High-resolution screening of metabolite-like lead libraries

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Section 6

Summary

Chapter 6.1

Conclusions and perspectives

This thesis aims at exploring new applications of high-resolution screening (HRS). Therefore, our goal was to improve the corresponding platforms and extend their possibilities, mainly by further integration of chromatography, mass spectrometry, bioassays and especially innovative synthetic methods. We developed HRS platforms for two targets, p38α mitogen-activated protein kinase (p38α) and soluble epoxide hydrolase (sEH), which were previously not accessible by HRS [Chapter 2]. Furthermore, by integrating four complementary modification methods with the p38α HRS platform, we achieved an efficient and integrated approach to generate and screen metabolite-like lead libraries, yielding structure-affinity relationships [Chapter 3]. We thoroughly analyzed the possibilities and limitations of structure elucidation of related substances by liquid chromatography–(ion trap–time-of-flight) high-resolution mass spectrometry (LC–HR-MS^n) within the framework of HRS on basis of the kinase inhibitor derived metabolite-like lead libraries [Chapter 4.1]. Finally, we peaked into the future of HRS which may feature miniaturized NMR for additional structure elucidation [Chapter 4.2] and/or inductively coupled plasma MS (ICP-MS) for absolute quantitation [Chapter 5] integrated directly into the HRS platform.

As far as the development of HRS bioassays is concerned, on the example of p38α, we demonstrated the first on-line post-column bioaffinity assay reported for any enzyme target and integrated it into an HRS platform [Chapter 2.2]. For the sEH HRS platform, we were mainly interested in making the possibilities of HRS for metabolism studies available to this novel target and therefore took a very classic approach [Chapter 2.3].

For the p38α HRS platform, we additionally introduced new solutions for the screening of highly lipophilic compounds. The use of fused silica capillaries demonstrates that there are still possibilities to improve upon current HRS bioassays technology, even in aspects that have been as extensively studied as post-column reactors. In this example, the enhanced peak shapes improve chromatographic resolution and dilution calculation. We achieved excellent performance of the p38α and the sEH HRS platforms. For the p38α HRS platform, this is indicated by excellent figures-of-merit, such as a *Z'*-factor of ca. 0.8 and an S/N of up to 100, and an affinity ranking in accordance with literature values. Unfortunately, the only tested compound of which affinity data on non-phosphorylated p38α was available (BIRB796), showed unconventionally slow binding kinetics, rendering a quantitative comparison of the IC_{50} values for validation purposes impossible, due to the limited incubation times achievable in HRS. Nonetheless, its affinity was detected as were those of representative compounds of all three types of ATP-competitive binding.

The sEH HRS platform featured an S/N>60, though quality would have certainly profited from using state-of-the-art chromatographic equipment. We could very reliably match affinity and small-molecule structure and even tackle isobaric and many isomeric compounds. This holds for both HRS platforms, but was more extensively demonstrated for p38α [Chapter 2.2, Chapter 3] than for sEH [Chapter 2.3]. The clear advantages of HRS over HTS with respect to the bioassay level are exemplified by a decreased p38α concentration and a similar sEH concentration at significantly reduced incubation time. Obviously, the ability to analyze mixtures is an important advantage of HRS over HTS as well.

For both HRS platforms, we successfully tackled issues with enzyme stability. In addition, for the sEH HRS platform, we overcame issues with low substrate solubility. Furthermore, during the development and the HRS campaigns [Chapter 2, Chapter 3], we observed a number of interferences which are clearly visualized by the HRS bioassays. Examples are the auto-fluorescence and peak tailing in the p38α HRS platform, which interfere with the IC_{50} measurements [Chapter 2.2, Chapter 3.2], and to some extend the precipitation issues during the development of the sEH HRS bioassay [Chapter 2.3]. While the visualization of interferences might be seen as a disadvantage, we believe that it in fact is infinitely preferable to spot and address these issues than to put confidence in faulty data. Therefore, we regard the ability of the HRS platform to resolve interferences as one of the major strength of this approach.

There are several interesting directions in which both the p38α and the sEH HRS platform may be further developed. Parallel bioassays (two or even multiple), similar to the one

developed for the estrogen receptors α and β [1], for binding selectivity analysis should be mentioned in this context. These could be applied to investigate selectivity over close family members or generally problematic off-targets. For example, in the case of p38α, the closely related kinases JNK-1, JNK-2 and JNK-3 could be simultaneously monitored for selectivity during lead optimization [2]. Additionally, detection of selectivity for the different p38 isoforms might lead to molecular probes which could be used to investigate the biological role of p38β, γ and δ in more detail [3]. Concerning off-targets, the increasing interest in the novel concept of translational research may lead to the exploitation of the large body of knowledge on p38 inhibitors in future drug discovery campaigns. For example, the suspected off-target interactions, related to certain side effects observed in the clinic, might be included in selectivity studies.

In any case, next to selectivity detection, future developments of HRS bioassays lie in miniaturization, because the reduction in both reagent consumption and sample volume has important advantages [4]. Using less target protein helps to control the cost of HRS and thus improves its competitive position versus HTS. Enhanced mass sensitivity, possibly in combination with pre-concentration through the integration of on-line SPE, might finally allow HRS analysis of clinical samples in the future.

We further advanced integration in HRS platforms, synthesizing metabolite-like lead libraries and analyzing them for structure and affinity in one platform or even in one hyphenated system. Combining synthesis and analysis in one workflow resulted in an efficient exploration of neglected chemical space around a substrate scaffold, consequently yielding structure-activity relationships with the input of no more than the substrates. These substrates were important p38α inhibitors, including a lead (DMPIP), a widely used molecular probe (SB203580) and clinical candidates (BIRB796and TAK-715). As we focused on metabolite-like lead libraries, it seemed prudent to additionally investigate, whether the platforms would be useful in selecting production strategies for actual metabolites. To this end, we included *in vitro* metabolism by human liver microsomes (HLM) into the method comparison.

The electrochemical conversion (EC) HRS platform was certainly the most interesting integrated synthesis approach from an analytical point of view [Chapter 3.1]. The EC setup employed had already been successfully used in EC–MS and EC–LC–MS setups and required no intermediate sample pre-treatment step. Therefore, a completely integrated EC–LC–p38α binding/MS platform was achieved. This proved to be especially useful for the detection of reactive EC products [Chapter 3.1] which were also (partly) observed later in human liver microsomal incubations [Chapter 3.3].

The electrophilic reactivity of the observed quinoneimine also offers the possibility for a new synthetic approach. A pre-column incubation step with a mixture of nucleophiles could be integrated into the EC–HRS platform, allowing the production of extensive lead libraries with a broad chemical diversity in a rapid and automated fashion as well as the direct analysis of the reaction mixtures in the same system. Especially interesting might be the integration of a second electrochemical (detection) step between column and MS which would serve to probe the redox stability of the new compounds. Through this approach, it might be possible to find leads with a reduced tendency to form reactive products [5]. This is especially interesting, if adverse effects caused by reactive metabolites are known or anticipated. The general approach of EC–HRS might also be interesting in stages of drug discovery other than lead optimization. When fitting an EC–HRS platform with a bioassay for a notorious off-target, such as hERG [6], it could be very useful as a rapid metabolic alert screening. Though EC is not a complete mimic of human metabolism, redox weak spots and the toxicological consequences of the associated modifications may be identified early on in drug discovery.

The use of biocatalysts to generate metabolite-like lead libraries proved very successful as well. The library of Cytochrome P450 BM3 (BM3) mutants applied produced a number of conversion products. The products showed increased hydrophilicity and one of them even retained full p38α affinity [Chapter 3.2]. Because large parts of the BM3 mutant library were “humanized”, a significant overlap with products from HLM incubation was found. The

fact that, through up-scaling and purification, IC_{50} determination and NMR measurements were enabled, greatly enhanced the detail of information obtained. In addition, we thereby demonstrated how the initial results of the HRS platform can be used to guide further structural and pharmacological investigation of promising conversion products.
Subsequently, we compared the EC and the biocatalytic approach to chemically (H_2O_2) and photochemically (visible light) induced transformations [Chapter 3.3]. H_2O_2 was very good in making many different, more hydrophilic products, e.g., numerous oxygenated isomers. Photochemical conversion showed some unique products and can induce isomerism in the core structure, which may result in novel pharmacophores. One consequence of that isomerism is a more limited success in structure elucidation by MS [Chapter 4.1].
In comparison, all methods have their specific profile of strength and weaknesses. For example, biocatalysis and H_2O_2 are most successful in increasing hydrophilicity. While biocatalysis is the most laborious method, but can also be the most selective, H_2O_2 is very straightforward but often produces the most (isomeric) products. Photochemistry and EC do not favor increased hydrophilicity in the products, but photochemistry yields the most unique products. However, the EC–HRS platform is very favorable for the analysis of reactive products, due to its direct analysis capabilities.
In conclusion, all conversion methods are highly complementary and thus together present an interesting toolbox for the generation of metabolite-like lead libraries. We could show the impressive value of integrating synthesis of mixtures into the HRS strategy. The p38α HRS platform rapidly delivered an initial structure-activity relationship for the lead libraries and can be considered as a solid starting point for further pharmacological and toxicological investigation.
Next to producing lead libraries with favorable properties, perhaps unsurprisingly, especially the biocatalytic and the chemical approach showed potential for the synthesis of metabolite standards. The novelty is that the implementation of the HRS platform allows a focus on active metabolites for further evaluation while at the same time still providing a comprehensive overview. Again, this might also be an interesting application for off-target HRS bioassays.
However, the biocatalytic and the chemical approach will require different strategies for metabolite standard production. By mutation, a biocatalyst can be generated which exclusively or predominantly produces one metabolite that is then easily purified. The strategy for H_2O_2 will rely on simultaneous purification of all (major) products, as conversion is undirected (high number of isomers) and there is little opportunity to tune the product profile. However, this will also provide standards which would be ignored in a focused approach, but might still be useful, for example when metabolites are detected in human that have not been predicted in *in vitro* or animal models.
Being an integral part of the HRS platforms, we closely investigated the possibilities and challenges of structure elucidation by LC–HR-MS^n under the special circumstances of a (HRS) screening paradigm [Chapter 4.1]. The resulting restrictions include the use of “generic” settings for the ionization and fragmentation experiments as well as prohibiting the use of elaborate MS experiments like stable isotope labeling and H/D-exchange experiments. Nonetheless, the attribution of modifications to specific parts of the molecule is often successful and in special cases allows absolute structure elucidation, for example for CP472, a conversion product of DMPIP in the EC experiments [Chapter 3.1].
The sometimes drastic changes in fragmentation initiated by comparatively minor modifications and unexpected intra-molecular rearrangements presented challenges in the LC–HR-MS^n structure elucidation. These rearrangements were also interesting examples of gas-phase chemistry, such as the electrocyclic rearrangement in the fragmentation of TAK-715, the rearrangement underlying the formation of CP305 (or its fragmentation) from SB203580, the hydride rearrangement leading to water elimination from the aromatic hydroxymethyl group in CP416A (derived from TAK-715), and the rearrangement of the whole *para*-fluoro-benzyl group in DMPIP. Additionally, we detected an unexpectedly high number of homolytic cleavages in violation of the even-electron rule. In the process, we

showed, using ion-trap fragmentation in combination with HR-MS readout by time-of-flight, the value of clearly assigning precursor-product ion relationships for structure elucidation [Chapter 4.1].
Next to the HR-MS^n structure elucidation, we pursued an intriguing possibility to incorporate NMR structure elucidation into an HRS platform in the future [Chapter 4.2]. As starting points, we took the reactive products of BIRB796 discovered in the EC–HRS platform [Chapter 3.1]. We set out to elucidate their structure by NMR. The logical choice for an approach that can be integrated with HRS was a miniaturized flow probe NMR in order to match the small sample volume and the flow-through EC system.
In the end, we combined an up-scaled electrode, an SPE step for pre-concentration and solvent exchange, and a stripline-NMR chip probe equipped to a 600 MHz NMR instrument. The system was well suited for the detection of the BIRB796 standard, and the solvent exchange was efficient enough to be able to compare the EC–SPE–stripline-NMR results to conventional (2D-)^{1}H-NMR. Unfortunately, the conversion rate of BIRB796 to its two reactive products was significantly lower than in the off-line experiments. Therefore, identification of the products could only be achieved by comparison with the conventional ^{1}H-NMR spectra, instead of by the desired independent structure elucidation. However, the platform showed promising results and there are numerous possibilities to close the existing sensitivity gap in order to allow direct integration into the HRS platform.
Integrated LC–MS/NMR platforms are already used in pharmaceutical and other applications [7,8]. As combined MS and NMR structure elucidation is crucial in HRS applications as well [1], HRS platforms featuring this combined structure elucidation power are certainly a worthwhile aim.
A major enhancement of the possibilities and a major step towards a wider applicability of HRS is the integration of absolute quantitation into HRS platforms. For example, this may enable a quantitative structure-activity relationship (QSAR) directly from mixtures generated by the described modification methods. A simple injection of a dilution series of the mixture might suffice in order to obtain IC_{50} curves. Mainly due to the lack of standards, detection techniques focused on atoms rather than on molecules will be the methods of choice when dealing with unknown compounds. Detection techniques like MS or UV/VIS, which focus on molecular properties, show a large variation in response, depending on the molecular structure. While the response also shows large variation between different atoms when atomic properties are detected, it is relatively easy to find a common atom in many different organic molecules.
One atom property based method, which was already discussed, is NMR. However, NMR is influenced by the chemical environment of the detected atom which has consequences for the response. Fortunately, these are much less pronounced than in molecular property based detection techniques and can be minimized by careful consideration of experimental parameters such as relaxation time. The real issue with NMR is its currently inadequate sensitivity. Yet, if this hurdle can be overcome, NMR promises quantitation and additional structure information at the same time.
ICP-MS is much more sensitive and its response is virtually independent of the chemical environment, due to the constant atomic ionization efficiency and the huge difference between molecular bond energy and the atomic ionization energy [9]. Unfortunately, the main elements of organic molecules, hydrogen, carbon, oxygen, and nitrogen, cannot be targeted by LC–ICP-MS for various reasons. However, many pharmaceuticals contain a halogen and/or sulfur atom and there are even examples containing metal atoms. While we measured bromine and iodine very sensitively by ICP-MS, this was not the case for sulfur and chlorine [Chapter 5]. The observed isobaric polyatomic interferences may be resolved using reaction/collision cells or an ICP-HR-MS instrument [9]. This might allow sensitive quantification of sulfur or chlorine containing molecules, where detection limits with unit mass resolution ICP-MS were a factor of 30 or 200, respectively, short of HRS compatible quantitation [Chapter 5]. ICP-MS relies on stable solvent conditions and is therefore not compatible with classic solvent gradients [10]. High-temperature LC (HTLC) presents an

elegant way to achieve efficient separation at isocratic solvent conditions [Chapter 5]. HTLC is not only interesting for ICP-MS compatibility, but may also present a solution to reduce the organic modifier influence on post-column bioassays. Though the ESI-MS response is decreased at low organic modifier concentrations, the high sensitivity of ESI-MS usually allows a favorable compromise.

In its application to drug discovery and related fields, we have demonstrated many strengths and possibilities of HRS and encountered some of its limitations. However, the role HRS platforms will play in the future depends on many factors. At the moment, the development status of HRS platforms limits their application to niches where separation of complex mixtures is a key element, for example screening of natural product libraries [11,12]. The specific niche will determine the HRS platform's outward appearance, e.g., whether pre-column affinity selection or post-column bioassays are employed, or whether on-line or at-line hyphenation is favored [Chapter 1.1.2]. Miniaturization may present a way to reduce HRS cost [4] and thus enhance its commercial competitive position versus HTS. However, knowledge presents a much greater hurdle towards the implementation of HRS platforms. Currently, a user has to be trained in all analytical and pharmacological disciplines related to the HRS platform. This will only change, if commercial solutions for robust and fully integrated systems as well as automated data analysis tools become available in the future. For HRS to be recognized as a real alternative in pharmaceutical screening and to create a demand for these solutions in the first place, next to maturity and user friendliness, one hurdle is potentially all-important. HRS does not smoothly fit the high-throughput batch approach dominating contemporary pharmaceutical industry and is thus very rarely seriously considered. However, this may very well change in the not so far future. The pharmaceutical industry is under great pressure to critically evaluate their R&D strategies and present more viable alternatives [13,14]. More flexible, quality-focused drug discovery campaigns and an increasing interest in flow chemistry as alternative to batch approaches may make HRS platforms an attractive tool. Flow chemistry might be integrated as demonstrated in the EC–HRS platform [Chapter 3.1] or with an intermediate on-line SPE step. With appropriate flow splitting and at substantially decreased cost, HRS might also be useful beyond discovery, for example for monitoring up-scaling or even for quality control of active pharmaceutical ingredient production based on the (side) effect rather than molecular composition. Thus, the "ideal" HRS platform of the future consists of a (multiple) modification method(s), state-of-the-art (multidimensional) separation, multiple bioassays for selectivity analysis, two independent structure analysis techniques like ESI-HR-MS (including MS/MS or MS^n capabilities) and NMR, and two independent quantitation techniques such as ICP-MS and NMR, operates at micro- or even (partly) nano-scale, and can be used by scientists with diverse backgrounds. Applications might include fully automated quantitative structure-activity relationship studies and, without a modification method, the quantitative elucidation of the pharmacokinetic contributions of all pharmacologically or toxicologically relevant metabolites for metabolic profiling.

References

1. de Vlieger JS, Kolkman AJ, Ampt KA, Commandeur JN, Vermeulen NP, Kool J, Wijmenga SS, Niessen WM, Irth H, Honing M (2010) Determination and identification of estrogenic compounds generated with biosynthetic enzymes using hyphenated screening assays, high resolution mass spectrometry and off-line NMR. J Chromatogr B 878 (7-8):667-674
2. Goldstein DM, Kuglstatter A, Lou Y, Soth MJ (2010) Selective p38alpha Inhibitors Clinically Evaluated for the Treatment of Chronic Inflammatory Disorders. J Med Chem 53 (6):2345-2353
3. Zhang J, Shen B, Lin A (2007) Novel strategies for inhibition of the p38 MAPK pathway. Trends Pharmacol Sci 28 (6):286-295
4. Heus F, Giera M, de Kloe GE, van Iperen D, Buijs J, Nahar TT, Smit AB, Lingeman H, de Esch IJ, Niessen WM, Irth H, Kool J (2010) Development of a microfluidic confocal fluorescence detection system for the hyphenation of nano-LC to on-line biochemical assays. Anal Bioanal Chem 398 (7-8):3023-3032
5. Samuel K, Yin W, Stearns RA, Tang YS, Chaudhary AG, Jewell JP, Lanza T, Jr., Lin LS, Hagmann WK, Evans DC, Kumar S (2003) Addressing the metabolic activation potential of new leads in drug discovery: a case study using ion trap mass spectrometry and tritium labeling techniques. J Mass Spectrom 38 (2):211-221
6. Piper DR, Duff SR, Eliason HC, Frazee WJ, Frey EA, Fuerstenau-Sharp M, Jachec C, Marks BD, Pollok BA, Shekhani MS, Thompson DV, Whitney P, Vogel KW, Hess SD (2008) Development of the predictor HERG fluorescence polarization assay using a membrane protein enrichment approach. Assay Drug Dev Technol 6 (2):213-223
7. Corcoran O, Spraul M (2003) LC-NMR-MS in drug discovery. Drug Discov Today 8 (14):624-631
8. Tang H, Xiao C, Wang Y (2009) Important roles of the hyphenated HPLC-DAD-MS-SPE-NMR technique in metabonomics. Magn Reson Chem 47 Suppl 1:S157-162
9. Ammann AA (2007) Inductively coupled plasma mass spectrometry (ICP MS): a versatile tool. J Mass Spectrom 42 (4):419-427
10. Gammelgaard B, Hansen HR, Sturup S, Moller C (2008) The use of inductively coupled plasma mass spectrometry as a detector in drug metabolism studies. Expert Opin Drug Metab Toxicol 4 (9):1187-1207
11. Heus F, Vonk F, Otvos RA, Bruyneel B, Smit AB, Lingeman H, Richardson M, Niessen WM, Kool J (2013) An efficient analytical platform for on-line microfluidic profiling of neuroactive snake venoms towards nicotinic receptor affinity. Toxicon 61:112-124
12. Potterat O, Hamburger M (2013) Concepts and technologies for tracking bioactive compounds in natural product extracts: generation of libraries, and hyphenation of analytical processes with bioassays. Nat Prod Rep 30 (4):546-564
13. Crommelin D, Stolk P, Besancon L, Shah V, Midha K, Leufkens H (2010) Pharmaceutical sciences in 2020. Nat Rev Drug Discov 9 (2):99-100
14. Kola I, Landis J (2004) Can the pharmaceutical industry reduce attrition rates? Nat Rev Drug Discov 3 (8):711-715

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