate. Samples (50 µL) were injected with a Gilson 235 autoinjector. The samples were separated on an XBridge C18 column (100 mm x 4.6 mm, 3.5 µm particle size; Waters, Milford, MA), which was preceded by an XBridge C18 guard column (10 x 2.1 mm; 3.5 µm particle size; Waters). The temperature of the
column was kept at 37°C by a Jones Chromatography model 7971 column heater. The column effluent was split in a ratio of 90/10. The 90% part of the flow was directed first to an SPD-20A UV-VIS detector and then to a GILSON 235P autoinjector, which was modified to operate as a nano fraction collector. The nanofractions were collected onto 96-well plates with a 6 or 12 s/well resolution. The 10% part of the eluent flow was directed to a Shimadzu SPD-20A UV-VIS detector followed by a Micromass Q-TOF Ultima mass spectrometer (Waters) equipped with an electrospray ionization source (ESI). The MS measurements were performed in positive- ion mode in the m/z range of 150 to 1400. The capillary voltage was set at 3 kV, source temperature was set at 150°C, the desolvation gas temperature was 200°C. The 96-well plates were collected on a Shimadzu SPD-M20A Prominence diode array detector and the column effluent was split in a 10/90 ratio. The 10% portion of the effluent was directed to MS and the remaining 90% was directed to the nanofractionation system. MS measurements were performed with a Shimadzu ion-trap time-of-flight (IT-TOF) mass spectrometer equipped with an ESI source operated in positive-ion mode. The instrument settings were: 4.5 kV spray voltage, 125 °C source heating block and curved desolvation line temperature, 66 kPa drying gas pressure and 1.5 L/min nebulizing gas flow. The scan range was set between m/z 200 and 1200.

2.2.2. Hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS)

The HILIC separation was performed on a UPLC system from Shimadzu ('s Hertogenbosch, The Netherlands) controlled via Shimadzu Lab Solutions software. The sample injection volume was 50 μL provided by a Shimadzu SIL-30AC autoinjector. The eluent gradient was delivered by two Shimadzu LC-30 AD pumps at a total flow rate of 0.6 mL/min using solvents A and B (see above). Separations were carried out on an Atlantis Silica HILIC column (150 x 2.1 mm; 3 μm particle size; Waters). The column was kept at 37°C in a Shimadzu CTD-30A column oven. The gradient was as follows: a linear decrease of solvent B from 50% to 0% in 10 min was followed by 2.5 min at 90% B and then a subsequent decrease of solvent B to 0% in 1 min. Column equilibration at 50% B was done for 5 min. After UV detection (Shimadzu SPD-M20A Prominence diode array detector) the column effluent was split in a 10/90 ratio. The 10% portion of the effluent was directed to MS and the remaining 90% was directed to the nanofractionation system. MS measurements were performed with a Shimadzu ion-trap time-of-flight (IT-TOF) mass spectrometer equipped with an ESI source operated in positive-ion mode. The instrument settings were: 4.5 kV spray voltage, 125 °C source heating block and curved desolvation line temperature, 66 kPa drying gas pressure and 1.5 L/min nebulizing gas flow. The scan range was set between m/z 200 and 1200.

2.3. Biological assay

2.3.1. Cell culture

The SH-SY5Y neuroblastoma cells were maintained in DMEM/F12 media supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 100 μg/mL geneticin as the stable expression selection marker. The cells were cultured in 75 cm² flasks at 37 °C/5% CO2 incubator and passaged every 3-4 days. One day before the Ca²⁺-flux assay was performed, SH-SY5Y cells were seeded in Matrigel-coated, black walled, and clear bottom 96-well imaging plates (Greiner Bio One, Alphen aan den Rijn, The Netherlands). These 96-well plates were pre-coated with 100 µL PLL and incubated for 30 min at 37°C. After the PLL was removed, 2 washing steps were performed with PBS and DMEM/F12. Subsequently, the ice-cold Matrigel (10 mg/mL) was diluted 10 times with DMEM/F12 and 50 µL of the diluted Matrigel was added to each plate well. After 1 h incubation at room temperature, the Matrigel was removed and the 96-well plates were washed two times with DMEM/F12. The cells were then seeded into the Matrigel-coated plates with the density of 80.000 cell/well. These plates were incubated overnight at 37 °C and used
for the Ca\textsuperscript{2+} flux assay the next day.

2.3.2. Ca\textsuperscript{2+}-Flux Assay

On the day of the experiment, the culture medium was removed from the cells incubated overnight in the 96-well plates and the cells were loaded with 100 µL/well assay mix consisting of Fluo-4 NW dye mix (2 times diluted compared to manufacturer’s protocol) and probenecid (2.5 mM final concentration) dissolved in the assay buffer (1X HBSS, 20 mM HEPES, pH 7.4). The cells were then incubated at 37 °C for 30 min in the dark. In the meanwhile, nanofractions were re-dissolved by adding 150 µL/well of assay buffer. The black-walled clear bottom 96-well plate containing the cells and assay mix was marked as the measurement plate, while the 96-well plate containing re-dissolved nanofractions was marked as the reagent plate. All the pipetting steps and measurement of the Ca\textsuperscript{2+}-flux in the cells were performed by a NOVOstar microplate reader (BMG Labtechnologies, Ortenberg, Germany). The response of the cells was measured by following the fluorescence (excitation and emission wavelengths, 485 and 520 nm, respectively) as a function of time.

Screening for agonists and PAMs was performed in two distinct assay formats. For the agonist assay, 30 µl of a known PAM (PNU12059; 10 µM), was first added to the measurement plate. After 90 s incubation time, 30 µl of the sample was added from the reagent plate to the measurement plate and the agonist signal was recorded for 60 s. As a final step, 22 µl 5% Triton-X100 was added to the cells to record the maximum fluorescence signal (F\textsubscript{max}). For the PAM assay the transfer of 30 µl of samples from the reagent plate to the measurement plate was followed by 90 s incubation time and the addition of 30 µl known agonist (nicotine; 100 µM), and after 60 s the addition of 22 µl of 5% Triton-X100.

The data gathered from the microplate reader was analyzed in Microsoft Excel and GraphPad Prism 5 Software (GraphPad Software Inc., San Diego, CA). The signal of each well was presented as the relative signal compared to the F\textsubscript{max} (%F\textsubscript{max}), which was calculated using the formula %F\textsubscript{max} = 100x(F-F\textsubscript{min})/(F\textsubscript{max}-F\textsubscript{min}), where F is the maximum fluorescence signal obtained by an agonist, F\textsubscript{min} is the baseline fluorescence, and F\textsubscript{max} is the maximum fluorescence obtained after the addition of 5% Triton X-100.

2.4. Mushroom extract preparation

Lyophilized \textit{Psilocybe McKennaii} fungi were extracted as described by Thomson\textsuperscript{26}, with modifications. The freeze-dried material was ground to a fine powder using mortar and chisel. Ten gram of this dry material was divided over five 50 mL Costar tubes. To each tube, 50 mL MeOH was added and roller-mixed for 24 hours. After centrifugation at 4000 rpm, the supernatants of the tubes were combined and filtered through a 0.45 µm Whatman cellulose filter (GE Healthcare, Zeist, The Netherlands). Approximately 230 mL MeOH from the initial 250 mL extract was then removed using a vacuum rotary evaporator. The remaining 20 mL extract was freeze-dried to dryness during 72 hours. For analysis, samples were made by dissolving the extract in water/ACN (99:1, \(v/v\)) with 0.1% TFA, followed by centrifugation at 13,400 rpm for 10 min. The supernatant was transferred to a sample vial for screening analysis.
3. Results and discussion

This work describes the development and optimization of an at-line cellular screening methodology for analysis of natural extracts in order to find new compounds acting on the α7-nAChR. For this purpose, LC was hyphenated to parallel MS detection and nanofractionation onto 96-well plates. The nanofractionated 96-well plates were freeze-dried and then used in the Ca\textsuperscript{2+}-flux assay to determine the functional activity of separated ligands binding to the α7-nAChR. The methodology developed was first optimized and validated for use in agonist and PAM assay format, since potentiating of α7-nAChR activity has a therapeutic relevance in CNS diseases. Then, the applicability of the developed methodology was demonstrated with the analysis of a *Psilocybe McKennaii* extract.

3.1. Optimization and evaluation of the cell-based at-line cellular screening setup with known ligands towards α7-nAChR

Before optimization of the bioassay conditions, it is important to consider the nature of the ion-channel activation. Since the α7-nAChR is a ligand-gated ion channel, binding of an agonist will evoke a large Ca\textsuperscript{2+} current. However, after rapid activation, the ion channel is desensitized in the timescale of seconds. The limitation of channel desensitization can be overcome by using type II PAMs, which increase the magnitude of the response and dramatically increase the opening time of the ion channel\textsuperscript{27}.

3.1.1. Optimization of assay conditions

The assay was first tested by seeding cells using uncoated plates or plates with either a PLL coating or a Matrigel coating. It was found that a Matrigel coating improved the attachment of the SH-SY5Y cells to the well-plates and prevented detachment of SH-SY5Y cells during washing steps before running the assay, as also observed in literature\textsuperscript{28}. Therefore, the 96-well plates were coated with Matrigel before seeding the cells. The cell-seeding density was then optimized in order to reach a 90-95% confluent monolayer of cells in 96-well plates after overnight incubation. This was reached by a cell seeding density of 80,000 cells/well. The concentration of the Fluo-4 NW fluorescent dye was optimized using the fluorescent dye mixture prepared according to manufacturer’s protocol. The dye was tested at the protocol concentration, a 2 times diluted, and a 4 times diluted concentration (Supporting Information Figure 1). For each dye concentration tested, the signal caused by 100 µM of nicotine injected (representing a full agonist response), and the signal caused by blank injections of assay buffer were measured seven times. With these data, the average maximum signal, the average blank signal, and their standard deviations were calculated to calculate the Z’ factor that is used to determine assay quality\textsuperscript{29}. The Z’ factors calculated were found to be 0.56, 0.60 and 0.52 for the three conditions mentioned above, respectively. As the highest Z’ factor was obtained from using the 2 times diluted Fluo-4 NW dye solution, and because a lower Fluo-4 NW concentration in the assay has a lower cytotoxicity, this concentration was used in further work. The incubation time of the PAM PNU120596 in the assay was evaluated subsequently. It was found that 90 s per well was minimally needed to obtain a good and repeatable signal.

3.1.2. At-line cellular screening setup in PAM assay format
In order to test the at-line cellular screening system in PAM assay format, a mixture of two PAMs of the α7-nAChR, PNU120596 and TQS, were separated and measured at-line with the Ca^{2+}-flux assay. Implementing UV detectors after the effluent split just before the nanofractionation and the MS inlet, respectively, allowed determination of the time difference between nanofractionation and MS detection. For the present setup using RPLC, this difference appeared to be 1.0 min. The suitability of the system for screening of PAMs was demonstrated using a mixture of PNU and TQS at two different concentrations (500 μM each and 100 μM each). The separated compounds showed allosteric modulation in the reconstructed bioassay chromatogram observed as positive peaks (Figure 2). These bioassay peaks could be correlated to their extracted ion chromatograms (XICs) plotted from the MS results obtained in parallel (Figure 2). Due to band broadening, assay dilution, and spreading of eluting peaks over multiple wells, the actual PAM concentrations in the assay are lower than the injected concentrations. This dilution is consistent and can be measured or calculated\textsuperscript{11}. The dilution factor from the chromatographic separation to the nanofractionation was determined to be 2.0. Further dilution - which is exactly known - occurs as freeze-dried well plates are re-dissolved and transferred to the measurement plate during assaying using known volumes. This extra dilution was 5.3 fold.

![Figure 2](image.png)

**Figure 2.** At-line cellular screening of a PAM mixture in the PAM assay format. The PAM mixture was injected for nanofractionation in two concentrations (500 μM PNU120596 and 500 μM TQS, and 100 μM PNU120596 and 100 μM TQS) in duplicate. The MS chromatograms (XICs; bottom) were correlated with the reconstructed bioassay chromatograms (top). The XICs of PNU120596 (m/z 313) and TQS (m/z 377) were plotted.

### 3.1.3. At-line cellular screening setup in agonist assay format

The suitability of the agonist setup of the cellular screening system was first demonstrated with acetylcholine (ACh), a natural agonist of the α7-nAChR. Again, the bioactivity peak observed in the reconstructed bioassay chromatogram could be correlated...
to the XIC of the ACh-ion from the MS data (Figure 3). In cases when relatively high concentrations of ACh are injected (e.g. resulting in 90% of the effective concentration (EC<sub>90</sub>) or a higher concentration), the chromatographic bioassay peak becomes wide due to band broadening in the chromatographic system. In reconstructed bioassay chromatograms, this is exaggerated as compared to linear responses as observed in for example UV detection or MS analysis, due to the intrinsically sigmodial-dose response of the bioassay. This phenomenon of excessive band broadening observed for high concentrations of ligand injected is common when working with on-line and at-line reconstructed bioassay chromatograms.

Figure 3. At-line cellular screening of an ACh serial dilution in the agonist assay format. ACh was injected for nanofractionation in four concentrations (500, 50, 5 and 0.5 μM). The wells with bioactive compounds from the bioassay were plotted as reconstructed bioassay chromatogram (top) and were correlated with the XICs of ACh (m/z 147; bottom).

ACh showed a strong and repeatable signal in the Ca<sup>2+</sup>-flux assay when relatively high concentrations were injected. ACh also elicited a Ca<sup>2+</sup>-signal in the nanomolar range (500 nM injected concentration, about 45 nM in the assay), which indicated that the signals might be caused not only by signaling via the α7-nAChR (pKi of ACh is 5.1 ± 0.1 for the α7-nAChR 30). To evaluate this, cells were pre-incubated with bungarotoxin, a specific α7-nAChR antagonist, prior to analysis. Indeed, ACh also showed a Ca<sup>2+</sup>-flux signal after pre-incubation with bungarotoxin, and both in the presence or absence of the type-II PAM (PNU 120596). In order to confirm the nature of the receptor, the cells were pre-incubated with scopolamine, a non-selective muscarinic AChR antagonist, which significantly blocked the Ca<sup>2+</sup>-signal evoked by ACh (Supporting Information Figure 2). The SH-SY5Y cells have an endogenously high expression level of M3
muscarinic ACh receptors, as described earlier\textsuperscript{31}. The presence of other receptors than the \(\alpha7\)-nAChR makes this assay applicable as a general screening tool to also identify bioactive compounds for these receptors. For specific \(\alpha7\)-nAChR responses, however, selective antagonists for the other relevant receptors have to be implemented in the assay setup.

In order to evaluate the cellular screening system for \(\alpha7\)-nAChR selective agonists, a mixture of PNU282987 (500 or 50 \(\mu\text{M}\)) and epibatidine (50 or 5 \(\mu\text{M}\)) was tested at two different concentrations (Figure 4). The fractions in which the two agonists were eluting from the HPLC, showed activation in the \(\text{Ca}^{2+}\)-flux assay, and the reconstructed bioassay chromatogram showed good correlation to the MS results.

Figure 4. At-line cellular screening of an agonist mixture in the agonist assay format. The agonist mixtures (500 \(\mu\text{M}\) of PNU 282987 and 50 \(\mu\text{M}\) of epibatidine, and 50 \(\mu\text{M}\) of PNU 282987 and 5 \(\mu\text{M}\) of epibatidine) were analyzed in duplicate. The reconstructed bioassay chromatogram (top) is correlated with the XICs of epibatidine (\(m/z\) 209) and PNU 282987 (\(m/z\) 265; bottom).

Notably, the effect of the PAM PNU120596 decreased in time. This effect was most pronounced when the temperature in the laboratory was higher than normal, 20-25 °C room temperature (due to warm summer days). The strong temperature dependency of the allosteric modulation of PNU120596, which decreases with rising temperatures, is described in literature\textsuperscript{32}. This temperature dependency makes measurements and correct interpretation of the \(\%F_{\text{max}}\) signal more time-consuming with non-cooled plate readers such as the one applied in this study. Freshly prepared PNU120596 had to be added to the reservoir every 1.5-2 hours to maintain similar responses. When refraining from this, the amplitude of the \(\text{Ca}^{2+}\)-signal caused by the allosteric modulator PNU120596 dissolved in the wells in the reagent plate is decreasing in time (See Supporting Information Figure 3). The efficacy of PNU120596 during measurements...
was controlled by frequently measuring positive controls.

3.2. At-line cellular screening of a Psilocybe McKennaii extract

In order to demonstrate the applicability of the at-line cellular screening setup for screening natural extracts, an extract from the *Psilocybe McKennaii* mushroom was analyzed in the agonist assay format. The followed workflow of screening and identification of the bioactives found is depicted in Figure 5. As one (fast) LC separation will never provide sufficient peak capacity to separate all components of a natural extract, we used a new approach to pinpoint accurate masses to the bioactives found. The extract was screened twice, that is, both by RPLC and HILIC employing two fundamentally different ('orthogonal') separation principles. Using RPLC, two bioactive peaks were found in the *Psilocybe McKennaii* extract. For both peaks, in the parallel MS traces multiple co-eluting compounds were observed for which the XICs of the matching \( m/z \) values were plotted (Figure 5A). Due to the accurate peak shape and retention time matching between bioactivity and MS chromatogram, many candidates that not exactly co-elute with the bioactive peak could be excluded. Still, there were several compounds with XICs that exactly overlapped with bioactive peaks so that the \( m/z \) value of the bioactive could not be determined unambiguously. The same extract was also analyzed by HILIC (Figure 5B), and the peaks observed in the bioassay chromatogram were correlated to potentially matching peaks in XICs constructed from the MS data. Slightly earlier or later eluting compounds were excluded and the resulting candidate \( m/z \) values from this analysis were now compared with the candidate \( m/z \) values obtained from the RPLC analysis. The \( m/z \) values that were correlated to a bioactivity peak in both the RPLC and in the HILIC analysis were considered to origin from an actual bioactive. The major bioactive compound observed at 3.6 min in the RPLC-MS trace was eluting at 7.1 min in the HILIC-MS analysis. The \( m/z \) of 258 was the only common value observed for these two large bioactivity peaks. The small bioactivity peak at 6.2 min in the RPLC separation was observed at 6.5 min in the HILIC separation. From the lists of \( m/z \) values observed at these retention times, the \( m/z \) of 221 was the only value observed with both the RPLC and HILIC analysis. The accurate masses for these compounds as derived from the IT-TOF data were \( m/z \) 258.110 and 221.185 (1 times charged ions), respectively. In addition, the accurate \( m/z \) values of psilocin and psilocybin, the most known alkaloids found in *Psilocybe* mushrooms were observed in the sample, but were found both not to be correlating to the bioactive peaks for the \( \alpha 7 \)-nAChR. Furthermore, this study also showed orthogonal HILIC separation of the crude extract instead of using traditional bioassay guided fractionation. It is the first time this straightforward and fast approach is demonstrated. It significantly reduces the chances of loss of bioactives (e.g. by denaturation, degradation and/or sticking/adsorption to tubes/tubing), as for every analysis fresh crude extract is used. After this fast screening procedure, the obtained \( m/z \) values can be used in follow-up studies for subsequent straightforward MS guided purification of the bioactives. After purification the chemical structure of the bioactives can be characterized using NMR, and they can be used for further biological studies, for example determining their affinity to the real membrane-bound receptor and their pharmacological profile.
Figure 5. At-line cellular screening of a Psilocybe McKennaii extract in the agonist assay format. The bioassay was performed after both an RPLC and a HILIC separation. The bioactive eluting in the RPLC separation at 3.6 min was eluting at 7.1 min in the HILIC separation. When the possible m/z values for the two peaks were compared by plotting XICs and matching peak shapes and retention times, an m/z value of 258 was found to be in common. The minor bioactive eluting at 6.2 min in RPLC was eluting at 6.5 min in the HILIC separation. After correlation of the m/z values observed in this case from the two different separations, an m/z value of 221 was assigned to the minor bioactive compound.

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References


Figure S1. Optimization of Fluo-4 NW dye concentration and reproducibility. For each dye concentration tested (protocol concentration, 2 times diluted, 4 times diluted), the signal caused by 100 µM of nicotine injected was used as the agonist signal. For testing reproducibility, 12 injections are displayed. After calculating the assay quality parameters, the 2 times diluted Fluo-4NW was selected as the optimal concentration for this assay.
Figure S2. Receptor specificity of acetylcholine (Ach). (a) The Ca\(^{2+}\)-signal of 100 µM nicotine can be blocked by the α7-nAChR selective antagonist α-bungarotoxin, whereas the Ca\(^{2+}\)-signal of 100 µM Ach is not blocked in presence of α-bungarotoxin. (b) 100 µM nicotine does not show a Ca\(^{2+}\)-signal without addition of the α7-nAChR selective PAM, 10 µM PNU120596 (without the addition of this type II PAM the receptor is desensitized). Ach shows a Ca\(^{2+}\)-signal without the addition of PNU120596, which means the Ca\(^{2+}\)-signal is caused not only by the activation of the α7-nAChR. (c) The Ca\(^{2+}\)-signal of 100 µM Ach was blocked by addition of the non-selective muscarinic AChR antagonist, scopolamine, indicating the presence of a muscarinic AChRs endogenously present on the SH-SYSY cells.
**Figure S3.** Example for the decreasing $F_{\text{max}}\%$ signal using PNU120596. (a) positive control (100 µM nicotine) and negative control (assay buffer) measured at the beginning of the day in agonist assay format. The positive control shows a 90% of $F_{\text{max}}$ signal (b). A positive control (100 µM nicotine) measured 2 hours after beginning of the measurement. The $F_{\text{max}}\%$ of the maximum signal from the positive control is significantly decreased. (c). The same positive control measured with a freshly prepared PNU120596. The signal of the positive control is again the same as at the beginning of the measurement.
At-line cellular screening methodology for bioactives in mixtures targeting the α7-nAChR
CHAPTER 6

Development of an on-line cell-based bioactivity screening method by coupling liquid chromatography to flow cytometry with parallel mass spectrometry

Abstract

This study describes a new platform for the fast and efficient functional screening for bioactive compounds in complex natural mixtures using a cell-based assay. The platform combines reversed-phase liquid chromatography (LC) with on-line flow cytometry (FC) and mass spectrometry (MS). As a model (an example or proof-of-concept study) we have used a functional calcium-flux assay in human neuroblastoma SH-SY5Y cells stably over-expressing the alpha-7 nicotinic acetylcholine receptor (α7-nAChR), a potential therapeutic target for central nervous system (CNS) related diseases. We have designed the coupled LC-FC system employing the neuroblastoma cells followed by analytical and pharmacological evaluation of the hyphenated setup in agonist and mixed antagonist-agonist assay modes. Using standard receptor ligands we have validated pharmacological responses and standardized good assay quality parameters. The applicability of the screening system was evaluated by analysis of various types of natural samples, such as a tobacco plant extract (in agonist assay mode) and snake venoms (in mixed antagonist-agonist assay mode). The bioactivity responses were correlated directly to the respective accurate masses of the compounds. Using simultaneous functional agonist and antagonist responses nicotine and known neurotoxins were detected from tobacco extract and snake venoms, respectively. Thus, the developed analytical screening technique represents a new tool for rapid measurement of functional cell-based responses and parallel separation and identification of compounds in complex mixtures targeting the α7-nAChR. It is anticipated that other fast-response cell-based assays (e.g. other ion flux assays) can be incorporated in this analytical setup.
1. Introduction

Natural products, such as venoms and plant extracts, are unraveled valuable resources to discover unknown bioactive compounds that could be developed into new drug lead molecules. However, discovery of new chemical entities from natural extracts is complicated because of the high costs and time-consuming nature of the screening processes to identify bioactive compounds from these complex samples. Generally, iterative bioassay-guided sample fractionation is used to pinpoint and purify bioactives from complex natural products. There is a need for new and improved analytical techniques allowing fast and efficient bioactivity screening of natural samples.

A powerful tool for revealing bioactive components in natural extracts is high-resolution screening (HRS), which combines biochemical assays in an on-line post-column fashion with liquid chromatography (LC) and mass spectrometry (MS). After LC separation, mixture components are individually evaluated for their bioactivity against a selected target. Splitting of part of the column effluent to MS allows direct determination of both bioactivity and mass-to-charge ratio ($m/z$) of the respective compound. A wide range of assay types and drug targets has been successfully applied in HRS systems. However, cell-based assays are often out of scope due to issues related to compatibility of living cells and organic solvents used in LC mobile-phases, and handling of cells in on-line systems.

Flow cytometry (FC) is widely used to measure physical characteristics of living cells. In FC, cells and particles are driven by a sheath-liquid stream and passed one-by-one through one or several laser beams in a flow cell. The scattered and emitted (fluorescence) light by each cell is measured, providing information on the size and granularity of individual cells. The ability to measure fluorescent signals from cells makes FC an excellent readout tool for cell-based assays. Apart from typical cytology-related assays, FC is often used as read out system for a broad spectrum of other assay types, including binding studies with fluorescently labelled antibodies. FC was shown a suitable tool in drug discovery screening programs. When cells are loaded with an appropriate calcium sensing or a membrane potential sensing dye, FC also allows the detection of cellular responses caused by binding of ligands to receptors, such as evoked calcium fluxes or changes in membrane potential.

This study describes the development and demonstration of a new analytical methodology for the initial screening of complex mixtures for bioactive compounds acting in a cell based assay. The platform consists of an FC-based cellular assay coupled on-line to LC with parallel MS detection. The assay measures functional calcium flux responses of the $\alpha_7$-nicotinic acetylcholine receptor ($\alpha_7$-nAChR) over-expressed in SH-SY5Y-cells. The $\alpha_7$-nAChR is an acetylcholine-gated ion channel, which has been implicated in diseases of the central nervous system, such as epilepsy, schizophrenia and Alzheimer's disease. Therefore, it is listed as an important medicinal target for the treatment of these diseases. As the $\alpha_7$-nAChR mediates high current of Ca$^{2+}$-ions, its activation and inactivation is commonly studied using fluorescence-based Ca$^{2+}$-flux assays in plate-reader formats, such as fluorescence imaging plate reader (FLIPR). Measuring Ca$^{2+}$-dynamics of cells can be performed by FC. However, without the combination with LC and MS, FC is not suitable for the analysis of complex mixtures. Until now, the combination of a cell-based assay using FC coupled on-line to LC and MS has not been established.
The analytical setup presented here consists of an FC, which serves as an on-line bioassay detector for reversed-phase LC that is parallel coupled to MS. The system allows the rapid identification of eluting bioactive compounds that evoke functional response in cell-based assay in the FC readout. The previously reported hyphenation of bead-based FC and LC for the detection of digoxins and its analogues using digoxin-coated beads and fluorescently labeled anti-digoxin was used as starting point of our study.

2. Materials and methods

2.1. Chemicals and biological reagents

LC-MS grade methanol (MeOH), acetonitrile (ACN) and 99.95% formic acid (FA) were purchased from Biosolve (Valkenswaard, the Netherlands). HPLC grade water (H₂O) was produced by a Milli-Q purification system from Millipore (Amsterdam, The Netherlands). Isoflow sheath fluid was obtained from Beckman Coulter (Breda, The Netherlands). Nicotine, epibatidine, PNU120596, PNU282987, methyllycaconitine (MLA), α-bungarotoxin (BTX) and acetylcholine (ACh) were purchased from Tocris Bioscience (R&D Systems Europe Ltd., Abingdon, UK), the chemical structures of these compounds are shown in Supporting Information Figure S-1. HEPES, ammonium bicarbonate (NH₄HCO₃), sodium chloride (NaCl) and Triton X-100 were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12), Dulbecco’s Phosphate Buffered Saline (PBS), Hank’s Balanced Salt Solution (HBSS), Fetal Bovine Serum (FBS), Penicillin-Streptomycin mix (5,000 U/mL), 0.25% Trypsin-EDTA, G418 (geneticin), propidium iodide (PI) and Fluo-4 NW assay kit were obtained from Life Technologies Europe (Bleiswijk, The Netherlands). The Fluo-4 NW assay kit consists of three components, A: Fluo-4 NW dye mix and B: 250 mM probenecid, in a starter pack-size component C: assay buffer: HBSS/20 mM HEPES (pH 7.4). *Naja nivea* freeze-dried snake venom was provided by Dr. Freek J Vonk (Leiden University, The Netherlands). *Dendroaspis polylepis* freeze-dried venom was obtained from African Reptiles and Venom (Bryanston, South Africa). *Ophiophagus hannah* freeze-dried venom was obtained from Venom Supplies (Indonesia).

2.2. Cell culture

Human neuroblastoma SH-SY5Y cells stably expressing the human α7-nAChR were obtained originally from Christian Führer (Department of Neurochemistry Brain Research Institute, University of Zürich, Zürich, Switzerland). The SH-SY5Y cells were cultured in T75 culture flasks and maintained in DMEM/F12 1:1 media supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 100 µg/mL geneticin as the stable expression selection marker. The cells were incubated in a 37 °C 5% CO₂ humidified atmosphere incubator; and were passaged every 3-4 days. On the day of an experiment, the cells from a nearly confluent culture flask were detached with trypsin and diluted with culture media to reach a 70,000 cells/mL concentration. The cells were centrifuged with 800 ×g for 4 min. After removal of culture media from the pelleted cells, the cells were re-suspended thoroughly in Ca²⁺-flux assay mixture, which consisted of Fluo-4 NW dye (2× diluted compared to the manufacturer’s protocol).
in HBSS supplemented with 20 mM HEPES (pH 7.4), 1% probenicid and 1% heat-inactivated FBS (in order to improve viability of the cells during measurements). This cell-suspension was incubated for 30 min at 37 °C, and after incubation it was filtered through a 100 µm Corning cell strainer (Sigma Aldrich) and transferred into a 10 mL superloop from GE Healthcare in order to pump the suspended cells continuously to the FC. In the superloop, a small magnetic stirrer bar (stirring at 250 rpm during flow cytometry measurement) was placed to keep the adherent cells in suspension. For the experiment to evaluate cell viability, 1 min before the cells were transferred to the superloop 2 µl per ml 1 mg/ml propidium iodide (PI) was mixed with the cell suspension.

2.3. Overall setup

The post-column cellular FC screening system consisted of a gradient LC instrument coupled on-line to an FC and to a high-resolution mass spectrometer using PEEK-tubing and superloops. A schematic representation of the overall system is shown in Figure 1. During initial assay optimization, the system was operated in LC-FC mode with parallel UV absorbance detection only (no MS detection).

**Figure 1.** Schematic of the post-column cellular flow cytometry screening system in two FC bioassay formats. (a) Agonist mode. (b) Mixed antagonist-agonist mode. The screening system consisted of an HPLC system coupled on-line to FC and to MS using PEEK-tubing and superloops for the infusion and mixing of the bioassay components. The system is described in details in the sections “LC instrumentation and settings” and “FC instrumentation and settings” of Materials and Methods.
2.3.1. LC instrumentation and settings.

The LC system (Figure 1. (1)) was from Shimadzu (s Hertogenbosch, The Netherlands) and consisted of two Shimadzu LC-20AD HPLC pumps, a SIL-20AC auto injector, a CTO-20AC column oven, an SPD-20A UV detector, and a CBM-20A communication module, controlled with Shimadzu LC-MS Solutions software. Reversed-phase LC separations were performed on a Waters X-Bridge BEH C18 column (2.5 \( \mu \text{m} \), 3.0×100 mm) at a temperature of 30 °C (Figure 1. (2)). Eluents A and B consisted of H2O/MeOH/FA (99/1/0.1 (v/v/v)) and (1/99/0.1 (v/v/v)), respectively. The flow rate was 120 µl/min. After the UV absorbance detector (Figure 1. (3)), the eluent flow was split: 116 µl/min was directed to the MS instrument (Figure 1. (4)) and 4 µl/min to the FC instrument (Figure 1. (5)).

During reproducibility testing and pharmacological evaluation by serial dilutions of known ligands, the LC instrument was operated under isocratic conditions using a mobile phase of 50% eluent B and 50% eluent A. For intraday and interday repeatability tests, 100 \( \mu \text{M} \) PNU282987 and 20 \( \mu \text{M} \) MLA was analyzed 9 times, two different experimental days. For the analysis of standard mixtures, a 15 min gradient from 0-60% eluent B was used, followed by equilibration to starting conditions. The Naja nivea and the Dendroaspis polylepis venoms were analyzed with a linear gradient of 0-60% eluent B in 30 min. The Ophiophagus hannah venom was separated with a linear gradient from 5-20% in 10 min, followed by a linear gradient to 70% B in 40 min.

The final assay concentrations of the standard solutions analyzed were calculated as described by Falck et al. (see Supporting Information). The calculation of assay quality parameters (Z’chrom, which is a modified version of Z’-factor that is more suited for describing chromatographic on-line assays, dynamic range, signal-to-noise ratio (S/N) and signal-to-background ratio (S/B) are described in the Supporting Information.

2.3.2. FC instrumentation and settings

The FC measurements were performed with a Beckman Coulter FC500 flow cytometer equipped with a Multi-Platform Loader (MPL) sample loader, controlled by MXP software (Beckman Coulter Nederland, Breda, The Netherlands). The FC was equipped with a single air-cooled argon laser (488nm). In order to allow a continuous flow of bioassay mixture into the flow cell of the FC, a few hardware modifications were made as shown in the SI Figure S-2. The sample pickup tubing from the sample probe to the flow cell was removed and directed into a switch valve. In order to start an FC measurement with data collection, the system requires an injection. Therefore, dummy samples were injected and directly sent to waste via the hardware modifications when data collection was needed. During the start and the end of a measurement, the FC instrument will pressurize the sample line applying a higher flow rate. To prevent the sample line tubing from disconnecting due to this pressure, the switch valve temporary allowed sheath liquid through the sample probe to be directed to the waste. During analysis, the flow of the sample line from the MPL was stopped by the switch valve to prevent wasting sheath liquid. The continuous flow cellular bioassay was connected to the inlet of the flow cell via PEEK tubing (i.d., 250 \( \mu \text{m} \)).

When using the system in the agonist mode, Fluo-4 NW loaded SH-SY5Y cells were placed in a 10 mL superloop 1 (GE Healthcare) and were continuously pumped at 50 \( \mu \text{l/min} \) with a Shimadzu 10AD HPLC pump (Figure 1. (6)). For a pulse-free and stable flow, an additional in-house built pulse damper operated at ~40 to 80 bar back.
pressure (with 0.064 i.d. natural PEEK tubing as restrictor to create the back pressure) was placed in between the LC pump and the superloop 1. From superloop 2 (50 mL), another Shimadzu 10AD HPLC pump with a pulse damper continuously infused (30 μL/min) of a solution of 36 μM PNU120596 (13 μM final assay concentration), a type II positive allosteric modulator (PAM) of the α7-nAChR (Figure 1. (7)). Addition of PNU120596 is necessary because the α7-nAChR is fast desensitized and by elongating the opening time of the receptor with a type-II PAM it is possible to measure receptor responses with the FC. The flow of cells and the PNU120596 solution were mixed in a PEEK T-piece, after which the cells were allowed to incubate with the PNU120596 for 1 min in a 175-cm blue PEEK tubing (i.d. 250 μm; internal volume 175 μL). Just before the FC flow cell, the flow of incubated cells was mixed via a PEEK T-piece with the 4 μL/min split-flow from the LC column and allowed to incubate for 3 s in a 20 cm of blue PEEK tubing (10 μL volume) before entering the FC flow cell for cellular analysis.

When using the system in the mixed antagonist-agonist mode, the Fluo-4 NW-loaded SH-SY5Y cells were placed in superloop 1 and a solution of 50 μM PNU120596 (13 μM final assay concentration) was placed in superloop 2. After the cells (continuous flow of 50 μL/min) were mixed with PAM PNU120596 (continuous flow of 30 μL/min) via a PEEK T-piece connector, 4 μL/min of the HPLC effluent was added to the total flow. After incubation for 2 min in 350-cm blue Peek tubing, a solution of the agonist nicotine (100 μM final assay concentration) was mixed via superloop 3 (10 mL) at 30 μL/min (Figure 1. (8)). Finally, the flow was directed into the FC flow cell via 20 cm of blue PEEK tubing (10 μL volume; 3 s incubation time).

The FC method used was set at 7200 s acquisition time with unlimited max events (set to 10,000,000). The following parameters were recorded in time during analysis: forward scatter (FS), side scatter (SS), as well as FL1, FL2, FL3, FL4, FL5 fluorescence channels. For the Ca2+-flux signals the signal detected from the FL1 channel, for PI staining the FL3 channel was evaluated.

The FC density plots obtained by the MXP software were converted into a chromatographic data format using Beckman Coulter Kaluza software, similar as previously described23. The data conversion process is described in detail in the Supporting Information. The figures presented here show both the FC plots and the chromatographic plots after conversion of the same data. All results are gated on the functional cells as described in the SI Figure S-3. The panels in figures marked with “FC raw” are density plots displayed by the Kaluza software, displaying time against intensity of fluorescence channel FL1 in logarithmic scale with in the third dimension (colors) the number of the cells. Panels marked with “FC converted” represent the same results in a chromatographic data format showing time against the averaged fluorescence intensity of each cell detected in FL1 channel per s.

2.3.3. MS instrumentation and settings

For MS measurement, 116 μL/min of the LC effluent was directed via the UV absorbance detector into the electrospray ionization (ESI) source of a Shimadzu ion-trap time-of-flight (IT-TOF) mass spectrometer. The MS instrument was operated in positive-ion mode using the following settings: 1.5 L/min nebulizing gas flow, 4.5 kV interface voltage, 200 °C source heating block and 200 °C curved desolvation line temperature. The scan range was set between m/z 145 and 2000.
2.4. Sample preparation

Standard stock solutions (10 or 100 mM) of PNU282987, acetylcholine, nicotine, BTX and MLA were prepared in DMSO and were diluted in eluent A to the required concentrations for analysis. For extraction of tobacco leafs, 1.2 gram of cigarette tobacco (equivalent of two cigarettes) was mixed with 5 mL eluent B and shaken for 10 min. The extract was centrifuged at 14,000 rpm for 5 min. The supernatant (20 μL injection volume) was directly injected for analysis. The freeze-dried snake venoms were stored at –20 °C and were freshly dissolved (10 mg/mL) in eluent A prior to analysis.

3. Results and discussion

3.1. Coupling of FC to LC

For setting up the LC-FC system, attention was paid to the compatibility of the LC eluents with the cell-based assays, and to the feasibility of infusing a continuous stream of cells into the system. For the connections of the continuous-flow bioassay at the path of the cells, different i.d. PEEK tubings (blue and orange, 250 and 500 μm i.d., respectively) were evaluated. With an orange tubing, the low linear flow velocity caused the cells to settle in the tubing. This conclusion was drawn by two observations: 1) decrease of the number of cells entering the FC in time; 2) upon shaking, ticking or moving the orange PEEK tubing, for a short moment an abrupt and large increase in the number of cells entering the FC was observed. When blue PEEK tubing was used, the number of cells entering the FC in time became stable and continuous, therefore blue PEEK tubings were used for the connections of the system.

To examine the compatibility of the LC eluent composition with living mammalian cells, different organic solvents and buffer salts were evaluated using a plate-reader Ca²⁺-flux assay, as described previously. ACN, MeOH, NaCl and NH₄HCO₃ were tested in different concentrations. The maximum tolerable concentration was defined as the concentration that caused a >5% decrease of the intensity of the Ca²⁺-flux assay signal for ACh, compared to the experiment without the evaluated eluent or buffer compound. The maximum tolerable concentrations were found to be 2% (v/v) for ACN, 4% (v/v) for MeOH, 100 mM for both NaCl and NH₄HCO₃ (SI Figure S-4). In this study, reversed-phase (RP) LC was used because of its general suitability for the separation of components of natural samples and its compatibility with ESI-MS. The effect of organic solvent (eluent B) from RP LC on the bioactivity peak detection in the on-line LC-FC assay was tested by injections of 7.45 μM nicotine under isocratic 0%, 70%, and 100% eluent B conditions. Final assay concentrations eluent B were 0%, 3.3%, and 4.6%, respectively, since after the split, the 4 μL/min HPLC eluent was diluted with 80 μL/min assay mixture (SI Figure S-5). The bioactivity peak height significantly decreased under 100% eluent B, whereas the signal was reproducible (and similar to 0% eluent B) under 70% eluent B conditions. The effect of a 15 min 0-70% eluent B HPLC gradient on the FC signal, and on the cells was tested in agonist and in mixed antagonist-agonist mode (SI Figure S-5.b and c, respectively). The HPLC gradient did not cause any significant change in the baseline signal of the bioassay. The viability of the cells was tested by using PI staining (SI, Figure S-6). PI is a fluorescent dye staining of only dead and apoptotic cells. Figure S-6 confirms that the gated cells, used deriving the FC bioassay data, are viable cells.
The possibility to gate, select and analyze only a specific cell population is an additional strength of using FC as bioassay detection.

3.2. Assay optimization and evaluation with standard ligands

The assay conditions i.e., type, pH and concentration of buffer, cell preparation, Fluo-4 NW dye concentration, PAM concentration, and incubation times, were transferred from a previously optimized Ca\textsuperscript{2+}-flux assay in plate-reader format\textsuperscript{24}.

**Figure 2**. Pharmacological evaluation of the LC-FC screening system. Serial dilutions of (a) nicotine, (b) PNU282987 and (c) MLA. Final FC bioassay concentrations: nicotine, 7.46 (I.), 3.73 (II.), 1.86 (III.), and 0.93 μM (IV.); PNU282987, 7.46 (I.), 1.86 (II.), 0.37 (III.), and 0.075 μM (IV.); MLA, 1.37 (I.), 0.275 (II.), 0.055 (III.), 0.011 (IV.), and 0.002 (V.) μM. (d) Correlation of the pEC\textsubscript{50} values of nicotine, PNU282987, epibatidine, pIC\textsubscript{50} of MLA and BTX derived from the FC experiments with pKi values from literature (r\textsuperscript{2}=0.82).

The intraday repeatability of the LC-FC system was evaluated in agonist and mixed antagonist-agonist mode by repeated injections of the agonist PNU282987 (0.76 μM final conc.), or MLA (0.1 μM final conc.) evoking a maximum agonist response (SI Figure S-7 and S-8). The calculated statistical parameters of the assay quality are shown in Table 1. It was observed that the dynamic range and the S/B of the assay were gradually decreasing with time. This decrease in signal might be caused due to the adherent cells that are kept in suspension without continuous fresh oxygen supply by the desensitization of the receptor, and/or by receptor loss/endocytosis. Separate experiments with fresh batches of cells carried out on different days (interday reproducibility experiments) showed that the signal decrease was reproducible (see SI Figure S-7). It is important to note that the LC-FC system was developed for detection screening and not for quantitative bioactivity determination. As a bioassay in general is considered as a good assay if the Z’ chrom or the Z’-factor is higher than 0.56, the assay...
quality was adequate during 1.5 hours of continuous measurement (Z’chrom 0.82, 0.70, 0.85 in agonist and Z’chrom 0.90, 0.87, 0.89 in mixed antagonist-agonist mode after 30 min, 1.0 h and 1.5 h).

Table 1. Statistical parameters of assay quality obtained during intraday and interday repeatability experiments in agonist and mixed agonist-antagonist mode. Z’chrom, dynamic range, S/B and S/N determined after 30 min, 1.0 h and 1.5 h from the repeated additions of PNU282987 or MLA.

<table>
<thead>
<tr>
<th>Time</th>
<th>Z’chrom</th>
<th>Dynamic Range (RFU)</th>
<th>S/B</th>
<th>S/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td>0.82</td>
<td>230</td>
<td>65</td>
<td>129</td>
</tr>
<tr>
<td>1 h</td>
<td>0.70</td>
<td>144</td>
<td>27</td>
<td>159</td>
</tr>
<tr>
<td>1.5 h</td>
<td>0.85</td>
<td>115</td>
<td>20</td>
<td>224</td>
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</tbody>
</table>

For further pharmacological evaluation, serial dilutions of nicotine, PNU282987, MLA (Figure 2), epibatidine and BTX were measured. In case an α7-nAChR agonist elutes from the HPLC, it can activate the receptor and evoke a high Ca²⁺ flow through the receptor resulting in a rise of intracellular Ca²⁺, which will bind to the Fluo-4 Ca-sensitive dye causing an increased fluorescence detected in the FC as a positive chromatographic peak. If an α7-nAChR antagonist elutes from the HPLC, the receptor channel will close and thus, preventing more Ca²⁺ to enter the cell. As a result the high fluorescence due the continuous agonist infusion in the mixed antagonist-agonist mode, will decrease, yielding a negative chromatographic peak in the FC readout. The signals obtained from dilutions of the standard ligands were dependent on the injected concentration and the affinity of the ligands. The pIC50 and pEC50 values calculated from the serial dilutions from LC-FC ( pEC50: nicotine, 5.85; PNU282987, 7.02; epibatidine, 7.19; pIC50: MLA, 9.14; BTX, 7.95) correlated well with the reported pKi values from ligand binding assays (nicotine, 5.15; PNU282987, 7.58; epibatidine, 6.66; MLA, 8.85; BTX, 8.79; r²=0.82)25-27. In the short incubation time of our cellular LC-FC system (2 min), BTX, a small protein ligand and antagonist of the α7-nAChR, showed a 10-fold lower affinity, i.e., pIC50 = 7.95, compared with pKi values reported (pKi = 8.79)27 for radioligand binding studies with 1–2 h incubation time. These lower pKi and pIC50 values observed with short incubation times are an intrinsic feature of the on-line screening approach in which analysis times are relatively short.

3.3. Evaluation of the on-line cell-based LC-FC system in agonist mode.

The on-line cell-based bioactivity screening system was further tested with the gradient separation of a mixture of the agonists PNU282987 and nicotine, injected at two concentration levels (Figure 3). The final assay concentrations for both agonists were 7.46 μM (injected concentration, 1.00 mM) and 0.37 μM (injected concentration, 50
micromolar). The two agonists were separated in the LC gradient separation and the observed FC traces were time-correlated with peaks observed during MS detection performed in parallel. At the higher concentration, both agonists induced activation of the nAChR receptor, whereas at the lower concentration only PNU282987 showed appreciable activation, which is in line with the relative affinity of both compounds to the receptor. The affinity of PNU282987 is in the nM range, whereas nicotine exhibits affinity in the micromolar range. This means that the 0.37 micromolar assay concentration is far below the EC50 of nicotine, and thus no bioactivity peak is observed at this concentration.

Figure 3. LC-FC screening of a mixture of nicotine and PNU282987 (7.45 or 0.37 micromolar each in the bioassay). The LC separated agonists showed bioactivation according to their affinity for the α7-nAChR.

3.4. Evaluation of the on-line cell-based LC-FC system in mixed antagonist-agonist mode

For the mixed antagonist-agonist assay mode, the order of infusion of bioassay solutions was modified, and an extra superloop was used for the continuous infusion of the agonist nicotine. The proof of principle experiment showing the functioning of the mixed antagonist-agonist mode is shown in the Supporting information (SI, Figure S-9). An important merit of the analysis in the mixed antagonist-agonist mode is that both antagonists, showing negative peaks, and agonists, showing positive peaks, can be detected, as long as they are not co-eluting. This is demonstrated by the analysis of a mixture of the agonist nicotine and the antagonist MLA (SI Figure S-10).

3.5. On-line cell-based LC-FC-MS of tobacco plant extract in agonist mode

Analysis of tobacco plant extract and snake venoms with potentially novel bioactive compounds was performed in agonist and mixed antagonist-agonist format,
respectively, with and without addition of an internal standard (IS; PNU282987, or nicotine). The IS provides a positive control during the FC bioassay. In addition, it allows the time alignment of the FC bioassay trace and the MS chromatogram, which is necessary for correctly correlating measured $m/z$ values to the respective bioactive compound. Without IS, potential bioactive compounds that would co-elute with the IS can be detected and identified. As proof-of-principle experiment for natural-extract screening in agonist assay mode, a tobacco extract was analyzed with the on-line cell-based bioactivity screening system (Figure 4). Extracted ion currents (XICs) for the protonated molecules of the agonist nicotine ($m/z$ 163.123) and the IS ($m/z$ 265.115) were nicely plotted. In the FC bioassay trace, the bioactivity peaks of nicotine and the IS are nicely observed. Nicotine was observed as a split peak in both the XIC and the FC bioassay chromatogram due to the injection of a high concentration in a strong solvent (MeOH). The tobacco extract in MeOH was directly injected in order to prevent potential losses of volatile compounds. Although peak splitting normally should be avoided, this result for nicotine nicely illustrates the capability of the overall system to confirm and characterize agonists by monitoring molecular mass and bioactivity simultaneously.

![Figure 4](image)

Figure 4. On-line cell-based LC-FC-MS of a tobacco leaf extract in agonist mode. (a) FC raw data, (b) FC converted data, (c) MS data. PNU282987 (100 µM; $m/z$ 265.115, black trace) was used as IS. The bioactive peaks observed in the FC bioassay were correlated to nicotine ($m/z$ 163.123, blue trace), which eluted as a split peak due to the high concentration injected and because the sample was prepared and injected in eluent B.

3.6. On-line cell-based LC-FC-MS of snake venoms in mixed antagonist-agonist mode

The use of the mixed agonist-antagonist mode in natural-extract screening was subsequently evaluated by the analysis of snake venoms. Many snake venoms contain
neurotoxins, such as for example α-BTX and α-cobratoxin, that virtually irreversibly block the α7-nAChR. Identifying such bioactive peptides was attempted by screening of the venoms of the cape cobra (Naja nivea), the king cobra (Ophiophagus hannah) and the black mamba (Dendroaspis polylepis) by LC-FC-MS.

The Naja nivea venom, which contains known neurotoxins, was analyzed with (Figure 5) and without the addition of nicotine as IS. The negative peak observed in the FC bioassay at ca. 20 min was correlated to m/z 1316.44 and 1128.53, corresponding to the 6+ and 7+ ions of a protein with a molecular weight of 7892.59 Da. Based on a Uniprot database search this mass could be assigned to Long Neurotoxin 1 (Uniprot No. P01390, 7902 Da). This protein was previously found to display high-affinity binding to the acetylcholine binding protein (AChBP) in a miniaturized HRS setup used for the screening of the Naja nivea venom. AChBP is a soluble binding protein that is homologous to the binding pocket of the α7-nAChR.

Two negative peaks were observed during LC-FC-MS of the Ophiophagus Hannah (Figure 6). By correlating the obtained bioassay chromatogram to the TIC, the first bioactive peak was annotated with m/z 1183.71 and 1014.76, corresponding to the 6+ and 7+ ions of a protein with a molecular weight of 7096.21 Da, which had no match in the Uniprot database. The second negative bioactive peak was attributed to ions with m/z 1256.72 and 1077.33 (6+ and 7+ ions) of a protein with a molecular weight of 7540 Da. Based on a Uniprot search, this bioactive matched with the homodimeric neurotoxin.
haditoxin (Uniprot.org number A8N286, 7537.32 Da). Further studies would be needed to confirm the identity of this protein.

When the venom of *Dendroaspis polyplepis* was analyzed by the on-line cell-based LC-FC-MS system, both a positive and a negative peak were observed in the bioassay chromatogram (SI Figure S-11). The positive peak corresponded to a compound with \( m/z \) 146.118, which was assigned to acetylcholine. African mambas have been described to contain high concentrations of acetylcholine\(^{33}\). The negative peak in the FC trace was correlated to ions with \( m/z \) 999.845 and 1142.530, corresponding to the 8+ and 7+ ions of a protein with a molecular weight of 7990.64 Da. A Uniprot database search indicated that this protein might correspond to the \( \alpha \)-elapitoxin-Dpp2d (Uniprot number C0HJD7, 7985.33±0.40 Da), which is a known high-affinity antagonist of the \( \alpha7 \)-nAChR. Further studies will be needed to confirm this assignment.

### Figure 6.

On-line cell-based LC-FC-MS of the *Ophiophagus hannah* venom. (a) FC raw data, (b) FC converted data, (c) MS data. The first negative peak (bioactive 1) observed in the bioactivity chromatogram was correlated to ions with \( m/z \) 1183.707 and 1014.76 (6+ and 7+, blue and black, respectively). The second negative bioactive peak (bioactive 2) corresponds to ions with \( m/z \) 1256.72 and 1077.33 (6+ and 7+, brown and green, respectively).

### 4. Conclusion

For the first time, we have described an on-line cell-based bioactivity screening system combines mammalian cell-based assays in FC format with LC separation and parallel MS. We envision that other fast cell-based assays can be applied in the current setup in order to obtain functional cellular response information on bioactive compounds from...
complex mixtures after chromatographic separation.

The LC-FC-MS system was successfully developed in both agonist and mixed antagonist-agonist assay mode. The latter mode gives the possibility of simultaneous detection of agonists and antagonists. Feasibility studies included assessment of intraday repeatability and interday reproducibility, analysis of serial dilutions of standard ligands, and analysis of a mixture of known ligands using gradient elution LC. Subsequently, applicability of the total system was demonstrated by the screening of tobacco plant leaf extracts in agonist mode and snake venoms in mixed antagonist-agonist mode.

The present study shows the viability of LC-FC-MS, but there is still room for improvement. For example, the current setup features a high post-column dilution due to the need of decreasing the organic solvent concentration in the LC mobile phase prior to FC bioassay in order to obtain living-cell compatible conditions. The dilution might be circumvented by using smaller i.d. analytical columns employing lower flow rates or by applying separation techniques requiring no or low volumes of organic solvents. The LC-FC screening concept is expected to be well applicable to other separation methods than RPC, such as ion-exchange chromatography or size exclusion chromatography. In these cases, however, the maximum tolerable buffer and salt concentrations have to be determined for both the living cells and the MS analysis.

Notably, the way of infusing the cells to the FC was not extensively optimized. In the current setup, mammalian cells are placed in a superloop without additional oxygen, presenting sub-optimal conditions for viability. We envision the development of oxygen pressurized containers with controlled conditions for continuous infusion of cells in suspension into the FC bioassay part of the system. Additional polydimethyl siloxane (PDMS) membrane systems under positive oxygen pressure could be used to allow fresh oxygen to diffuse into the superloop and to the flow streams containing the cell suspension prior to FC readout. Hence, advancements to the here described methodology, which efficiently combines biology and analytical chemistry, are anticipated.

Under the selected LC conditions, at least 20% of the cells are viable. However, using the gating function of FC allows the selective analysis of the response of viable cells only for the bioassay readout. Due to the experimental conditions and the continuous-flow character of the bioassay, significantly longer incubation times as currently used, will probably pose the issue of increasing percentages of cell death next to increased peak broadening. Therefore, the type of screening system presented is limited to assays with intrinsically short incubation times.

The proposed method requires fast binding ligands for attaining optimal signals. Ligands with slow receptor onset will show low affinity in the on-line screening setup (incubation times, <2 min) yielding low intensities. For such ligands, at-line microfractionation HRS with subsequent plate-readers measurements is more suitable as it allows long incubation times but at the cost of reduced component resolution and slower speed of analysis.

The short incubation time is a general disadvantage of every on-line post-column screening setup which combines liquid chromatography with a continuous-flow bioassay. The incubation time can be prolonged to 2-5 minutes, however, the longer the incubation time, the more peak broadening occurs. Fortunately, in case of ion channel targets most of the agonists and allosteric modulators which are interesting
for the pharmaceutical industry, have fast onset rates. Therefore screening setups with short incubation times are excellent tools for bioactive compound identification from mixtures. Although the bioactivity signals measured do not provide accurate information on compound concentration, affinity and binding kinetics, the current screening analytics aim at a fast “yes or no” (bioactivity is present or not) answer.

Compared to traditional bioassay-guided fractionation (BGF), the LC-FC system has the advantage of rapidly and straightforwardly obtaining a high-resolution bioassay readout directly after an analytical separation with parallel MS detection. In contrast to BGF approaches, this allows direct identification of bioactive compounds. This rapid and selective initial screening of complex mixtures, allows straightforward subsequent MS-guided LC-purification of the bioactives. As a consequence sample throughput is significantly higher as compared to BGF in which typically multiple rounds of elaborate low-resolution fractionation and bioassay steps are required.

**Acknowledgements**

The work of Reka Otvos was supported by the AIMMS PhD project “Identification of novel bioactive substances on brain receptors” (project number 10-001-203). Arthur Kool, Geert Weijers and Tom Weenink from Beckman Coulter Nederland are acknowledged for their help and advice on using the FC instrument and on the data processing with the Kaluza software. We would like to thank Dick van Iperen for his contributions in constructing the LC-FC system.
Development of an on-line cell-based bioactivity screening by coupling LC to FC with parallel MS


Supporting information

Process of converting FC raw data into chromatographic format

After gating all points representing living and functional cells (see SI Figure S-2), the raw data from the MXP software was exported into .csv files using the Export compensated data function of the Kaluza software (Beckman Coulter). Subsequently, the 1-s average of the FL1 channel was calculated using an in-house written R Studio software (Boston, MA, United States) algorithm (Supporting information). Since the MXP software displays and stores events in time as a 1024 channel format, the following calculation was used to transform this format into measured events per second for each channel:

\[
timesec = \text{time}_{1024} \times \left( \frac{\text{time}_{acq}}{\text{timelast channel}} \right) ;
\]

where \( \text{timesec} \) is the real time displayed in seconds, \( \text{time}_{1024} \) is the time displayed in the exported .csf file, \( \text{time}_{acq} \) is the real acquisition time of the measurement, and \( \text{timelast channel} \) is the time of the last channel in the exported .csf file. The final converted results were displayed in time against the mean of relative fluorescence in FL1 channel per second using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA).

Calculation of final assay concentrations

The final concentration in the assay was calculated as described by Falck et al. 2010.1 In short, the final concentration is determined by the mixing dilution (\( D_M \)) and the chromatographic dilution (\( D_C \)).

\[
D_M = \frac{u_F}{u_E};
\]

where \( u_F \) is the final flow rate in the bioassay detector and \( u_E \) is the flow rate of the LC eluent mixing with the other components of the assay.

\[
D_C = \frac{\text{FWHM}}{2 \cdot \sqrt{\pi \ln 2}} \cdot \frac{u_C}{V_i};
\]

where FWHM is the full width at half maximum, \( u_C \) is the LC flowrate and \( V_i \) is the injection volume.

\[
c_F = \frac{c_i}{(D_M \cdot D_C)};
\]

Where \( c_F \) is the final assay concentration, \( c_i \) is the injected concentration.

Calculation of assay statistical parameters

For determination of the assay quality, a modified equation of \( Z' \)-factor, the \( Z'_{\text{chrom}} \) (equation 4) value was used, which is well suited for describing on-line assays. The reasons for the suitability of \( Z'_{\text{chrom}} \) instead of \( Z' \)-factor for describing assay quality of chromatography-based on-line assays is described in detail by Falck et al.1.

\[
Z'_{\text{chrom}} = 1 - \frac{(3 \cdot \text{SD}_H + 3 \cdot \text{noise})}{|\mu_H|};
\]

Where \( \text{SD}_H \) is the standard deviation of the peaks obtained from full activation signal, \( \mu_H \) is the average of the peak height of full activation (which is equivalent to the assay window, or \( \mu_c - \mu_s \), the difference of the average of full activation and no activation.
Noise refers to the chromatographic baseline deviation.

Dynamic range is calculated as the difference between the average of peak height of maximum signal (μH) and the average of noise.

The signal to noise ratio (S/N) is defined according to Zhang et al.\(^2\) as equation 5.

\[
S/N = \frac{\text{mean signal} - \text{mean background}}{\text{standard deviation of background}}
\]  

(5)

The signal to background (S/B) is defined according to Zhang et al.\(^2\) as equation 6.

\[
S/B = \frac{\text{mean signal}}{\text{mean background}}
\]  

(6)

The R script used for the generation of data points for converted FC bioassay chromatograms

```r
convert.data<-function(filename,directory){
library(plyr)
data = read.csv(paste(directory,filename,sep=""))
acc.time = as.numeric( gsub("\.act|\.csv\",\"\",str_extract(string=filename,pattern="\"\.act\d+\.csv\"\") )
lastbucket = data$TIME[length(data$TIME)]
scale.factor = acc.time/lastbucket
data$TIME.sec = data$TIME * scale.factor
summary.data = ddply(data,.(TIME.sec),summarise,avg.FL1=mean(FL1))
new.filename = paste(directory,paste("converted",filename,sep = " "),sep="")
dev.off()
write.table(summary.data,file = new.filename, sep = ",", col.names = NA, qmethod = "double")
}
file.directory = "U:/flow cytometer/" # mid the trailing '/' it must be present here
files = list.files(file.directory, pattern="*.csv",full.names=F)
for (file in files) {convert.data(file,file.directory)}
```

References


Figure S-1. Chemical structures of (a) nicotine, (b) PNU120596, (c) PNU282987, (d) MLA, (e) BTX.

Figure S-2. Hardware modifications of the FC instrument. (A) The original sample flow in the FC instrument. The samples are placed in the sample rack (1) of the FC instrument. After injection by the sample probe (2), the sample is directed into the flow cell (4) through the sample pickup tubing (3). (B) Modified sample flow in the FC instrument with the continuous flow on-line FC bioassay. A dummy sample is placed in the sample rack (1) of the flow cytometer. The sample pickup tubing (3) is disconnected from the flow cell (4) and directed to a switch valve (5). During the start/end of the measurement the switch valve is open and the flow is directed to waste (6). During measurement time the switch valve is in stop position (7). The tubing of the continuous flow on-line bioassay (8) is directly connected to the flow cell of the FC instrument (4).
Figure S-3. Effect of using different gating during FC data acquisition. (A) shows the forward scatter (FS) vs side scatter (SS) during a regular measurement. Most of the cell population belongs to the viable SH-SY5Y cells (red circle), however fragmented (green circle) and aggregated cells (above the limit of FS) are also present in the mixture. (B) shows Time vs FL1 without using gating. The peak of a positive control is observed, however, the noise is much higher. (C) Gating as used during all data acquisition in this study. The cells outside this defined gating region are fragmented, aggregated or dead cells (aggregated cells are above the FS detection limit, fragmented cells are smaller than the normal cells in the FS detector and are also more granulated, and cells that are smaller than the SH-SY5Y cells and not showing fluorescence are also fragmented cells). Gating on the functionally responding cell population reduces the noise. (D) FL1 by time, gating on the fragmented cells. These cells are not showing a calcium response.
Figure S-4. Testing the effect of organic solvents and salts on the SH-SY5Y cells in plate-reader format. Various concentrations of (A) ACN, (B) MeOH, (C) NaCl, (D) NH₄HCO₃ were added before the addition of 100 μM of acetylcholine (ACh). Maximum tolerable concentration was defined as a decrease in the assay window of the Ca²⁺-flux signal for ACh of more than 5 percent as compared to the same experiment without the addition of the organic solvent or salt. For ACN the maximum tolerable concentration was found to be 2%, for MeOH 4%, for NaCl 100 mM and for NH₄HCO₃ 33 mM.
Figure S-5. Testing the effect of organic solvents on the SH-SYSY cells in the on-line FC-LC assay format. (a) Duplicate injection of 7.45 μM nicotine (final assay conc.) was tested with isocratic 0%, 70% (3.4% final assay conc.), and 100% (4.6% final assay conc.) eluent B. The results were in correlation with the 4% max eluent conc. determined using plate reader experiments (Figure S-4), since the bioactivity peak height significantly decreased with 100% eluent B, while the bioactivity signal was repeatable (and similar to using 0% eluent B) with using 70% eluent B. (b) and (c) Effect of a 15 min 0-70% eluent B HPLC gradient on the FC signal and on the cells in agonist mode (b) and in mixed antagonist-agonist mode (c). On Figure C the HPLC gradient starts after reaching the increased baseline by the continuous agonist infusion. The HPLC gradient did not cause any significant change on the baseline of the assay.
Figure S-6. Determination of cell viability using propidium iodide (PI) staining. PI is a fluorescent dye staining only dead and apoptotic cells by binding to PI DNA and RNA which causes fluorescence enhancement with the excitation/emission maxima of 533/617 nm, therefore it is excitable by the argon blue laser (488 nm) and the emitted fluorescence can be detected by the FL3 channel (620 nm) of the FC. The dye is membrane impermeable, so viable cells are not stained by PI. The cell viability assessment is demonstrated with a blank 0-70% eluent B HPLC gradient. (a) Ungated density plot of forward scatter (FS) per side scatter (SS); the third dimension (color) represents the number of cells. Based on the size and granularity of the cells, groups of cells (gate A, C, D, and E) can be separately gated. (b, c, d, e) Figures show histogram plots of gated cells in the FL3 channel (dead cells show fluorescence): gate A, fragmented cells (b), gate C, apoptotic cells stained with PI (c), gate D, aggregated cells (d), gate E, viable, functionally responding cells (e). These histogram plots display the fluorescence intensity in the FL3 channel on the x-axis and the number of events (cell count) on the y-axis. Figure (e) shows that when gating on the cell population under gate E, the cells are not stained with PI, demonstrating that they are viable. Therefore during every experiment in this study gate E was used to select the viable cells for plotting the FC bioassay data. In (f) the percentage of viable cells (Gate E/Ungated, number of viable cells/all cells) is displayed in time. There is a very minor decrease in viable cells, however the HPLC gradient did not affect the number viable cells in the FC assay.
Figure S-7. Intraday repeatability and interday reproducibility in agonist mode. Repeated injections (n=9) of 0.746 μM (injected concentration ci, 100 μM) PNU282987. Experiments performed on two different days are shown. The raw (a) and the converted (b) FC bioassay chromatograms are displayed. The signal window is decreasing during the measurement, however even after 1.5 hours a bioactivity peak can be still clearly distinguished from the baseline noise.
Figure S-8. Intraday repeatability and interday reproducibility in mixed antagonist-agonist assay mode. Repeated injections (n=9) of 0.1 μM (injected concentration ci, 20 μM) MLA. Experiments performed on two different days are shown. The raw (a) and the converted (b) FC bioassay chromatograms are displayed.
Figure S-9. Proof-of-principle experiment showing the functioning of the mixed agonist-antagonist setup. The raw (a) and the converted (b) FC bioassay chromatograms are displayed in the figure. At time 0 min the flow of continuous nicotine was not connected. At 13 min, nicotine (1 mM injected concentration, 7.45 \( \mu \text{M} \) final assay concentration) was injected as positive control. From 30 min, nicotine (1 mM injected concentration, 7.45 \( \mu \text{M} \) final assay concentration) is continuously infused, which results in the permanent activation of the cells and a constant increased fluorescence signal. When an antagonist eluted from the LC column (70 and 88 min, 50 \( \mu \text{M} \) of MLA was injected) it temporarily blocked the continuous activation of the receptor resulting in a negative peak in the FC bioassay. When the continuous infusion of nicotine was stopped, the baseline returned to the original fluorescence level as in the beginning of the experiment without continuous nicotine infusion.

Figure S-10. Parallel agonist-antagonist measurement in antagonist mode. Gradient LC separation of nicotine and MLA. The raw (a) and the converted (b) FC bioassay chromatograms are displayed.
**Figure S-11.** On-line cell-based bioactivity screening of the Dendroapsis polylepis snake venom. The raw FC (a), the converted FC (b), and the MS (c) chromatograms are displayed. The positive peak in the bioactivity chromatograms was correlated to an ion with an m/z value of 146.118, which corresponds to acetylcholine. The negative bioactive peak corresponds to an m/z value of 1142.530 and 999.845 (+7 and +8 charged ions, respectively).
CHAPTER 7

Summary and future perspectives
This thesis describes the development of new analytical screening platforms, which can be used as tools in the field of discovery of biologically active compounds from natural extracts. Nature provides a rich source of active compounds that are produced by microbes, plants, mushrooms and animals, and these active compounds are known to interact with many different types of biological targets, such as receptors, ion channels and enzymes. Therefore, bioactive compounds derived from natural extracts can be valuable drug leads in drug discovery processes. The first phase in active compound discovery from natural extracts is traditionally performed by bioassay-guided fractionation (BGF). This methodology has been applied successfully for the discovery of many important drugs and other active compounds in the past. However, BGF has limitations, which amongst other reasons has caused a decrease in natural-extract based drug discovery programs over the last 20-30 years. BGF requires large quantities of initial sample, is labor intensive and time consuming, and is susceptible to loss of bioactives because of the repeated bioassay steps needed. New analytical methods, which are less labor intensive, require less rounds of bioassaying, and which only need low amounts of sample for analysis, are in demand. This thesis deals with the development and application of on-line high-resolution screening (HRS) analytics for natural extracts, with a focus on venom. HRS setups commonly combine liquid chromatography (LC) with both mass spectrometry (MS) and a post-column continuous flow bioassay. This hyphenated technology provides separation of components of complex mixtures followed by parallel detection of their specific bioactivity and chemical identity. An important advantage of HRS compared to BGF is that from a single HRS analysis information can be obtained on the bioactivity of individual mixture components with recorded molecular masses.

The drug targets studied in this thesis are the α7-nicotinic acetylcholine receptor (α7-nAChR) and the serotonine type 3 receptor (5-HT3R). These receptors are known to be involved in many central nervous system (CNS) diseases, such as cognitive deficits, schizophrenia, anxiety, depression, epilepsy, Alzheimer’s disease, Parkinson’s disease and pain. Therefore, identification of new active compounds interacting with these receptors can be a start for further development of new medicines or lead to their development into pharmacological and diagnostics tools. In this thesis different types of bioassays were developed and applied in HRS systems to identify active compounds interacting with the α7-nAChR and the 5-HT3R.

Chapter 1 aimed to give a comprehensive overview on the background of this thesis, discussing the lacks and needs in fields of neuroscience related to ligand-gated ion channels, drug discovery and screening of natural extracts and other complex mixtures. Chapter 1 was divided into three topics: targets, sources and approaches. The “Targets” section gives an overview of the relevance of the drug targets studied in this thesis. The structure, localization, function and involvement in different diseases of the α7-nAChR and the 5-HT3R were discussed in detail. The “Sources” section handled the relevance of compounds from different natural sources for drug discovery. Finally, the “Approaches” section discussed the classical and the new analytical approaches, which are used for the identification of new active compounds targeting ion channels. The venom-based drug discovery workflow was treated and actual examples leading to bioactive compounds were given. Finally, new analytical approaches using hyphenated systems in the endeavor of natural extract screening for bioactive compounds were discussed.

The aim of Chapters 2-4 is to overcome the limitation of using large sample volumes in traditional BGF and HRS systems employing normal bore LC column dimensions. For
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this, miniaturized, or microfluidic, on-line HRS systems were developed which require only nanoliters or micrograms of sample, allowing screening of small-size samples. Examples are venom samples of rare species or animals producing only small quantities of venom. Animal venoms turn out to be rich sources of bioactive compounds that are able to activate, inhibit or modulate receptors, ion channels or enzymes. Therefore, venom-based drug discovery is an important tool for finding new leads for new medicines. The microfluidic on-line HRS setups developed employs nano-LC coupled to MS equipped with nano-electrospray ionization (nano-ESI) and in parallel a microfluidic chip-based continuous flow bioassay with an in-house built microfluidic fluorescence detector.

In Chapter 2 an analytical workflow is described in which the acetylcholine binding protein (AChBP) was used as screening target. The AChBP is a soluble binding protein, which is homologous to the extracellular domain of the α7-nAChR. This workflow enabled the bioactivity assessment of peptides and proteins from complex natural mixtures by an initial screening using the miniaturized on-line HRS platform, followed by a subsequent MS-guided purification and chemical identification using proteomics approaches. The workflow was demonstrated with the venom of the African spitting cobra, \textit{Naja Mossambica}. From this venom, medium- and low-affinity ligands of the AChBP were identified. After purification of the bioactives by MS-guided fractionation, the bioactivity of the purified toxins was confirmed by rescreening using the microfluidic on-line HRS system. From in-solution tryptic digestion experiments the amino-acid sequence of the bioactive peptides was confirmed and yielded Cytotoxin 1 and Cytotoxin 2 as the bioactives. These cytolytic and carditoxic peptide toxins were not known to bind to the AChBP.

Chapter 3 describes a follow-up study of the analytical workflow described in Chapter 2. In this study the analytical methodology was demonstrated for the screening of small molecules and peptides from toad skin extracts and cone snail venom, respectively. Next to MS and MS/MS identification of the bioactives found, additional NMR analyses were performed on the bioactives discovered in the toad skin extract in order to achieve their full structural elucidation. A bioactive peptide in the \textit{Conus textile} snail venom was pinpointed amongst >1,000 other peptides. This identification process was rapidly performed employing only two duplicate analytical runs (60 min each). The number of cysteine bridges in the bioactive peptide was subsequently uncovered by reduction experiments using dithiothreitol (DTT). In this experiment, after reduction of the cysteine bridges, the number of cysteine bridge pairs can be determined by MS measuring an increase of the accurate mass of target peptide with $m/z +2.016$ (2x1.008; the total mass of 2 H+ ions arising from reducing an S-S group to SH). The analytical workflow to screen for non-peptide small molecule bioactives (molecular weight of 200 to 1,000 Da) was demonstrated by the screening of \textit{Bufo alvarius} and \textit{Bufo marinus} toad skin extracts. Several tryptamine-like and steroidal binders of the AChBP were detected in the crude skin extracts. After MS-guided purification, the chemical identity of the compounds was assessed by NMR and MS/MS, and their biological activity was tested and confirmed in conventional radioligand binding assays.

Chapter 4 described the development of a fluorescence enhancement assay for the serotonine binding protein (5HTBP) in a microplate reader format followed by the development and application of a microfluidic on-line HRS format. The 5HTBP is an engineered binding protein which has the ligand recognition properties of the 5-HT$_3$R in the scaffold of the AChBP. This robust and straightforward fluorescence enhancement
assay is a good initial screening method of complex mixtures for finding novel bioactives targeting the 5-HT₃R. Main strengths of this fluorescence-based assay are its cost-effectiveness and ease of operation compared to the radioligand binding variant, and the ability to perform the assay in microfluidic on-line HRS format when analyzing mixtures. Application of this analytical screening format for screening mixtures was demonstrated using *Pseudonaja inframacula*, *Pseudonaja affinis* and *Dendroaspis polylepis* snake venoms. It was shown that the bioactives found could rapidly be correlated to their accurate masses obtained by MS.

A typical characteristic of the microfluidic on-line HRS system developed and applied in Chapters 2-4 is the possibility to perform the analysis with very small sample volumes. This is a great advantage in natural extract screening in cases that only low amounts of sample are available. In this thesis, microfluidic on-line HRS methodologies are described with two different assays: one for AChBP and the other for 5HTBP, both based on the fluorescence enhancement assay principle. The generic microfluidic on-line HRS setup is intrinsically adaptable to many other assay formats. Examples of other microfluidic on-line HRS formats, that are not described in this thesis, focus on screening for activators and/or inhibitors of thrombin and for factor Xa. These assays are based on the enzymatic cleavage of a fluorogenic substrate by thrombin or factor Xa. We foresee other types of assays, mainly fluorescence based binding assays and enzymatic cleavage based homogenous assays, that are applicable to the microfluidic on-line HRS setup for screening of animal venoms for new drug leads. A generic limitation of on-line HRS setups is that only homogenous assays, with short (seconds to minutes range) incubation times, can be implemented in a straightforward manner. Assays that for example require long incubation times, filtration based assays, and/or cellular assays with adherent cells are out of scope for the microfluidic on-line HRS screening.

The new analytical workflow introduced in this thesis - with the subsequent LC-MS guided purification of the accurate masses of bioactives identified using the microfluidic on-line HRS - was able to efficiently purify bioactives from animal venoms. This workflow is much more straightforward compared to the traditional BGF approaches, since accurate mass is directly obtained by the microfluidic on-line HRS screening. With this workflow the repeated bioassay steps after each purification step in BGF could be circumvented. This is beneficial only small sample quantities are available and also cost-effective, since bioassays are often quite expensive.

In Chapters 2-4 the developed and applied bioassays screen for affinity towards soluble binding proteins, which mimic real receptors. Only ligand binding affinity information is obtained from such assays. For measuring functional information (i.e. whether a ligand is an activator or a blocker of an ion channel), cell based assays that include measuring ion channel activity are needed. Coupling cell-based assays in on-line HRS is a complex endeavor due to the technical difficulty of combining living cellular systems with LC eluents. Therefore, as an alternative, the recently introduced at-line nanofractionation methodology can be used when screening complex mixtures with cell based assays. The nanofractionation approach uses automated high-resolution fractionation of LC column effluents on high density well plates. Chapter 5 describes the development of an at-line cell-based screening methodology encompassing LC with nanofractionation followed by a functional fluorescence-based calcium-flux assay of the α₇-nAChR. As for the on-line HRS analytics, in the nanofractionation approach, parallel MS detection is carried out in order to simultaneously assess accurate masses
of eluting bioactives. The applied assay uses a human neuroblastoma cell line stably overexpressing the α7-nAChR and was used for screening agonists and allosteric modulators of the α7-nAChR. After method development, the applicability of the new methodology was demonstrated by screening a hallucinogenic mushroom extract (from *Psilocybe mckennaii*). This study also demonstrated that two orthogonal separations of the crude extract can facilitate the identification of bioactive compounds when extracts are very complex and bioactives co-elute in one dimension. In this orthogonal separation approach the same crude mixture was analyzed two times; the first analysis using reverse phase LC and the second analysis using hydrophilic interaction LC (HILIC). Accurate masses correlated to bioactivity in the first dimension, should also show bioactivity in the second dimension, thereby strongly reducing the bioactive candidates. As follow-up research we envision screening approaches using direct 2D LC separation in combination with on-line or at-line bioassays and parallel MS detection. These systems would be powerful for profiling very complex mixtures.

The cell-based screening approach described in Chapter 5 combines high-resolution nanofractionation with a functional cell-based assay. This at-line nanofractionation methodology has high potential for screening natural extracts, and it improves the traditional BGF screening approach by collecting fractions in the resolution of seconds instead of minutes. The at-line nanofractionation approach can be combined with many different assay formats performed in microplate readers. Recent examples are at-line nanofractionation methodologies with radioligand binding assays for screening chemokine receptor ligands, fluorescence-based assays screening for thrombin and factor Xa modulators, and cell-based assays for screening G-protein coupled receptor (GPCR) targets. Next to natural extract screening, the nanofractionation approach has also been applied in other fields, such as in metabolic profiling of drugs and in environmental analysis. As future perspective we see the further miniaturization of the nanofractionation approach which would allow performing the bioassay in array format and which would require only minute sample amounts. We furthermore foresee the continuation of developing cell-based assays combined with at-line nanofractionation for various types of drug targets. Looking beyond the cellular level, biological assays at the whole organ level, or even at the animal model level, might be combined with at-line nanofractionation approaches. Assays in organ-on-a-chip microfluidic format using three dimensional (3D) cell cultured tissues, neurons-on-a-chip, liver cells or kidney cells could potentially be combined with at-line nanofractionation. Zebrafish embryo assays in microfluidic HTS format comprise a whole animal model which is widely used for drug lead discovery, drug target discovery, disease modeling and toxicology, and could be adapted for, and implemented to, nanofractionation screening. Advantages of zebrafish embryo assays are that they are in vivo assays that are both ethically sound and cost-effective compared to more conventional animal models, and that they are easy to automate for HTS screening. Combining at-line nanofractionation with organ-on-a-chip assays or zebrafish embryo assays for screening campaigns of natural extracts can give not only cellular readouts, but also functional responses on whole living organisms of individual bioactives in complex natural extracts.

Chapter 6 introduced the concept of a post-column continuous flow cell-based bioactivity screening assay. The system combines LC separation with an on-line mammalian cell-based assay using flow cytometry (FC) as readout with parallel MS. In this system the same functional calcium-flux assay was applied as in Chapter 5 using
human α7-nAChR expressing SH-SY5Y neuroblastoma cells. The advantage of using on-line screening compared to the at-line nanofractionation approach is that on-line assay in continuous flow is much faster and that the bioassay readout obtained has higher resolution. The LC-FC-MS screening system was developed in two assay modes: agonist and mixed antagonist-agonist mode. The latter mode gives the possibility of simultaneous detection of agonists and antagonists. The applicability of the system was demonstrated in two proof-of-principle experiments by the screening of tobacco plant leaf extracts in agonist mode, and snake venoms in mixed antagonist-agonist mode, as we expected to detect agonists from the tobacco extract (i.e. nicotine) and antagonists (i.e. three finger toxins) in snake venoms.

Follow-up research should concentrate on overcoming some shortcomings of the prototype system. The current screening setup in principle is well suited for suspension cell-lines (for example blood cells), which do not need to be mechanically kept in suspension. In the prototype system an adherent cell line had to be kept in suspension using a small stirr bar in a superloop (i.e. a pressurized container) and cells were continuously infused into the flow cytometer. In future setups, the cell viability of adherent cell lines could be improved by using cell-culture microparticles or beads. We also foresee development of advanced superloops with oxygen-pressurized compartments allowing long-time continuous infusion of viable cells into the FC bioassay part of the system. The setup described in Chapter 6 is using an LC system with a normal bore column. However, because of the low compatibility of mammalian cells with organic solvents, the LC effluent had to be highly diluted before mixing with the continuous flow bioassay. The unfavorable dilution could be decreased by using nano-LC or micro-LC columns that use down to nl/min flow rates instead of the 120 μl/min used in the current setup. Other chromatographic separation techniques, such as ion-exchange chromatography (IEC) or size-exclusion chromatography (SEC) could be used instead of reversed-phase LC. For these alternative separation techniques, however, other limitations arise connected to MS and bioassay compatibility. For instance, salt concentrations should still be tolerable for living cells and not cause ion suppression in MS. Flow cytometry can give additional bioassay readout information in HRS screening setups. First of all, responses of individual cells are measured. Secondly, modern flow cytometers can monitor multiple fluorescence and scattering signals, which allows performing different assays simultaneously as well as measurement of cellular morphology and/or clustering changes over time and upon mixing with bioactives eluting from the chromatographic system. As an example, the measurement of calcium fluxes by a green fluorescent dye (detected in one channel of the flow cytometer) in combination with the simultaneous recording of cell viability by a specific fluorescent dye (detected in another channel of the flow cytometer) is demonstrated in Chapter 6. Finally, software algorithms of most FCs allow gating, or selecting and analyzing only a group of cells detected by the FC.

A general concern regarding cell-based assays in any new cell-based screening setup is that cells express many different receptors and ion channels. This means that a signal, such as a calcium flux, can be caused not only by the receptor studied (which is usually over expressed by the cell), but also by other receptors. Therefore, it is crucial to use receptor selective antagonists and allosteric modulators as controls to confirm that signals observed are caused by the activation or inhibition of the specific receptor under study. This might be used in an advantageous manner if the cell line is used for
screening multiple drug targets expressed by the cell at the same time\textsuperscript{10-12}. On the other hand, performing assays using target-specific modulators and/or inhibitors allows to selectively modulate one receptor for the screening. Other receptors present in the cells, which are not aimed to be studied in the assay, can be inhibited thereby, ensuring that the signals observed are specifically caused by the receptor under study.

Altogether, the screening methodologies described in this thesis are new analytical tools that are sensitive, fast and straightforward, and as such valuable additions to the field of natural extract screening and venom based drug discovery programs. The analytical methods in this thesis were applied to natural extract based drug discovery for CNS diseases, but these methods show great potential for screening other types of complex mixtures and other drug targets.
Nederlandse Samenvatting
De natuur is een rijke bron van actieve stoffen geproduceerd door allerlei micro-organismen, paddestoelen, planten, en dieren. Deze verbindingen kunnen een interactie aangaan met biologische 'targets', zoals bijvoorbeeld, receptoren, ionen-kanalen en enzymen, en daarmee hebben zij effect op het functioneren van cellen en organismen. Bioactieve verbindingen uit natuurlijke bronnen zijn daardoor ook waardevol in de ontwikkeling van nieuwe geneesmiddelen. Dit proefschrift beschrijft de ontwikkeling van nieuwe analytische screeningsmethoden die kunnen worden gebruikt bij het ontdekken van biologisch actieve verbindingen uit natuurlijke bronnen.

De eerste fase in het onderzoek naar nieuwe bioactieve substanties vanuit natuurlijke extracten wordt traditioneel uitgevoerd middels zogenoemde 'bioassay guided fractionation' (BGF). Deze methode is reeds met succes toegepast bij de ontwikkeling van diverse geneesmiddelen gebaseerd op natuurlijke extracten. BGF heeft echter beperkingen, welke er mede voor hebben gezorgd dat de ontwikkeling van geneesmiddelen uit natuurlijke extracten de laatste 20-30 jaar is afgenomen. De redenen hiervoor zijn dat de ontwikkeling van geneesmiddelen middels BGF grote hoeveelheden natuurlijk extract gebruiken, dat de methode arbeidsintensief is, en dat verlies van bioactiviteit kan optreden vanwege de herhaalde stappen in het proces. Er is daarom een duidelijke behoefte aan minder arbeidsintensieve methoden, die bovendien minder bronmateriaal nodig hebben.

Dit proefschrift beschrijft de ontwikkeling en toepassing van 'on-line' hoge resolutie screening analyse (HRS) voor natuurlijke extracten, waarbij voornamelijk gebruik is gemaakt van dierlijke giffen. HRS-opstellingen combineren over het algemeen vloeistofchromatografie (LC) met massaspectrometrie (MS) en met een continue flow biosassay. Deze technologie maakt het mogelijk componenten in complexe mengsels te detecteren, gevolgd door een parallele bepaling van hun specifieke bioactiviteit en chemische identiteit. Een belangrijk voordeel van HRS in vergelijking met BGF is dat middels een enkele analyse de bioactiviteit van afzonderlijke componenten kan worden gedetecteerd en direct kan worden gecorreleerd aan hun moleculaire massa. Dit bespoedigt de identificatie van de bioactieve stof.

In dit proefschrift zijn de α7-nicotine acetylcholine receptor (α7-nAChR) en de serotonine type 3 receptor (5-HT3R) gebruikt als aangrijpingspunt voor screening van bioactieve stoffen. Deze receptoren worden in verband gebracht met ziektes aan het centraal zenuwstelsel, zoals cognitieve gebreken, schizofrenie, epilepsie, Alzheimer en Parkinson. De identificatie van nieuwe verbindingen die effect hebben op deze receptoren zijn interessant in het kader van de ontwikkeling van nieuwe geneesmiddelen en diagnostische gereedschappen. In dit proefschrift zijn verschillende typen bioassays ontwikkeld en toegepast in HRS systemen om actieve verbindingen te identificeren die werken op de α7-nAChR en de 5-HT3R.

Hoofdstuk 1 geeft een overzicht van de achtergronden van dit proefschrift waarbij de behoeften in de neurowetenschappen met betrekking tot metingen aan ligand-gestuurde ion kanalen, geneesmiddelontwikkeling en het screening van natuurlijke extracten en andere complexe mengsels centraal staan. Hoofdstuk 1 is verdeeld in drie onderwerpen: Doelen, Bronnen en Benaderingen. In de sectie Doelen wordt een overzicht gegeven van de relevante geneesmiddeldoelen die zijn bestudeerd in dit proefschrift. Hierbij wordt de structuur, detectie, functie en betrokkenheid van de α7-nAChR en de 5-HT3R bij

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Het doel van de Hoofdstukken 2-4 is de ontwikkeling van nieuwe analytische technieken die de beperkingen wegnemen van traditionele BGF- en HRS-systemen ten aanzien van de grote monstervolumina die nodig zijn. Om dit te realiseren zijn geminiaturiseerde on-line HRS-systemen ontwikkeld die nanoliters of microgrammen van het monster kunnen analyseren. Voorbeelden van monsters waar dit van grote waarde kan zijn, zijn giften van zeldzame diersoorten. Dierlijke giften blijken rijke bronnen van natuurlijke bioactieve verbindingen, die in staat zijn om receptoren, ion-kanalen en enzymen te activeren, te inhiberen of te moduleren. Om deze redenen is gif-gebaseerd onderzoek een belangrijk hulpmiddel in de ontwikkeling van nieuwe geneesmiddelen. Het nieuw ontwikkelde ‘microfluidic’ on-line HRS-systeem gebruikt nL-LC gekoppeld met MS via nano-electrospray ionisatie (nano-ESI). Parallel gekoppeld is een continue vloeistofstroom bioassay op chip-formaat met een fluorescentie detector.

In Hoofdstuk 2 wordt een analytische workflow beschreven waarin het acetylcholine bindende eiwit (AChBP) werd gebruikt als screening target. AChBP is een oplosbaar eiwit en is homoloog met het extracellulaire domein van de α7-nAChR. De analytische workflow maakte het mogelijk om de bioactiviteit van peptiden en eiwitten van complexe natuurlijke mengsels te beoordelen met behulp van het geminiaturiseerde on-line HRS platform. Hierna werd een MS-gestuurde zuivering gevolgd door chemische identificatie van de ACHBP-bindende stoffen met behulp van proteomics technieken uitgevoerd.

De werking van de geïdentificeerde stoffen werd aangetoond in het gif van de Afrikaanse spugende cobra, Naja Mossambica. In dit gif werden matige en lage affiniteit-liganden van AChBP geïdentificeerd. Na isolatie van deze bioactieve verbindingen met de MS-gestuurde fractionering werd de bioactiviteit van de gezuiverde gifstoffen bevestigd middels her-screening met het microfluidic on-line HRS systeem. Met een enzymatische digestie met trypsine werd de aminozuur volgorde van de bioactieve pepitiden bevestigd als overeenkomend met Cytotoxine 1 en Cytotoxine 2. Van deze cytolytische en cardiotoxische pepitiden was niet bekend dat ze binden met AChBP.

Hoofdstuk 3 beschrijft een vervolgstudie van de analytische workflow beschreven in Hoofdstuk 2. In dit hoofdstuk wordt de analytisch methode gedemonstreerd met de screening van kleine moleculen en peptiden afkomstig van een extract van de huid van een pad en van het gif van een Conus slak. Naast MS- en MS/MS-analyses van de bioactieve stoffen in het paddenhuideextract werden NMR-analyses uitgevoerd om zo tot een complete structurele opheldering te komen. Een bioactief peptide van het Conus textile slakkengif werd gevonden tussen meer dan 1.000 andere pepitiden. Het identificatieproces was relatief snel; slechts twee duplicaat analyses van elk 60 min waren nodig. Het aantal cysteïne bruggen in de aminozuurketens werd bepaald door reductieve experimenten met dithiothreitol. De analytische workflow voor het screenen van niet-peptiden (kleine organische bioactieve stoffen met een molecule negent...
van 200 tot 1000 Da) werd gedemonstreerd met de analyse van *Bufo alvarius* en *Bufo marinus* paddenhuidextracten. Verscheidene tryptamine analogen en steroïde binders van AChBP werden gedetecteerd in deze extracten. Na MS-gestuurde opzuivering werd de chemische identiteit van deze moleculen geëvalueerd door NMR- en MS/MS-analyse, en werd de biologische activiteit getest en bevestigd met een conventionele radioligand binding assay.

Hoofdstuk 4 beschrijft de ontwikkeling van een fluorescentie assay voor het serotonine bindende eiwit (5HTBP) in een microplaat-formaat en in een microfluidic on-line HRS-formaat. Het 5HTBP is een eiwit met gelijksoortige eigenschappen als het ligand bindende gedeelte van de 5-HT₃R. Het 5HTBP is gemaakt met behulp van cellen waarvan het gen dat codeert voor AChBP aangepast is, zodat het 5HTBP produceert. De ontwikkelde fluorescentie assay voor het 5HTBP is robuust en eenvoudig en een goede eerste screening-methode van complexe mengsels voor het vinden nieuwe bioactieve verbindingen die binden aan de 5-HT₃R. De belangrijkste voordelen van deze fluorescentie assay zijn de lage kosten van de assay en de eenvoudige protocollen in vergelijking met de conventionele radioligand binding assay. Ook geeft het de mogelijkheid om de assay in microfluidic on-line HRS-formaat uit te voeren voor mengselanalyse. De toepassing van deze analytische screening voor mengselanalyse werd aangetoond met gif van de peninsula bruine slang, de gevlekte bruine slang en de zwarte mamba (resp. *Pseudonaja inframacula*, *Pseudonaja affinis* en *Dendroaspis polylepis*). De gescreende bioactieven konden met de analytische techniek snel worden gecorreleerd met de accurate massa verkregen met parallel uitgevoerde MS.

De in de Hoofdstukken 2-4 ontwikkelde bioassays, screenen voor biologische affiniteit voor oplosbare bindende eiwitten, welke varianten zijn van de humane receptoren. Van dit type assays kan alleen informatie over bindingsaffiniteit worden verkregen. Voor het krijgen van functionele informatie (zoals het bepalen of een ligand een activator of een blokker van een ionkanaal is) zijn cel-gebaseerde assays nodig die de ionkanaal-activiteit meten. Het koppelen van een cel-gebaseerde assay in on-line HRS is technisch en biologisch gezien complex door de analytische uitdagingen met betrekking tot het combineren van levende celsystemen met LC chemicaliën. Daarom wordt de onlangs geïntroduceerde ‘at-line nanofractionation’ methode gebruikt als een alternatief voor het screenen van complexe mengsels met cel-gebaseerde assays. De nanofractionering benadering gebruikt geautomatiseerde hoge-resolutie fractionering van LC effluenten over microplaten met hoge ‘well’-dichtheid.

Hoofdstuk 5 beschrijft de ontwikkeling van een at-line cel-gebaseerde screening methode waarin LC-nanofractionering gevolgd wordt door een functionele calcium-flux fluorescentie assay voor de α₇-nAChR. Zoals in de on-line HRS analytische technologie, wordt in de nanofractionering benadering MS-detectie in parallel uitgevoerd om de accurate massa van de bioactieven te kunnen bepalen. Deze bioassay gebruikt menselijke neuroblastoma cellen die de α₇-nAChR stabiel tot overexpressie brengen. De bioassay is gebruikt voor het screenen van agonisten en allosterische modulatoren voor de α₇-nAChR. Na analytische methode-ontwikkeling werd de toepasbaarheid ervan gedemonstreerd met het screenen van een extract van hallucinogene paddenstoelen (*Psilocybe mckennaii*). Deze studie toont ook het nut van het achter elkaar uitvoeren van twee orthogonale scheidingen van ruwe extracten, waardoor de identificatie
van bioactieve verbindingen in zeer complexe extracten met veel co-eluerende componenten mogelijk wordt. In deze benadering wordt hetzelfde ruwe extract twee keer geanalyseerd. De eerste analyse met een ‘reversed-phase’ LC-scheiding en de tweede analyse met een ‘hydrophilic interaction’ LC (HILIC) scheiding. Verbindingen die correleren met bioactiviteit in zowel de eerste als de tweede scheiding kunnen worden gematched op grond van hun accurate massa.

Hoofdstuk 6 introduceert het concept van een continue on-line ‘post-column flow cell’-gebaseerde bioactiviteit screening assay. Dit systeem combineert LC scheiding met een on-line humane cel-gebaseerde assay door middel van flow cytometrie (FC) als biologische detectie met in parallel daaraan MS voor identificatie. Dit systeem gebruikt dezelfde functionele calcium-flux assay als beschreven in Hoofdstuk 5, waarin α7-nAChR expresserende SH-SY5Y neuroblastoma cellen werden gebruikt. Het voordeel van on-line screening in vergelijking met at-line nanofractionering is dat een on-line assay sneller is en de uitgelezen bioassay een hogere resolutie heeft. Het LC-FC-MS screening systeem is ontwikkeld in twee assay-formaten: agonist-formaat en duale antagonist-agonist-formaat. Het duale assay-formaat geeft de mogelijkheid om gelijktijdig de detectie van agonisten en antagonisten uit te voeren. De toepasbaarheid van het systeem werd gedemonstreerd in twee ‘proof-of-principle’ experimenten, namelijk voor de screening van extracten van tabaksplantbladeren in agonist-formaat en slangengif in duale antagonist-agonist-formaat.

Samenvattend zijn de screening methoden beschreven in dit proefschrift nieuwe analytische gereedschappen die gevoelig en snel zijn. Ze vormen daarom waardevolle toevoegingen aan het veld van het screenen van natuurlijke extracten en op gif-gebaseerde geneesmiddelontwikkeling programma’s. De analytische methoden ontwikkeld in dit proefschrift vinden toepassing in de naturextract- en gif-gebaseerde geneesmiddelontwikkeling voor ziektes aan het centraal zenuwstelsel. Verder hebben deze methoden ook een groot potentieel voor het screenen van complexe mengsels en/of het screenen van bioactieve verbindingen voor andere targets dan die gebruikt in dit proefschrift.

Korte Nederlandse samenvatting

In de natuur komen verschillende mengsels van stoffen voor die mogelijk bruikbaar zijn in de ontwikkeling van geneesmiddelen. Voorbeelden zijn slangen- en slakkengif, en extracten van planten en paddenstoelen. De identificatie van de actieve componenten in deze mengsels is echter uitdagend, kost veel tijd, en er zijn relatief grote hoeveelheden van de vaak schaarse mengsels voor nodig. In dit proefschrift zijn nieuwe analytische technieken ontwikkeld die de identificatie van bruikbare componenten in giften en extracten sneller en makkelijker maken en waarvoor veel minder materiaal nodig is. Nieuwe bioassay-technieken zijn ontwikkeld gebruikmakend van combinaties van vloeistofchromatografie, massaspectrometrie, flow cytometrie en bioassays. Deze nieuwe methoden zijn toegepast voor het ontdekken van waardevolle componenten in natuurextracten.
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Magyar nyelvű összefoglaló
E doktori disszertáció célja új analitikai biológiai szűrő (screening) platformok fejlesztése, amelyekkel biológiaiak aktiv molekulákat lehet felfedezni természetes anyagok kivonataiban. A természet gazdag forrása aktiv vegyületeknek, amelyeket mikróbák, növények, gombák és állatok szervezete állít elő. Ezek az aktiv vegyületek gyakran kölcsönhatásba lépnek különböző biológiai célpontokkal, mint például receptorokkal, ioncsatornákkal és enzimekkel. Emiatt a természetes előfordulási formájukból kivont biológiaiak aktiv vegyületek fontos kiindulási pontjai a gyógyszerkutatásnak.

Az aktiv vegyületeket a természetes kivonatokból hagymányosan bioassay irányított frakcionálással (Bioassay-guided fractionation, BGF) azonosítják. E módszert sikeresen alkalmazták a múltban sok fontos természerves forrásból kivont vagy kifejlesztett gyógyszer felfedezésére. Habár a BGF-nek vannak limitációi, ami más okok mellett a természetes kivonat alapú gyógyszerkutatási programok csökkenését okozta az elmúlt 20-30 évben. A BGF-hez nagy mennyiségű kiindulási kivonat szükséges, munka- és időigényes, és az aktiv molekula gyakran elvész a megismételt bioassay lépések miatt. Emiatt új analitikai módszerekre van szükség, amik kevesebb munkaigényesek, kevesebb ismételt bioassay lépést használnak, és kisebb kezdeti természetes kivonat mennyiséget igényelnek. Ez a tézis on-line nagy felbontású screening (high resolution screening, HRS) analitikai technikákat ír le természetes kivonatok szűrésére, a természetes kivonatokon belül az állati mérgekre (venomokra) fókuszálva. A HRS technikákat általában a nagy hatékonyságú folyadékkromatográfiát (high performance liquid chromatography, HPLC) kombinálják tömegspektrometriával (mass spectrometry, MS) és a kolonna után installált folyamatos áramlású bioassayakkal. Ez a kombinált technológia szét tudja választani a vegyületeket komplex keverékekből (mint például a természetes kivonatok), és a szétválasztás után parallel detekcióval tudja a vegyületek biológiai aktivitását és azok molekuláris tömegét. A HRS technológia előnye a BGF-nal szemben, hogy egyetlen mérésből információt lehet vele szerezni a keverék egyes komponenseinek a biológiai aktivitásáról és az aktiv vegyületek molekuláris tömegéről.

Ennek a tézisnek a molekuláris célpontjai az α7-nikotinos acetilkolin receptor (α7-nAChR) és a hármastipszis szerotonin receptor (5-HT₃R). Ez a két receptor számos központi idegrendszeri betegségben szerepet játszik, mint például skizofréniaban, szorongásban, depresszióban, epilepsziában, Alzheimer betegségben, Parkinson betegségben és mentális hanyatlásban. Ebből kifolyólag szükség van új aktiv molekulák felfedezésére, amelyek modulálják ezeket receptorokat, és új gyógyszekeket vagy diagnosztikai eszközöket lehet belöőük fejleszteni. Ebben a tézisben különböző fajta bioassayeket kombináltunk HRS technológiával, így olyan aktiv molekulákat lehet azonosítani, amelyek hatnak az α7-nAChR-ra és a 5-HT₃R-ra.

Az 1. Fejezet egy átfogó összefoglalást ad a munka tézis hátteréről, megvitatja a hiányosságokat és igényeket a neurobiológia ligand vezérelt ioncsatornák szakterületén, és a természetes kivonat alapú gyógyszerkutatás területén. A fejezet három alfejezetre van felosztva: Célpontok, Források és Módszerek. A "Célpontok" alfejezet áttekintést ad a munkám során alkalmazott molekuláris célpontok fontosságáról: részletesen áttekinti az α7-nAChR és a 5-HT₃R szerkezetét, lokalizációját, funkcióját, és különböző betegségekben való részvételüket. A "Források" alfejezet áttekinti a természetes eredetű aktiv molekulák fontosságát. Végül a "Módszerek" alfejezet a klasszikus és új analitikai módszereket tekinti át, amelyeket új aktiv molekulák felfedezésére használnak az ioncsatorna kutatás területén. A venom alapú gyógyszerkutatás folyamatát aktuális példák
mutatják be. Az alfejezet utolsó része új analitikai módszereket tekint át, amelyek kapcsolt technikákat használnak a természetes kivonatok szűrésére.

A 2-4. Fejezetek célja, hogy legyőzze a tradicionális BGF és HRS technikák (amik normál méretű HPLC kolonnákat használnak) azon limitációját, hogy nagy mennyiségű kezdeti mintát igényeljen. Ehhez miniatúrizált, vagy mikrofluidikai on-line HRS technikákat fejlesztettünk, amelyek csak nanoliternyi, vagy mikrogramnyi mintát igényelnek az analízishez. Ez olyankor lehet nagyon hasznos, amikor ritka állatfajok vagy szűrőszőrű és szűrők egyéb eszközök működését. Ezen okból a venom alapú gyógyszerkutatás egy fontos módszert új gyógyszer vezérmolekulák azonosítására. A könyvbén bemutatott mikrofluidikai on-line HRS technika nano-folyadékkromatográfiát (nano-LC) használ nanoelektrospray ionizációt alkalmazó MS-el és paralel mikrofluidikai chip alapú folyamatos áramlású, mikrofluidikai fluoreszcens detektort alkalmazó bioassayekkel összekapcsolva.

A 2. Fejezet egy olyan analitikai munkafolyamatot mutat be, ami az acetilkolin kötő fehérjét (Acetylcholine binding protein, AChBP) használja a biológiai szűrés (vagy screening) célpontjaként. Az AChBP egy vízoldékony fehérje, ami homológ az α7-nAChR extracelluláris doménjével. Az analitikai munkafolyamat első lépésében komplex természetes eredetű keverékeket analizáltunk a mikrofluidikai on-line HRS technikával, majd a bioaktív peptideket és fehérjéket MS-irányított frakcionálással tisztítottuk és proteomikai módszerekkel azonosítottuk. A munkafolyamatot az afrikai köködőkobra, Naja mossambica venomján mutattuk be. Ebből a venomból közepes és alacsony affinitású AChBP ligandokat azonosítottunk. Miután MS-irányított frakcionálással kinyertük a bioaktív toxinokat, a tisztított fehérjéket bioassayokkal összekapcsolva.

A 3. Fejezet a 2. Fejezetben bemutatott analitikai munkafolyamat folytatása. Ebben a tanulmányban az analitikai módszer kis méretű molekulák és peptidek szűrésére fókuszál varangy bőr kivonatokból és tengeri kúpcsiga venomjából. Az bioaktív molekulák MS és MS/MS azonosítása mellett NMR (nukleáris magrezonancia) analízissel értük el a teljes szerkezeti elucídálatást. A kúpcsiga venomjából egy bioaktív peptidet ezer másik peptiddel mellett azonosítottunk. Ehhez a gyors azonosítási folyamatot csak két ismételt analitikai mérés (egyenként 60 perces) volt szükséges. A cisztein hidak számát dithiothreitol-os (DTT) redukciós kísérellel azonosítottuk. Az analitikai munkafolyamatot a nem-peptid típusú kis molekula bioaktivok szűrésére a Bufo alvarius és a Bufo marinus varangy bőr kivonatokban demonstráltuk. Számos triptamin-szerű és szteroid típusú AChBP ligandot detektáltunk a varangy bőr kivonatokból. Az MS-irányított tisztítás után a molekulák kémiai identitását NMR és MS/MS technikákkal azonosítottuk, illetve a biológiai aktivitást hagyományos radioligand kötő assayekkel igazoltuk vissza.

A 4. Fejezetben egy fluoreszcencia fokozó bioassayt fejlesztettünk a szerotonin kötő fehérjéhez (5-hydroxytryptamine binding protein, 5HTBP) való kötődés méréséhez,
mikroplate olvasó formátumban és mikrofluidikus on-line HRS formátumban. A 5HTBP egy megtervezett kötő fehérje, aminek a 5-HT₃R ligand felismerő helyei vannak az AChBP vázába tervezve. A megbízható és direct fluoreszcencia fokozó bioassay egy alkalmaz kezdeti szűrő módszer új bioaktív molekulák felfedezésére komplex keverékhekből, amelyek a 5-HT₃R-ra hatnak. A fluoreszcencia alapú módszer fő erősségei, hogy olcsó és egyszerű a radioligand kötő assayhez képest, illetve lehetőséget nyújt a mikrofluidikai on-line HRS technikában történő alkalmazáshoz, amely előnyös keverékek analizálásához. Az technika gyakorlati alkalmazását a Pseudonaja inflamacula, Pseudonaja afferens és a Dendroaspis polylepis kígyómérgek analízisével demonstráltuk. Ezekben a kísérletekben a biológiai aktivitást gyorsan és direkt korrelálni tudtuk az aktív molekulák pontos tömegéhez.

A 2-4. fejezetben leírt bioassayek a biológiai aktivitás szűréshez vízoldékkony kötő fehérjéket használtak, amelyek az igazi receptorok mimikái. Ezekből az assayeből csak a ligandok kötő affinitásáról lehet információt nyerni. Ha a ligandok funkcióját akarjuk mérni (a ligand aktiválja -e vagy blokkolja az ion csatornát), sejt-alapú assaykra van szükség. A sejt-alapú assayek kombinálása az on-line HRS technikákkal egy komplex törekvés, mivel technikailag nehéz kombinálni az élő sejt alapú rendszereket a HPLC eluensekkel. Eggyel alternatív módszerként az úgynevezett at-line nanofrakcionálást lehet használni, ha komplex keverékeket akarunk sejt-alapú assayekkel szűrni. A nanofrakcionálás egy automatizált technika, ami a HPLC-n átfolyó eluenst nagy sűrűségű, 96-, 364- vagy 1216-lukú plate-ekre frakcionálja. Az 5. fejezet egy olyan sejt-alapú szűrő technikát ír le, ami az at-line nanofrakcionálást a7-nAChR funkciót mérő fluorescens calcium áramlásti assayekkel kombinálja. Ugyanúgy, mint az on-line HRS technika, a nanofrakcionálási módszer is alkalmaz parallelnál MS-t a bioaktív molekulák tömegének az elemzéséhez. Az agonistákat és allostérikus modulátorokat szűrő assay-hez a7-nAChR-t stabilan expresszáló humán neuroblasztóma sejtvonalat használtunk. A technika optimalizálása után a módszer alkalmazását a hallucinogén gomba Psilocybe mckennaii kivonatán demonstráltuk. Ebben a fejezetben azt is bemutattuk, hogy két ortogonális elválasztó technikával olyan kivonatokból is azonosítható a bioaktív molekulát, amelyekből egydimenziós elválasztással a bioaktív több másik molekulával együtt eluálódik. Az ortogonális elválasztással ugyanaz a kivonat volt kétszer analizálva, egyszer fordított fázisú kromatográfiával, egyszer hidrofil interakciós kromatográfiával.

A pontos molekulatömeg és a bioaktivitás korrelálni fog mindkét dimenzióban, amivel megbízhatóbban lehet meghatározni a bioaktív molekula tömegét.

szűrésén kimutattuk a nikotint (agonista), illetve a kígyómérgekből különböző ismert 3-ujjú toxin típusú antagonistákat.

Összességében, ebben a könyvben bemutatott biológiai szűrő technikák érzékeny, gyors és direkt analitikai eszközök, amik értékes új fejlesztések a természetes kivonatok szűrése és a venom alapú gyógyszerkutatás szakterületéhez. A leírt analitikai technikák természetes kivonatokból a központi idegrendszer célzó molekulákat szűrnek, viszont a technika sikeresen alkalmazható más komplex keverékek és más biológiai célpontok vizsgálatára is.

Rövid magyar nyelvű összefoglaló

A természet gazdag forrása olyan molekuláknak, amelyekből új gyógyszereket lehet fejleszteni. Ilyen természetes források a különböző állati eredetű kivonatok (mint például a kígyómérgek), illetve a növények és gombák kivonatai. Ezeknek a természetes eredetű molekuláknak, melyekből a jövő gyógyszereit lehet fejleszteni, a jelenlegi azonosítási technikák nagyon időigényesek és tetemes mennyiségű küldelési mintát igényelnek. Ezért, ennek a doktori dolgozatnak a célja olyan új analitikai módszerek fejlesztése, amelyekkel a biológiaiak aktiv molekulák azonosítása természetes kivonatokból gyorsabb, egyszerűbb és kisebb mennyiségű küldelési mintát igényelnek. Ezek az új analitikai módszerek direktt, egy időben működő szűrő rendszerként kombinálják a keverékek vegyületekre való elválasztását (folyadékkromatográfia), az elválasztott ismeretlen molekulák tömegénak meghatározását (tömegspektrometria), a molekulákkal kezelt sejtek multiparaméteres vizsgálatát (áramlási citometria), és biológiai vizsgálatokat (bioassay-k). Ezeket az új kombinált analitikai technikákat sikeresen alkalmaztuk új biológiaiak aktiv molekulák azonosítására.

(The Hungarian Summary and Hungarian Short summary was reviewed by Dr. Anna Pósfalvi and Zoltán Fekete)
List of publications
Publications in this thesis:


Other publications, not in this thesis:


Final Words
This is the final part of this book to thank everyone who have helped, influenced and supported me during my PhD years. First of all, before mentioning any names, I have to say it was fantastic four years at the VU, my PhD was an experience for a lifetime. I really loved to be a VU PhD student, and I am happy that I could meet so many great people like you all.

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