Bioorganic & Medicinal Chemistry 20 (2012) 1973–1978

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Chemoproteomic approaches to drug target identification and drug profiling

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ARTICLE INFO

Article history:
Received 26 July 2011
Revised 21 October 2011
Accepted 2 November 2011
Available online 9 November 2011

Keywords:
Affinity chromatography
Chemical proteomics
Drug discovery
Drug target
Mass spectrometry
Target deconvolution
Quantitative proteomics

ABSTRACT

Chemoproteomics represents a new research discipline at the interface of medicinal chemistry, biochemistry, and cell biology focused on studying the molecular mechanisms of action of drugs and other bioactive small molecules. Research strategies frequently combine phenotypic screening with subsequent target identification, and aim at a proteome-wide characterization of drug-induced changes in cellular protein expression and post-translational modifications. In recent years quantitative mass spectrometry has taken center stage in many of these approaches. This review describes experimental strategies in current chemical proteomics research, discusses recent examples of successful applications, and highlights areas in drug discovery where chemical proteomics-based assays using native endogenous proteins are expected to have substantial impact.

1. Introduction

Despite major investments in 'Omics' technologies, drug discovery and Development still is an inefficient and increasingly uneconomical process.[1,2] The dominating paradigm in the pharmaceutical industry is still target-based drug discovery, despite an increased perception of the role of signaling networks in homeostasis and in disease.[3,4] Because proteins represent the major type of drug targets, proteomics-based approaches, which enable the analysis of a wide variety of proteins under relatively physiological conditions, harbor great promise. However, these approaches need to be reduced to practice such that they successfully complement more traditional drug discovery strategies.[5,6] Industry standard assays of drug action assess the biochemical activity of the purified target protein in isolation. In many cases recombinant enzymes or protein fragments are used instead of the full-length endogenous proteins. However, an isolated recombinant protein, or protein fragment, does not necessarily reflect the conformation and activity of the target in its physiological context, because of absent regulatory domains and interacting proteins, or incorrect protein folding and post-translational modifications. Hence data generated in such assays may not be predictive for the efficacy of a compound or drug in cell-based or in vivo models. Chemoproteomics techniques enable the study of native proteins in cell extracts or cell fractions, under conditions carefully optimized to preserve protein integrity, folding, post-translational modifications, and interactions with regulatory proteins. These approaches can be broadly categorized into two types (Fig. 1): (i) global proteomics strategies, directed at a cell-wide characterization of cellular response to the small molecule, for instance with respect to protein expression levels or defined post-translational modifications; and (ii) targeted chemoproteomic approaches such as activity- or affinity-based target profiling, employing chemical probes engineered to capture protein targets, or entire sub-proteomes.[6–11] In this review, we briefly discuss recent applications using global proteomics for small molecule profiling, followed by a discussion of affinity- and activity-based chemoproteomics techniques, with a view of complementing and in some instances replacing the more standard recombinant protein assays employed in drug discovery.

2. Global profiling of drug effects by quantitative proteomics

Global proteomic strategies involve drug treatment of cells or animals followed by whole-cell or organ-wide proteome analysis and are attractive because of the unbiased nature of the analysis, their conceptual simplicity, and the increasing technical feasibility.[12–15] For cell-based profiling, there are no specific requirements for the small molecule apart from cell permeability, and protein mass spectrometric techniques are now becoming more widely available.[16–18] In this type of studies, the changes amenable to detection are often limited by the analytical depth of the analysis, that is, the number of proteins which can be differentially quantified in the experiment, and hence the results tend to be biased towards the more abundant proteins. As a consequence, the proteins which

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doi:10.1016/j.bmc.2011.11.003

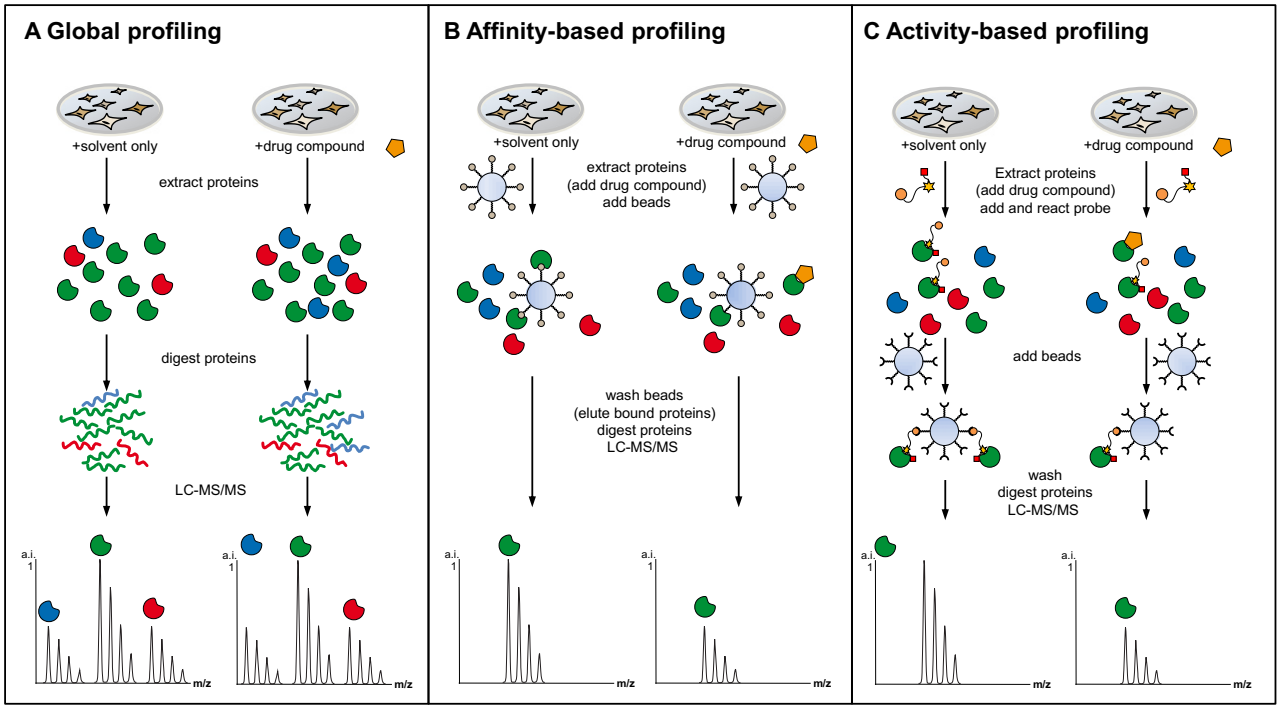

Figure 1. Experimental workflows in chemoproteomics. (a) Global proteomics approaches: cultured cells are treated with the compound of interest, followed by the preparation of a cell extract, digestion (typically with trypsin) to peptides, and analysis by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). (b) Affinity-based profiling: The drug or compound under study, or more often, a functionalized derivative of the compound of interest, is immobilized covalently, or via a biotin tag, on a solid support. The immobilized drug is subsequently incubated with a cell extract to specifically enrich for target proteins that are subsequently identified by mass spectrometry. Competition with free excess inhibitor added to the cell extract reduces the abundance of captured target proteins, but not of nonspecific interacting proteins. (c) Activity-based profiling: a reactive probe is designed to specifically target the active site of an enzyme family. Following protein extraction, the cell extract is incubated with the probe to covalently attach to its targets. In the second step, probes and targets are purified using affinity chromatography before digestion and LC–MS/MS analysis. Pre-treatment of cells with a small molecule compound binding to the active site of the investigated enzyme family leads to reduced capturing of the target enzyme via the reactive probes.

display altered expression levels are not necessarily direct targets or downstream of the affected signaling cascade, but often represent the more abundant proteins involved in stress response and/or housekeeping functions.[15,19] In order to minimize the abundance bias inherent to such analyses, experimental strategies typically employ multidimensional protein and peptide separation strategies. For separation on the protein level, electrophoretic methods such as 2-dimensional gel electrophoresis offer excellent resolution and continue to be the preferred option. When combined with fluorescent labeling techniques such as DIGE, 2D-gels enable differential analysis of protein expression and modification patters at relatively high throughput.[20] Typically, only regulated protein spots of interest are then identified by methods like MALDI-TOF(/TOF) mass spectrometry. However, a number of inherent limitations are associated with 2D-gel electrophoretic methods.[20,21] These include difficulties in analyzing membrane proteins and very basic proteins such as histones, and relatively limited protein coverage—albeit more than one thousand spots can be detected, these typically represent only a few hundred abundant proteins. A recent extensive study aiming at delineating a protein signature for liver carcinogenicity highlights many of the specific advantages and disadvantages of this approach.[22] The authors evaluated the effects of 63 carcinogenic and noncarcinogenic chemicals on protein expression in rat liver. 2D-DIGE and statistical analysis of enabled identification of carcinogen characteristic spot patterns. Approximately 1000 spots were matched to a master gel (pI range 3-–0), these spots where then excised and subjected to protein identification via LC–MS/MS. This resulted in 728 identified protein spots corresponding to 356 abundant different gene products.

More recently, the development of fast scanning mass spectrometers and efficient multidimensional chromatographic methods for peptide separation enabled identification of several thousand proteins from a single trypsin digested sample. When combined with stable isotope labeling techniques[23], these bottom-up approaches enable the quantitative analysis of proteins and their modifications with unprecedented coverage and sensitivity and with much less of a bias against membrane proteins or proteins with extreme pI as compared to electrophoretic protein separation methods.[24,25] Recent examples include reports on the differential effects of the beta(1)-adrenergic antagonist atenolol, and the nonsteroidal anti-inflammatory drug ibuprofen on different cell types,[26,27] and a study revealing altered protein expression of 73 proteins after treatment of K562 cells with the BCR-Abl inhibitor imatinib.[28] Strategies involving sub-cellular fractionation or affinity enrichment allow a more directed analysis of drug-induced changes in protein expression and post-translational modifications, with less of an abundance bias. Recent exemplary work employed immunopurification or chromatographic phosphopeptide enrichment methods to analyze drug-induced changes in protein phosphorylation, by mapping network-level responses to inhibitor treatment, enabling the inference of signaling network topology.[8,13,29,30] In particular, mass spectrometry-based phosphoproteomics technologies have achieved a remarkable analytical depth allowing the study of drug action on thousands of phosphorylation sites. In a recent study, triple labeling by stable isotope labeling by amino acids in cell culture (SILAC) was used to quantify the effect of kinase inhibitors on phosphorylation sites in growth factor-stimulated cells. Among thousands of phosphopeptides, fewer than 10% were affected by MAPK inhibitors whereas almost 1000 phosphopeptides were affected by treatment of a leukemia cell line with the unselective Abl kinase inhibitor dasatinib.[30] A similar strategy was applied to quantify changes in protein lysine acetyla-

tion by clinical histone deacetylase inhibitors and identified multiple nonhistone targets.[31] Other recent work used cell fractionation and histone extraction to analyze histone acetylation and methylation patterns in response to treatment with histone deacetylase or demethylase inhibitors.[14,32]

3. Chemoproteomics-based target profiling

The basis of proteomics-based target profiling is the use of chemical probes specifically engineered to capture and identify protein targets, or classes of structurally or functionally related proteins often referred to as sub-proteomes. Hence the major considerations in any chemoproteomics strategy are concerned with the design of the chemical probe, which can be viewed as the 'fishing bait', and the analytical strategy used to detect and quantify the proteins captured by the bait. The two types of bait probes are noncovalent tagged or immobilized drugs or tool compounds,[5,7,33] and covalent active site-labelling probes, often referred to as activity based protein profiling (ABPP).[9,11,34] In its simplest form, a compound of interest known to bind to the target, or class of targets, is conjugated to a suitably derivatized biotin agent or immobilized directly on a resin, for example, agarose beads which are available with different coupling chemistries and spacer lengths. If the compound of interest does not contain a suitable functional group (usually an amine, carboxyl, hydroxyl, or sulfhydryl group), chemical synthesis of a suitable functionalized analog of the compound will be required, and the resulting analog needs to be evaluated as to whether it retained its target affinity or biological activity. The resulting fishing probe is then incubated with cell extracts and mass spectrometry can be employed in order to identify captured proteins.[33] Proteins are captured in noncovalent fashion and can be eluted from the affinity matrix by excess probe compound. Successful applications of the noncovalent approach include the selective enrichment of kinases[18,35–40] and other nucleotide-binding proteins,[41–43] phosphatidylinositol-binding proteins,[44–46] phosphodiesterases,[47] and histone deacetylases.[48] Other drug target classes may be more amenable to activity based protein profiling (ABPP). These methods, pioneered by the Cravatt laboratory, employ reactive probes conjugated with biotin which are designed to specifically bind to active sites of enzymes. In the first step, the probe is incubated with the cell or tissue sample for a defined amount of time. Subsequently, the covalent probe–protein conjugates are isolated via the biotin tag. Probes were initially developed for proteases but are now available for a variety of enzyme classes.[11] Probe compounds labelled with fluorescent groups enabled the substrate-independent identification of inhibitors of uncharacterized enzymes.[49] The design of the probe is a crucial step in ABPP assays and requires detailed structural information to ensure the selective binding of the probe in the catalytic site of the enzyme target and the efficient reaction of the probe with reactive amino acids in proximity of the catalytic site. Activity-based probes can be adapted for in situ and in vivo labelling by introducing a 'bioorthogonal chemical handle', such as an alkyne. Probe-labelled enzymes are then captured by click chemistry conjugation to azide-containing reporter tags.[50–52] A generic strategy for probe design was recently suggested based on a trifunctional design comprising a reversibly interacting selectivity group, a reactive group and a biotin ('sorting') function.[54] This strategy was exemplified for the profiling of cAMP-binding proteins, methyltransferases, and kinases.[53–55] However, the number of kinases identified with these reactive probes was substantially lower compared to capturing ligands based on noncovalent multi-kinase inhibitors,[18] indicating a limited impact of the bond-forming reaction on target class coverage.

A common consideration in all these approaches is that qualitative protein binding profiles obtained in activity- or affinity-based enrichment experiments yield only limited information, if any, regarding binding potencies of targets and off-targets detected. Moreover, the fact that reactive or immobilized analogs, rather than the compound of interest by itself, are employed as fishing baits to identify targets, bears the risk that the structural alterations introduced for fishing probe generation may alter potency and selectivity compared to the original compound under investigation. Consequently, each identified protein requires additional validation, typically using the standard repertoire of enzymatic assays.[38,56,57] Hence there is a risk that a substantial amount of laborious validation efforts is invested in false positive or physiologically nonrelevant candidates, especially when, owing to the sensitivity of current mass spectrometers, tens or even hundreds of proteins are identified from a proteomic target identification experiment. Therefore it is of utmost importance to discriminate significant, high affinity interactions with protein targets of low abundance, which are typically caused by relatively more abundant proteins exhibiting low affinity interactions with the fishing probe. For instance, albumin and hemoglobin are known to exhibit low affinity for many small molecules and many NADH/NADPH binding proteins bind to immobilized ATP-mimetics.[36,38,56–58] False-positive identifications may also be caused by proteins binding to the resin or to additional groups introduced to compounds for probe generation, for example, linkers, reactive groups, biotin etc. Moreover, if the resin was derivatized with a chemical ligand at a relatively high concentration, its hydrophobic and charge properties may be altered, which is likely to contribute to nonspecific protein binding. One simple way to address these issues is by excluding those proteins from further analysis that have been frequently observed in independent experiments using similar probe matrices.[59] A more elaborate strategy to avoid false positive target identifications is to design active and inactive analogs of the fishing probe.[60,61] Parallel experiments with active and inactive versions of the probe may allow to shortlist candidate target proteins by differential display of quantitative mass spectrometric results. The drawback of this approach is that inactive analogs are often not available and synthesizing additional probes is laborious. Therefore, the best way to address false positive identifications is the adoption of a quantitative, competition-based experimental design whenever possible. It should become standard procedure to perform the incubation of the cell extract with the fishing probe in presence and absence of an excess of the free parental (i.e., nonderivatized) compound. Subsequent identification and quantification of proteins enables the unambiguous identification of the targets of the parental compound, as those proteins for which captured amounts were reduced by the presence of free compound compared to the vehicle control.[18,39,48,62,22] Stable isotope labelling-based quantitative mass spectrometry techniques for precise and accurate relative quantification of proteins are an ideally suited tool in this type of proteomic experiments,[17,61] since the ability of combining from three (using SILAC) to eight (using *i*TRAQ) differentially treated biological samples allows the direct determination of the potency of the parental compound, by performing the experiment across a range of compound concentrations. This type of experiments directly yield IC_{50} values for up to 100 or more protein targets, from which apparent dissociation constants (K_D) values can be derived by taking into account the amount of target sequestered by the fishing probe, or probe matrix.[40,48,63] In a recent report, this approach was applied to the profiling of inhibitors binding to native megadalton HDAC complexes in cell extract. Under the conditions of the experiment, the catalytic and regulatory subunits of protein complexes remained associated after the catalytic subunit binds to the bait probe, and therefore subunits of stable complexes could be delineated by matching inhibitor K_D values.

Notably, unexpected differences in inhibitor binding to class I HDAC complexes were observed, despite the fact that these complexes are formed around the same catalytic subunits, suggesting that that the selectivity of HDAC inhibitors should be evaluated in the context of HDAC complexes rather than purified catalytic subunits.[48,64]

Finally, Chemoproteomic analysis of inhibitor targets can be ideally combined with the mapping of post-translational modifications. When cells are treated with a protein kinase inhibitor, direct kinase targets may be revealed by their reduced binding to the affinity matrix, however, protein kinases downstream of the respective target kinases will display an altered phosphorylation state due to the reduced signalling by the target kinase. For example, in the case of imatinib-treated CML cells, several kinases not directly targeted by the drug, including CSK, ERK2, RSK1 and RSK3, exhibited a significantly down-regulated phosphorylation state on known regulatory residues.[18,65]

4. Applications of chemoproteomics-based target identification in phenotypic drug discovery

In target-based drug discovery, a project typically begins with the nomination of a target, defined as a protein which should be (i) tractable, that is, its biochemical activity can be modulated by the desired therapeutic agent in a dose-dependent fashion, and (ii) validated, that is, the target mediates a pathophysiological process such that modulation of its function affects a disease-relevant outcome, which can be determined in disease-relevant cell or animal models of disease. Targets are classified as 'clinically validated' and 'druggable', if the modulation of the target was shown to lead to the desired clinical outcome. Almost all clinically validated targets belong to a small number of classes, with a bias towards cell surface proteins (e.g., G protein-coupled receptors, ion channels, or transporters), and a small number of intracellular protein families (e.g., nuclear receptors, metabolic enzymes, kinases, or phosphodiesterases). It was estimated that the entirety of approved small molecule drugs acts through approximately 200 human proteins as targets,[66] a small number compared to the more than 20,000 protein-coding human genes.[67] Therefore, ten times as many suitable drug targets may be waiting to be discovered.[68] There are numerous proteins in pathways with a strong disease implication, for example, based on pathobiochemical and human genetic evidence, which are not accessible by target-based small molecule-based approaches. Phenotypic screening provides an alternative to target-based approaches with the potential to discover therapeutic agents and new drug targets,[69] and many approved drugs were discovered by this approach.[70] However, the development of a compound discovered by a phenotypic approach can be difficult if the molecular target is not established.[71] Hence the combination of phenotypic screening with target deconvolution by chemical proteomics provides a means to enable the identification of targets without the dependence on traditional target classes and assay types. This type of target deconvolution was pioneered by Schreiber and colleagues in their seminal work on the molecular targets of immunosuppressants[72,73] and inhibitors of histone acetylation.[74] More recent approaches employed a combination of screening of structurally diverse compound collections in cell-based assays with subsequent chemoproteomics-based target identification. Huang et al. discovered the tankyrase enzymes as tractable targets in the Wnt signaling pathway, which plays an important role in colorectal cancers and is characterized by a dearth of tractable drug targets.[60] Using a similar strategy, Nicodeme et al. performed cell-based screening for modulators of Apolipoprotein AI production, and proteomic profiling of hit compounds led to the unexpected discovery of bromodomain proteins as tractable targets for the modulation of apolipoprotein transcription. The inhibitors exhibit a novel mechanism of action by blocking the protein–protein interaction formed between acetylated histones and the bromodomains of BET-family proteins, which were not previously regarded as tractable targets.[75] Fleischer et al. used affinity-based proteomics to delineate the target of the potent and selective cytotoxic agent CB30865 as nicotinamide phosphoribosyltransferase, an enzyme in the NAD biosynthetic pathway which helps cancer cells to sustain their increased energy metabolism.[76] Besides the potential of these strategies for the discovery of drugs, these studies support the notion that there is a general need for new types of small molecules as research tools to study protein function, particularly for proteins which are not classical drug targets.

5. Applications of chemoproteomics in drug selectivity profiling

Regardless of the fact that compounds are typically optimized against a single target, many drugs exhibit polypharmacology, that is, they act on multiple targets. These off-targets often cause toxic liabilities, a major reason causing drugs to fail in clinical development.[77] However, many oncology drugs exhibit polypharmacology, leading to increased efficacy and lower incidences of drug resistance. Such drugs often target proteins from large target classes with a high degree of structural conservation around the active site, like kinases and HDACs. Obviously drugs with polypharmacology are more likely to produce toxic side effects, but in oncology the increase in efficacy often outweighs the disadvantages.[6] Industry standard profiling strategies rely on large assay panels using different purified enzymes to address potential off-target liabilities.[78] The progress in affinity-based proteomic techniques has enabled the profiling of small molecule drugs under close to physiological conditions. In addition to strategies involving the immobilization of the compound of interest itself, as described in the previous section, which in many cases remains a technically demanding and resource intensive undertaking, there are now powerful strategies based on tagged or immobilized probes designed to bind to defined subsets of proteins. To this effect, the probes are designed to bind to tens to hundreds of proteins defined by structurally related active sites, representing chemically tractable subproteomes.[42] This type of probes can be designed for covalent or noncovalent protein binding. Successful application include purine-binding proteins,[79] protein kinases,[18,40,80] lipid kinases,[45,46] GTPases,[81] methylases,[55] dehydrogenases,[82] phosphodiesterases,[47] serine-, cysteine-, metallo-, and proteasomal proteases,[11,83,84] and histone deacetylases.[48,85] In case of the 'kinobeads' for protein kinases and the hydroxamate matrix for histone deacetylases developed by Bantscheff et al., up to 1000 proteins were found to bind to the probe matrix, and were routinely quantified using a competition binding assay format coupled to protein quantification by quantitative mass spectrometry.[48,61] Notably, this method of drug profiling can be applied directly to patient-derived primary cells or biopsies.[35,36] Some target classes are more amenable to activity-based profiling (see also next section). Cravatt et al. designed a probe which captured around 80% of the mammalian serine hydrolases, enabling the profiling of 70 serine hydrolases against 140 structurally diverse carbamates, followed by selectivity profiling using the very same approach.[49,22] Protein kinases are also amenable to activity-based profiling, based on acyl phosphate-containing nucleotides conjugated with biotin.[62] These probes covalently capture kinases and other ATP-binding proteins from cell extracts, and were used in a competitive binding assay to determine inhibitor potencies, similar to the approaches based on noncovalent capturing described above. Finally, in addition to the applications described above, activity-based chemical proteomics strategies can be adapted to enable the identification of targets in vivo.[86]

6. Applications of chemoproteomics in the screening of compound libraries

Many drug discovery assays rely on the ability to express and purify the target protein in active form in substantial amounts—typically milligrams to grams of protein for high throughput screening of compound libraries. There are many types of protein targets, including entire target classes, where this is not easily achieved, for instance because the target protein cannot be expressed at high levels, does not fold properly, or requires interacting proteins for proper activity. Methods based on immobilized probe compounds to capture the target directly from a cell or tissue extract without further purification can be used in competition-binding formats and provide a viable alternative strategy. Fadden et al. employed the capturing of purine-binding proteins from porcine tissue with ATP-derivatized Sepharose and performed affinity elutions with 5000 small molecule compounds, resulting in the identification of 463 compounds eluting a total of 77 distinct proteins. Among these, novel and structurally diverse inhibitors of the chaperone HSP90 were identified, which were further optimized to enter clinical development as anticancer agents.[86] A different strategy was used by Bantscheff et al. who screened a compound library for histone deacetylases inhibitors in a human cell extract, using an immobilized hydroxamate-based probe. The compounds were not used for protein elution but instead were added to the cell extract, such that each compound was assayed for the inhibition of four different HDACs binding to the immobilized probe.[48] A common feature of these approaches is that the entire complement of proteins binding selectively to the immobilized probe is screened simultaneously. In particular for target classes with a substantial number of structurally related targets, like protein kinases or deacetylases, this is an advantage over traditional approaches, since undesired off-targets are monitored early in the project.

7. Conclusions

Recent advances in chemical proteomics and in mass spectrometry instrumentation have promoted new drug discovery strategies based on assays with increased content and better appreciation of the molecular context in which protein targets operate. These methodologies are providing complementary approaches to drug target identification, selectivity profiling, and lead finding, and have the potential to substantially contribute to in vivo studies and clinical studies of drug-target interactions. The first therapeutic agents discovered by chemical proteomics strategies are progressing towards clinical development[86,87], and the increasing number of applications are likely to further add to the track record of this new field.

Acknowledgements

We would like to thank our colleagues at Cellzome AG, in particular Drs. C. Hopf, G. Neubauer, and M. Savitski, for many stimulating discussions, and F. Weisbrodt for help with the figure.

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