Integrated Analytical Strategies for Drug Discovery

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2011

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

Chapter 9

Summary, Conclusions and Perspectives

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The drug discovery and development process is a complex and time consuming trajectory with many subdivisions of sciences involved. Analytical chemistry is one of the key scientific disciplines that is and will always remain involved throughout the whole process. In this thesis, the development and application of innovative analytical technologies in drug discovery and development is described. In **part I**, the development and application of techniques in the field of drug metabolism for chemical analysis is described. With the publication of the Metabolites in Safety Testing (MIST) guidelines by the US Food and Drug Administration, the development and implementation of new analytical technologies for this purpose has increased significantly. The main questions to be answered in drug metabolism studies are: what is the structure of the metabolites and in which quantity are they made? Although very simple questions, the answers to these questions require state-of-the-art analytic and synthetic approaches. There are many challenges such as metabolite quantification without reference standards, full structural identification of low abundant metabolites, and the biological activity assessment of the metabolites formed. In the work described in this thesis several of these challenges were addressed and evaluated.

Formation

The metabolites and other pharmaceutically relevant molecules described in this thesis were generated in several ways. This included the formation of metabolites by conventional *in vitro* incubations with rat and human liver microsomes (RLM and HLM) as well as with a set of drug metabolizing mutants of cytochrome P450 BM3. **Chapters 2-5** describe the use of these enzymatic systems for different purposes. In **Chapters 2-4**, they were used for the generation of human relevant metabolites of kinase inhibitors, steroids and the antibacterial agent trimethoprim. In **Chapter 5**, the additional use of these biosynthetic enzymes enabled the formation of focused screening libraries for the estrogen receptors hERα and hERβ. In **Chapters 2 and 7**, electrochemical oxidation and reduction was applied for the formation of drug related molecules. Although electrochemistry has been applied extensively in the formation of metabolite standards to facilitate the metabolite identification process, a direct comparison with human relevant metabolites was not performed in this thesis since this was not within the scope of the present use of electrochemical methods. The comparison of

electrochemical oxidation products in with e.g. microsomal incubations should always be done with great caution since electrochemistry cannot replace the stereoselective and catalytic effects of enzymes. Another approach for the formation of pharmaceutically relevant molecules is described in **Chapter 8**. In an one-pot reaction, a complex mixture of 6 regioisomers of *N*-alkylated derivatives of the antibiotic neomycin was made, structurally characterized, and used to assess their antibacterial effect in an at-line micro fractionation approach.

Identification and quantification strategies

For the identification of products, the most important technique used in this thesis is hyphenated electrospray ionization high-resolution multistage mass spectrometry (ESI-HR-MS^n). Metabolite identification was performed in **Chapters 2-5** and included different strategies. In **Chapter 2**, quantification of metabolites without the use of synthetic standards was achieved by hyphenating high temperature liquid chromatography (HTLC) to electrospray mass spectrometry (ESI-MS) and/or inductive coupled plasma-mass spectrometry (ICP-MS). The use of ICP-MS allows the quantification based on several marker atoms that should be present in drug molecules and metabolites. Next to the platinum-containing anti-cancer drugs, drugs and metabolites can be quantified if they contain sulfur, iodine, chlorine and/or bromine. The hyphenation of a separation method with any kind of detector can have an effect on the detector response, especially when gradient LC methods are applied. In the case of ICP-MS, ELSD, ESI-MS and many other detection methods, the detector signal is significantly influenced by changes in the mobile phase composition and can therefore lead to under- or over-estimation of analyte quantities. In this thesis, this issue was addressed by developing an isocratic HTLC separation combined with temperature gradients up to 200 ˚C to enable the use of ICP-MS detection. The elemental specificity and large linear range of the ICP-MS showed to be valuable features to be used complementary to, e.g., ESI-MS. Recent developments in ICP-MS hardware include the implementation of high resolution MS analyzers which increases the application area of this valuable technique in the drug discovery arena.

Chapters 3 and 4 describe typical metabolite identification studies. Based on *in silico* prediction tools, high resolution mass spectrometry and the use of multiple enzyme systems for the formation of metabolites, a comprehensive dataset was obtained on the metabolism of trimethoprim. In both chapters, interspecies

differences were observed between rat and human liver microsomal incubations. Moreover, the drug metabolizing mutant of P450 BM3 showed to be applicable for the generation of human relevant (reactive) metabolites. Challenges in this particular field are the detection of low abundant metabolites and the characterization of all reactive metabolites formed. Recent developments of new strategies such as Br-labeled glutathione to enable high resolution isotope filtering significantly contribute to increase the coverage of all metabolites formed. The use of specific labeling of glutathione could also enhance its detection limits for ICP-MS and provide absolute quantification opportunities as well as additional profiling tools.

The limitations of mass spectrometry in the structure identification of metabolites were encountered in **Chapter 5**. Steroidal structures were metabolized by different enzymatic systems and subsequently profiled by LC-ESI-HR-MS. Fast polarity switching of the ESI-MS was used to obtain both positive and negative ESI-MS data on the metabolites. This showed to be essential for the detection of these steroidal structures because modification of the parent compound significantly altered the ionization properties of the molecules as well as the UV-absorption characteristics. Although HR-MS data was obtained on the metabolites, the exact position of modification could not be determined in an effective way. Therefore, NMR studies were performed to elucidate the structures of norethisterone metabolites generated by P450 BM3 mutants. This resulted not only in the identification of the modification site, but also in the information on the regioselectivity of the metabolism reaction. Norethisterone was mainly hydroxylated at C15 and C16 positions in a β orientation. Interestingly, the 16β-OH metabolite showed to have selectivity towards the hERα over hERβ. The second-order metabolism of this drug involved aromatization of the steroid A ring to 16β-OH ethinylestradiol, which had a major positive effect on the binding affinity towards the receptor. This metabolite was not published before and was found to be present in the HLM in vitro experiments as well.

In **Chapter 8**, ESI-HR-MS^n experiments allowed the identification of 6 regio-isomers of *N*-alkylated neomycin derivatives. After LC separation of this complex mixture, HR-MS^n experiments were performed. Whereas the MS and MS^2 spectra were identical for all six regio-isomers, differences were observed in MS^3 and MS^4 experiments. Based on the fragmentation patterns of the glycoside bonds in the

core structure of the derivatives, the site of alkylation in rings 1 and 4 could be identified. Determination of the specific site of modification in ring 2 of neomycin was not possible since it produced symmetrical MS^2 fragments.

Screening for biological activity

Part II of this thesis covered the implementation of biological interactions in an analytical chemical environment. The multidimensional screening approaches described in **Chapters 5-8** truly add valuable information to the more conventional chemical analysis methods. In a fast and efficient way, data was generated on both structure and biological activity of the metabolites, synthesis products, electrochemical conversion products, or any other category of analytes. An important aspect of the concept is the integration of separation techniques and bioaffinity assessment. This allows the screening of complex mixtures such as metabolic incubations, crude synthesis mixtures or natural extracts. In this thesis, two distinct types of assays were used for three different target proteins. **Chapter 5** discussed the use of nuclear receptors, *i.e.*, the human estrogen receptor α and β subtypes, in an on-line binding assay. Binding was monitored through the detection of the fluorescence enhancement of a tracer-receptor complex. The same approach was also presented for the mitogen activated protein kinase p38α in **Chapter 6**, where the development of a new assay was described including the optimization of signal-to-noise ratios. The advantage of a binding assay for on-line screening purposes is that the interaction between the target protein and ligand is relatively fast and the readout for this reaction can be performed in the same timescale as the chromatographic separation and MS based detection of the ligands. In **Chapter 7**, this assay was applied in an on-line formation, identification and biological characterization strategy of kinase inbititors. The alternative for a binding assay would be on-line activity assays where product formation by the active enzyme has to be monitored. Although already performed for several enzymes, it significantly increases the complexity of the assay and influences the robustness of the total system. On a totally different timescale, the antibiotic action of several neomycin derivatives was assessed in an at-line approach in **Chapter 8**. After separation of the derivatives and addition of all reagents necessary for the biological assay, microfractionation into a 384 well plate was performed. In this way, the on-line format was adapted to facilitate the use of conventional plate reader assays and allowing also relative long incubation times

needed for, e.g., observation of antibacterial effects (up to 18 hrs). This approach significantly broadens the application area of hyphenated screening assays since the main limitation until now was the limitation of incubation times. Next steps definitely should include the development of functional assays against complicated targets such as membrane bound receptors (G-Protein coupled receptors, GPCRs) and ion channels.

The development of the tools described in the individual chapters of this thesis contributes to the overall toolbox available to researchers in the drug discovery process. Figure 1 gives an overview of the tools developed in this thesis, earlier by others, or that might be developed in near future.

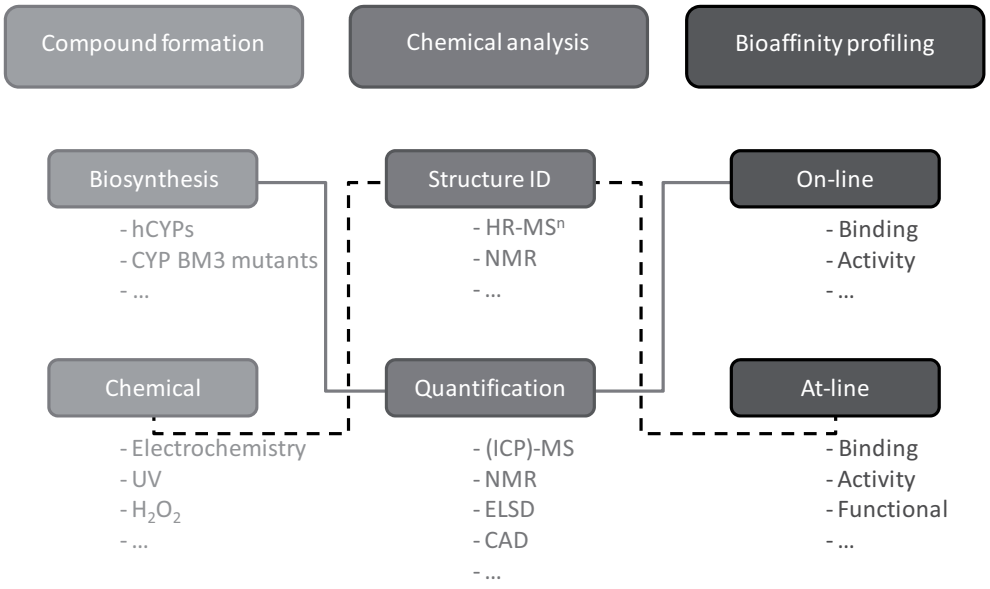

Figure 1: Modular approach for the integration of compound formation, chemical analysis and bioaffinity profiling for drug discovery.

Ideally, this concept leads to a modular approach where the different tools can be hyphenated to each other. Two lines were drawn to highlight some possibilities. Several of these hyphenations have been described in this thesis, but one of the important next steps would be to establish quantification of the bioaffinity assessment. This will lead to quantitative affinity assessment of, *e.g.*, metabolites without reference standards. The first steps in this process were taken in **Chapter 2**, where metabolites of kinase inhibitors were quantified without reference

standards. The integration of*, e.g.*, the kinase affinity assay developed in **Chapter 6** with ICP-MS based quantification should provide quantitative affinity data on newly formed compounds such as metabolites or crude synthesis mixtures.

The impact and innovation of new analytical technologies facilitating drug discovery largely depends on the interaction of the different sciences involved. This requires truly multidisciplinary research projects where input is delivered on the challenges encountered in each individual sub-discipline. It is in these kind of projects where the analytical strategies can significantly contribute to the efficiency and quality of the drug discovery and development process.