Chapter

7

Summary, Conclusions and Future Perspectives

The aim of the research described in this thesis was to further develop and subsequently apply the at-line nanofractionation methodology for bioactivity/bioaffinity profiling of complex mixtures and its integration into drug discovery. More specifically, this research aimed at metabolic profiling with parallel bioaffinity assessment of lead candidates targeting chemokine receptors CXCR1, CXCR2, and CXCR3, and screening of natural products such as snake venoms and mushroom extracts for novel bioactive compounds towards thrombin, factor Xa, angiotensin converting enzyme (ACE) and the α7 nicotinic acetylcholine receptor (α7-nAChR). To achieve this, a reversed phase (RP) or a hydrophilic interaction liquid chromatography (HILIC) separation was coupled to both MS detection for identification of eluting compounds and parallel bioassays using at-line nanofractionation as linking technology. The subsequent freeze-drying of the plates containing nanofractions enabled hyphenation of different types of bioassays avoiding interferences of the solvents used for separation. For the bioaffinity assessment of ligands targeting the chemokine receptors, a radioligand binding assay was applied. In the screening of natural products, the bioactivity was assessed by using either a fluorescence-based enzymatic assay (for enzyme targets thrombin, factor Xa, ACE) or a cell-based Ca^{2+} flux assay (for the α7-nAChR). The responses of the bioassay were plotted versus time to construct a so-called bioaffinity/bioactivity chromatogram. The high-resolution nanofractionation, mostly 6s/well, allowed for the resolution of the LC separation to be retained in the respective bioaffinity/bioactivity profiles. In this way, correlation based on retention time and peak shape could be established between peaks detected in the bioaffinity/bioactivity profile and extracted ion chromatograms of the corresponding *m/z*-values plotted from the parallel MS measurement.

In **Chapter 2**, the decoupled at-line nanofractionation methodology, as described above, was optimized and applied to bioaffinity assessment of mixtures of ligands targeting the CXCR3 receptor. It was shown that both 6 and 12 s fractionation resolution yielded good results in terms of bioaffinity peak resolution provided by the RPLC separation and subsequent nanofractionation onto 96-well plates. The limits of detection in the bioassay were found to be in the range of IC_{50} concentrations of the compounds tested when injected with a 100 µL injection-loop volume implying the ability to detect high-affinity binders in low concentrations. Furthermore, the comprehensive metabolic profiling of the important CXCR3 lead compounds, NBI-74330 and VUF11211, using 6 s nanofractionation resolution, was reported. Two separate chromatographic runs of each sample were acquired for MS identification and bioaffinity testing. Accurate MS and MS^2 measurements using an ion-trap–time-of-flight (IT–TOF) MS allowed (partial) structure elucidation of metabolites present in the metabolic mixtures. The bioaffinity was assessed in a radioligand binding assay based on displacement of [^{3}H]- VUF11211, a tritiated allosteric low molecular weight (LMW) CXCR3 ligand (1). In total, nine metabolites of NBI-74330 and eight metabolites of VUF1121 were detected and their structures were (partially) elucidated. A pyridyl-*N*-oxide of NBI-74330 and two VUF11211 hydroxylated metabolites were found to possess high affinity for the CXCR3 receptor. Except for the NBI-74330 pyridyl-*N*-oxide, which is

a known active metabolite of NBI-74330, no other metabolites of both lead compounds were described previously. Pharmacological relevance of the bioaffinity found for two VUF11211 metabolites was confirmed in another radioligand binding assay, where both metabolites displaced the ^{125}I-CXCL10 chemokine, an endogenous ligand of CXCR3. The methodology can also be applied to the analysis of chemokine ligands, as demonstrated with CXCL10.

In **Chapter 3**, the method developed for CXCR3 ligands was transferred and advanced for bioaffinity and selectivity assessment of both LMW molecules and chemokines binding to the CXCR1 and/or CXCR2 receptor. An important advancement was the simultaneous bioaffinity assessment and MS identification using a quadrupole–TOF instrument, which was enabled by introduction of a direct post-column split. The rapid bioaffinity screening of the metabolic mixtures generated from eight LMW CXCR2 ligands demonstrated that the method is able to identify the presence of bioactive metabolites binding with high affinity, and to determine their selectivity for the two receptors tested. Finally, the metabolic profiling with parallel bioaffinity assessment was applied to MK-7123, an allosteric modulator of both CXCR1 and CXCR2, resulting in detection of three active metabolites. One of these active metabolite, detected in the bioassay, could not be detected in the MS due to the low concentration in the mixture or too low ionization efficiency.

In **Chapter 4**, the at-line nanofractionation approach was developed and successfully applied for the screening of 39 snake venoms for thrombin and factor Xa inhibitors. A direct split enabled hyphenation of the LC separation to the parallel Q–TOF-MS analysis and bioactivity assessment in 384-well plates. The bioassay is based on the formation of a fluorescent product after the cleavage of a Rhodamin 110-based substrate by thrombin or factor Xa. In the presence of an inhibitor, a decrease in the fluorescence was measured by the plate reader. Many snake venoms showed an increase in the fluorescence at some time points in the chromatogram, which was attributed to snake protease activity. Furthermore, a strategy for the rapid identification of bioactive compounds was demonstrated on the example of three factor Xa inhibitors found in the venom of *Daboia russelii russelii*. Finally, the possibility of using this approach to study functional phylogeny or ontogenetic changes based on the comparison of the snake protease activity profiles was explored.

The strategy for the identification of the bioactive components of snake venoms was further advanced in the analysis of 30 snake venoms for the presence of ACE inhibitors, which is described in **Chapter 5**. Two innovations were incorporated to allow comprehensive bioactivity profiling and identification of the bioactive constituents. An orthogonal HILIC separation was introduced and applied on a crude snake venom showing the presence of ACE inhibitors. Since HILIC uses the same mobile phase constituents as RPLC, its implementation was easily achievable by reversing and adopting the gradient used in the RPLC separation and exchanging the column. Switching between the RPLC and HILIC separation resulted in a different elution order of (bioactive) compounds present in the mixture, which could be traced back in the bioactivity profile. In this way, narrowing down or confirmation of the accurate masses corresponding to the bioactive compounds was

attained. The other innovation relates to the direct nanoLC–MS/MS analysis of a bioactive nanofraction for the (partial) peptide sequence determination. These advancements resulted in the identification of one ACE inhibitor in the snake venom of *Cerastes cerastes cerastes* and two ACE inhibitors in the snake venom of *Crotalus adamanteus*.

In **Chapter 6**, the at-line nanofractionation approach was developed, optimized and applied to hyphenating an LC separation, MS detection and a functional cell-based assay for compounds targeting α7-nAChR. Two different assay setups with the SH-SY5Y cells overexpressing the α7-nAChR were used so that both agonists and positive allosteric modulators (PAMs) could be identified. The approach developed was applied in the screening of the extract of a hallucinogenic mushroom, *Psylocybe McKennaii*, resulting in the detection of two bioactive compounds. The use of complementary RPLC and HILIC separations facilitated the identification of *m/z* values corresponding to the bioactivity.

The results reported in this thesis, briefly summarized above, show the high potential of the at-line nanofractionation methodology and its suitability for implementation in drug discovery platforms for the analysis of drug metabolites and the screening of natural products for new leads.

The formation of bioactive metabolites formed may compromise the safety and/or efficacy of a drug candidate, which may result in significant losses of time and money within the pharma industry. Therefore, an early-stage bioactivity/bioaffinity profiling of both lead compounds and their metabolites using the at-line nanofractionation methodology would be highly beneficial. Current early-stage studies provide information on metabolic stability of lead compounds and eventual changes of bioaffinity/bioactivity of crude metabolic mixtures, while full chemical and pharmacological characterization of the metabolites is generally postponed to a later stage as it requires time-consuming and elaborate purification and/or synthesis of metabolites. Besides the possibility for metabolic stability studies, the at-line nanofractionation approach developed in this thesis allows the simultaneous qualitative bioaffinity assessment of the lead compound and its metabolites. Consequently, it provides a rapid insight in the metabolic and pharmacological 'hotspots' of the leads analyzed, which in turn gives valuable information for further improvement of their chemical structure and drug-like properties (lead optimization). Furthermore, the methodology is easily adoptable to different drug targets and applicable to study the selectivity towards different receptors, as was shown for the highly homologous CXCR1 and CXCR2 chemokine receptors. However, there is also enough space left for improvements of the methods currently developed, so that some limitations may be overcome. As, already mentioned, the method developed currently provides information on relative metabolic stability and qualitative bioaffinity. The possibility of quantification of the metabolites formed in terms of concentration and binding affinities would contribute significantly to the implementation of the at-line nanofractionation methodology in metabolic studies at an early stage of drug discovery. In this respect, UV/VIS and MS are valuable techniques. Assuming that absorbance or ionization efficiency of the metabolites do not considerably differ from those of the corresponding parent compounds, calibration curves for the

parent drug can be plotted and used for the quantification of the metabolites. However, even small changes introduced by biotransformation can cause significant differences in properties of a compound yielding unreliable quantitative data, especially due to changes in MS ionization efficiency. This could possibly be overcome by implementation of an on-line or off-line radiodetection using ^{14}C-labeled compounds, although most likely not in the discovery phase but in an early development phase (2). Working with radiolabeled compounds at the development stage is common, but the benefit of radiolabeling many compounds at an early stage in drug discovery may be questioned. It is important to note that in the case ^{14}C-labeled compounds are used, the radioligand binding assays need to be performed with the radioligands emitting radiation other than β-radiation (which is characteristic for ^{14}C) to allow parallel bioaffinity assessment. Besides the lack of quantitative data on concentrations of metabolites formed and consequently their affinity or potency, LC–MS analysis alone often cannot provide full structural characterization, as was for example described in Chapter 2 and 3 of this thesis. A special hurdle represents the identification of the positional isomers such as *o*-, *m*-, and/or p-substituted metabolites with aromatic rings. This might be overcome by implementation of a parallel off-line or on-line NMR analysis, similar to what has been applied before in combination with the on-line post-column screening setups (3). However, the main obstacle for this remains insufficient sensitivity of current NMR spectrometers and the difficulty to obtain sufficiently pure fractions. The developments and advancements in ion-mobility MS might offer an excellent alternative tool for identification of positional isomers. Currently, only glucuronide derivatization-based analysis provides results due to the resolution issue. However, further advancements in respect to resolution in ion-mobility cells (primarily prolonging the length of the cell) are expected in near future, which will enable for analysis of non-derivatized positional isomers.

Quite often, the at-line and especially the on-line bioaffinity screening is limited to establishing the binding of a ligand to a target receptor. Even though this binding to the target receptor represents a pre-condition for a compound to cause a desirable biological effect, only by the implementation of the actual bioactivity assessment information would be obtained on the type of the ligand-target interaction (i.e., agonism, partial agonism, or antagonism) and its possible changes/effects. Therefore, the development of a cell-based assay, although difficult to achieve for all drug targets, would be an important development. Results in this direction are possible using the at-line nanofractionation approach developed, as was shown in the studies with histamine receptors (4,5) and with α7-nAChR receptors (Chapter 6 of this thesis).

Another important point one should address is the specificity of the response measured. Cells co-express many receptors that might be activated causing a false-positive bioactivity detection. In the study of ligands towards the α7-nAChRs (Chapter 6), for example, a significant contribution to the agonistic signaling was observed from mAChRs endogenously expressed on the surface of SH-SY5Y cells. Therefore, the signaling of related co-expressed receptors should be blocked using corresponding specific antagonists in order to study a specific ligand-receptor interaction.

The re-dissolving of the nanofractions in the bioassay buffer can represent an important limitation in the case of highly lipophilic compounds. Such compounds are more difficult to dissolve in the hydrophilic buffers used in bioassays, so the effective concentrations of the compounds may be too low, even for very sensitive detector systems such as radioligand binding assays. This is an especially important issue in metabolic profiling, since the concentrations of metabolites generated are usually very low. During the analysis of CXCR2 ligands, significantly higher IC_{50} concentrations were determined for MK-7123 and SB265610 after pipetting and freeze-drying serial dilutions on a plate in comparison to the IC_{50} values previously reported using a conventional radioligand binding assay. However, the analysis of freeze-dried CXCR3 ligands, NBI-74330 and VUF11211, yielded similar results to conventional radioligand binding assay. This is most probably due to the difference in lipophilicity between these compounds. The CXCR2 ligands are more lipophilic than CXCR3 ligands and therefore more difficult to re-dissolve in the bioassay buffer resulting in lower concentrations and a shift of the IC_{50} curve to the right. Additional pipetting of a DMSO solution to the nanofractions prior to freeze-drying, which was recently demonstrated in analysis of estrogen-like compounds in environmental samples (6), may be implemented to tackle this problem.

In last two decades, the pharma industry has put a significant effort in drug discovery and development programs of biopharmaceuticals. This resulted in continuous growth of the share of these drugs on the market. The main reason for this shift is the high potency and high target specificity of biopharmaceuticals and consequently the increased safety due to minimized off-target side effects. In the future, special interest might be in the development of relatively small peptides (up to 3000 Da), which would allow possibilities for alternative administration routs, even oral administration while retaining high specificity, efficacy and safety. Furthermore, smaller peptides have a lower immunogenicity potential than the current large biopharmaceuticals, which makes them more attractive for the pharma industry. The SAR studies of the large peptides have been successfully used to design highly potent smaller molecules. Current research in the field of peptide drugs aims to investigate the influence of modification of the peptide backbone on its oral bioavailability and metabolic stability. An especially important role in this is played by the type of modifications (such as cyclizations and *N*-methylations) that mimic the structures of peptides isolated from microorganisms, which are known to possess physicochemical properties favoring them for development into drugs. With this in mind, one can easily understand that animal venoms as a source of highly specific, potent and fast-acting peptides might grow in an importance as a source of lead compounds for drug discovery. Since venoms are very complex mixtures, advanced analytical methodologies allowing rapid screening and identification of bioactive peptides are highly desirable. The at-line nanofractionation methodology can represent an obvious choice in this case.

In Chapter 4 and 5, the advantages of the at-line approach in drug discovery from natural products were clearly demonstrated in small screening programs of snake venoms towards inhibition of thrombin, factor Xa and ACE. These reflect in rapid detection of

the bioactivity and simultaneous MS identification of the bioactive compound(s). The identification strategy introduced in Chapter 5, which relies on the use of RPLC and HILIC as complementary separation methods to narrow down the number of candidates for bioactivity and pinpointing the corresponding masses seems to be a promising tool facilitating the identification of bioactive compounds. Furthermore, the direct nanoLC-MSMS analysis of the content of the wells containing the bioactive fraction, using high-resolution MS, showed to be successful in the final identification of the small bioactive peptides. These separate nanoLC–MSMS runs could be avoided if the data-dependent MSMS measurements were recorded during the initial screening phase. However, nanoLC–MSMS provides a more sensitive peptide analysis in general. Furthermore, the identification of large peptides and proteins could be implemented by analyzing the content of the well with the bioactive after in-gel or in-solution tryptic digestion. Subsequently, the analytical results can be processed by database search engines such as MASCOT or interpreted manually. Also, the number of disulfide bridges could be determined by analyzing the content of the bioactive well before and after the reduction using DTT, for example, as demonstrated before.

A possible addition to the current set-up is the use of alternative ionization and fragmentation techniques for both peptide and protein analysis. For example, the current approach does not allow a differentiation between isobaric amino acids, leucine and isoleucine, unless database searching resolve this issue. If not, the use of high-energy CID in MALDI-TOF-TOF could be an interesting approach to overcome this hurdle. More information could also be obtained by complementing the information obtained from collision induced dissociation (CID) by information obtained from electron transfer dissociation (ETD) as fragmentation technique. The use of newly developed MS instruments allowing for alternating CID and ETD data acquisition could provide more information within the timescale of just one chromatographic run and thereby facilitate the identification of the bioactive peptides and proteins. Furthermore, the implementation of a complementary on-line or off-line NMR analysis could be beneficial for full structural elucidation. Other possible improvements mentioned above for analysis of active metabolites apply here as well.

Finally, the miniaturization of the whole set-up could make the pharma industry more interested in the implementation of the at-line nanofractionation methodology in the drug discovery screening platforms and enable its wider application. As seen for the on-line approach, except for significant decrease in costs, the miniaturization brings efficient identification of bioactive compounds even in very small amounts of sample. This is especially important for the analysis of venoms, since these precious samples are not always available in large quantities. The miniaturization could be achieved by implementation of nanoLC separation and nanospotting technology for nanofractionation on microplates and subsequent bioassaying. An additional fractionation device could be installed for the fractionation on a MALDI plate, prior to the MALDI-TOF-TOF analysis, for example.

Altogether, the at-line nanofractionation methodology described in this thesis promises a high potential for implementation in drug discovery screening platforms. The current developments already show that this strategy represents an excellent tool for the screening of bioactive mixtures and the identification of the bioactive compounds. The possibility to use different kind of bioassays, i.e., both slow enzyme assays, frequently applied radioligand binding assays, and even functional cell-based assays, in combination with analytical techniques makes this approach very attractive. If the suggested improvements, as discussed above, could be successfully implemented, the at-line nanofractionation methodology could certainly become the first-choice methodology for the bioactivity profiling of mixtures in drug discovery. Furthermore, other fields with interest in the analysis of bioactive mixtures could also benefit from the implementation of the at-line nanofractionation methodology in their routine analysis.

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