**Revealing promiscuous drug-target interactions by chemical proteomics**

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**Abstract**

Molecular understanding of the (poly-)pharmacological activities of a drug cannot be achieved without a global characterization of its interactions with cellular components. Mass spectrometry based chemical proteomics approaches have recently been demonstrated as powerful tools for characterization of drug-target interactions directly from cells and cell extracts. At the same time, undesired cross-reactivities with other proteins within or outside the target class can be identified. This information can aid in the prediction of side effects and contribute towards optimization of candidate drug molecules. In this review, we describe recent advances in chemical proteomics and outline potential applications in drug discovery.

**Teaser**

Chemical proteomics enables characterization of drug-target and off-target interactions from native proteomes.

## Introduction

At present, considerable effort in the pharmaceutical industry is directed at the generation of drugs specifically inhibiting individual proteins out of protein families such as protein and lipid kinases, histone deacetylases, proteases and heat shock proteins. Structural conservation within these enzyme families makes the design of mono-selective inhibitors particularly challenging. Moreover, the pharmacological activity of therapeutics is not necessarily restricted to the target protein class since inhibitors are often designed as mimetics of ligands commonly used by several different enzyme families, e.g. ATP. In conventional strategies, compound selectivity and off-target liabilities are typically addressed by assay panels comprising a subset of the target protein family as purified enzymes [1-3]. However, correlation of assay results with efficacy in cellular or animal models often turns out to be difficult which might be at least partially explained by the biased nature and the limited physiological relevance of recombinant enzyme assays.

Recent developments in chemical proteomics, a multidisciplinary research area integrating biochemistry and cell biology with organic synthesis and mass spectrometry, have enabled more direct and unbiased studies of a drug's mechanism of action, directly in the proteome as expressed in the target cell or the tissue of interest. Advances in quantitative mass spectrometry, now enable the detection and quantification of drug-induced changes in protein expression and activation with unprecedented proteome coverage and affinity enrichment strategies using immobilized drugs or tool compounds have enabled the direct determination of protein-binding profiles of small molecule drugs under more physiological conditions.

In this review, we will briefly outline typical chemical proteomics work-flows and particularly focus on affinity based proteomics technologies using immobilized capture ligands for proteome-wide detection of drug targets and off-target liabilities. Potential applications of chemical proteomics in the drug discovery process will be discussed.

## Chemical Proteomics Approaches

Chemical proteomics approaches employ a variety of different experimental procedures of which the most commonly used can be categorized into three major experiment types: i) global proteomics approaches, in which changes in protein abundance and activation are determined upon drug treatment; ii) activity, or affinity, based protein profiling (ABPP), for whichsmall molecular probes are employed to covalently capture a distinct class of proteins; and finally iii) immobilized inhibitors that can be used for affinity enrichment of interacting proteins (figure 1). While all of these methods can provide valuable information on the mode-of-action of drug candidates, each method has distinct advantages and disadvantages, which will be briefly described in this section.

Recently, several studies applied global proteomics approaches to evaluate the cellular response upon drug treatment. In this approach cells or animals are treated with a drug before system-wide proteome analysis to evaluate globally induced changes in protein abundance. A particular advantage of this approach is that it is unbiased as the unmodified drug interacts with its endogenous targets. However, since typically no target enrichment is used, the observed changes are limited by the analytical depth of the analysis, often restricted to the most abundant proteins. Also, proteins found to be changed in abundance are not necessarily part of inhibited signaling pathways but often represent high abundant proteins involved in stress response and/or housekeeping [4,5]. Hence, identification of direct target proteins is rarely accomplished by global proteome profiling approaches. Illustrative examples of such approaches by Chen *et al.* [5,6] evaluated the differential effect of the R- and S-enantiomers of atenolol, a β1-selective adrenoreceptor blocker, and the nonsteroidal anti-inflammatory drug ibuprofen on two different cell types by a combination of two-dimensional peptide separation of trypsin digested cell extracts and quantitative mass spectrometry. 27 and 13 proteins, respectively, were identified as differentially expressed. The large majority of these can be classified as high abundant [4]. Yamanaka et al. showed that this approach can be extended to toxicological studies in animals as well [7]. The authors studied effects of 63 chemical compounds on protein expression in rat liver after 28 daily dosings using 2-dimensional differential gel electrophoresis (2D-DIGE) and employed statistical methods

to detect proteins characteristic for carcinogenicity. The proteins showing the best correlation with Ames-test positive compounds included structural proteins like lamin A, enzymes like methyltransferases and dehydrogenases, albumin and HSP60 and were suggested as biomarkers for prediction systems to detect genotoxic and non-genotoxic carcinogens in short-term bioassays.

Also cellular global changes in posttranslational modifications, such as phosphorylation, can be evaluated upon drug-treatment as reported for key players in the BCR-ABL kinase pathway [8]. In a more targeted variation of this approach, Lee et al monitored histone modifications in response to treatment of cells with histone deacetylase inhibitors [9]. In this study, human colon cancer cells were treated with HDAC inhibitors of varying degrees of selectivity followed by a simple prefractionation method to enrich for histone proteins. By employing a quantitative mass spectrometric approach the authors could identify HDAC-1 specific histone acetylation patterns and quantify these in response to inhibitor treatment.

A second, more focused, strategy in chemical proteomics is activity or affinity based protein profiling (ABPP). In this approach small molecular probes are targeted against a distinct class of proteins to which they can covalently attach through a reactive group. Typically, the reactive probe is fused via a spacer to an affinity tag such as biotin. In a first step, the small molecule probe is incubated with the biological sample and allowed to react. Subsequently, the probe is captured on a solid support along with the proteins, or protein complexes it associated with. For some enzyme classes, e.g. cytochrome P450 proteins, the reactive group can be tailored in such a way that it only reacts with the protein in its active conformation [10]. Detailed structural information is required to design an activity-based probe as such that the reactive group is in close proximity to susceptible amino acid residues in the catalytic site that are only exposed upon activation. The technique is most powerful when applied to probing differential protein activity in health and disease to reveal specific activity of target class members in human pathologies. Examples of this are, however, still scarce [11]. The main advantage of the ABPP approach is its ability to directly assess enzyme activity [10]. It does, however,

require a significant amount of time and resources to optimize the probes to acquire specificity. ABPP is a very powerful means to specifically enrich and characterize drug target classes and could be a valuable tool in drug discovery. Detailed ABPP requirements and target classes have recently been reviewed [12,13] and will therefore not be further discussed in this review.

Combining affinity chromatography with mass spectrometry is a third strategy often applied in chemical proteomics. In this case, the compound of interest is immobilized on a solid support to allow the solid-phase extraction of interacting proteins in a single step. Although, this approach is as generic as ABPP, the number of target classes addressed with this method is, at the moment, still limited. The majority of publications in this area, so far, focused on protein kinase inhibitors (see below). In other reports endogenous signaling molecules such as ATP/ADP [14], phosphatidylinositols [15,16] and cyclic nucleotides [17] have been used for affinity enrichment of interacting proteins. Also the use of specific peptide sequences as baits has been reported [18,19].

In the next section we describe the main experimental aspects of affinity based chemical proteomics approaches.

**Experimental considerations in affinity chromatography based chemical proteomics**

Chemical proteomics provides a very targeted approach that has the potential to identify specific interactors of small signaling molecules, drugs or toxins in comprehensive analyses. The affinity purification step, based on a highly specific reversible interaction of proteins with the tagged- or immobilized compound, aids critically in decreasing the complexity of the "drug-interactome" prior to mass spectrometric analysis allowing the identification of lower abundant proteins, which are generally missed in more global proteome analyses. However, the efficiency of this approach is dependent on many factors and has some limitations that need to be addressed. Some of these challenges, and how they have recently been addressed, are discussed below.

*Immobilization of the small molecule.* Immobilization of compounds on a solid support requires the presence of functional groups such as amines, carboxylic acids or hydroxyls. Drug and signaling molecules do not necessarily exhibit such functional groups, which makes the synthetic design of the drug-support linker moiety an essential part of chemical proteomics. Moreover, special care must be taken that modifications to the molecule do not impede its bioactivity. Therefore, it is helpful to have high-resolution structural data available of protein-drug complexes, which can reveal the exact nature of the interaction and indicate which part of the drug molecule is available for chemical modification. The small molecule derivative made for linkage to the beads should be tested for its bioactivity preferable in a validated enzymatic assay. Effects of the specific linkage induced problems were described by Scholten et al. who used modified cyclic nucleotides with a flexible linker molecule at either the 2' or 8' position [17]. These immobilized analogs revealed a distinct protein profile in affinity pull-downs (see also figure 3A). Tanaka and co-workers noted that also the linker between the resin and the compound can affect the binding of affinity-enriched proteins significantly [20].

*Solubility of target proteins in cell extracts.* Chemical proteomics experiments should be executed under non-denaturing conditions to preserve proper folding and assembly of proteins. While this tends not to be a problem for soluble cytosolic proteins, extraction of

intact membrane proteins and membrane protein complexes, which are often primary targets of drug molecules (e.g. receptors) is more challenging because of the generally more aggressive conditions required. Consequently, membrane proteins tend to be underrepresented in the list of identified interactors. For instance, although Aye et al. [21] were able to identify more than a dozen of PKA-AKAP complexes in their affinity pull-down using immobilized cAMP, they were unable to detect several well-known membrane associated PKA-AKAP complexes. However, recent reports using immobilized kinase inhibitors showed good coverage of receptor tyrosine kinases [22,23] and Winkler et al. reported on affinity capturing experiments of the active γ-secretase complex with a biotinylated transition-state analogue inhibitor[24].

*Abundance of the target protein.* Many target proteins of successful drugs are low abundant proteins that have high affinity (nM range) for the compound. However, in every affinity pull-down experiment there is a competition for binding to the immobilized compound between these low abundant high-affinity binders and much higher abundant albeit lower affinity (μM) binding proteins. In particular, high abundant proteins such as albumin and hemoglobin are known to have (medium) affinity for quite a range of small molecules. Other examples are the large families of CoA and NADP(H) binding proteins, which tend to have also affinity for nucleotide like compounds such as ATP-mimetics. Consequently, a direct correlation between the amounts of individual proteins captured in a chemical proteomics experiment and the affinity of these proteins to the immobilized compound is not possible [25,26].

*Background binding of proteins to the affinity matrix.* In addition to genuine target proteins, affinity chromatography enriches also for a large number of “contaminant” proteins, including those that bind non-specifically, or with low affinity to the matrix or the used linker molecule (background proteins). To a certain degree, the level of background proteins can be reduced by using more stringent purification methods. However, this can also remove lower abundant specific proteins and their secondary interactors, i.e. proteins interacting with the drug target. Recently, Trinkle-Mulcahy *et al*.

[27] identified a large set of proteins that bind non-specifically to several commonly used affinity matrices, revealing important differences that may affect the chosen experimental design. Such data provide a specificity filter to distinguish specific protein binding partners from background proteins in chemical proteomics experiments.

Identification of target proteins is facilitated by introducing an appropriate “negative” control for each affinity pull-down experiment. Suitable controls are e.g. inactive analogs of the compound of interest [28]. By comparing proteins captured with both the active and the inactive molecule, relevant target proteins can be identified. It is, however, often not feasible or expensive to have inactive structural analogs, hence other strategies are required to limit, or identify, background binding. To reduce background binding pre-clearing of the beads with specific washing-steps can be used [29]. Yamamota et al. [30] introduced an elegant serial affinity chromatography strategy for distinguishing specific from non-specific binding. First the lysate was incubated with the immobilized ligand beads, which were then removed. Fresh immobilized beads were then incubated with the “left-over” lysate. Both resins should capture the same amount of non-specific high-abundant background proteins, however, the first resin should be enriched for the more specific target(s) [31]. Alternatively, competition binding approaches using free compounds have been reported. In such experiments affinity enrichment is performed in the presence or absence of a free compound of interest. Upon differential display, e.g. using quantitative mass spectrometry, target proteins can be identified by their reduced abundance on the affinity matrix in the presence of the free compound [14,22,32]. In general, whenever serial or differential affinity pull-downs need to be quantitatively compared, stable isotope labeling strategies have proven particularly advantageous for the discrimination of genuine interactors from non-specific binders.

*In vivo interactions.* In general, chemical proteomics experiments suffer from the fact that they do not fully represent *in vivo* interactions. Each experiment is initiated by making first a lysate, wherein DNA, RNA, lipids and other metabolities are often first removed before performing the pull-downs. Although, it may be argued that the affinity of the drug for its target protein may be largely preserved from *in vivo* to in the lysate,

secondary binders may behave differently as they are likely more influenced by the cellular context. E.g. the presence of detergents as well as the reduction in protein concentration during lysate preparation might influence the stability of noncovalent protein complexes. Therefore, any putative primary or secondary interaction partner identified through affinity purification must be validated to confirm its physiological relevance *in vitro* and *in vivo*.

**Target identification of protein kinase inhibitors by chemical proteomics**

Protein kinases are increasingly recognized as drug targets not only in oncology but also for a variety of non-oncology diseases such as inflammation, autoimmune disease [33-35], metabolic disorders [36] and central nervous system disorders[37]. 518 protein kinases and more than 2000 other ATP- and purine-binding proteins are estimated to be encoded by the human genome [38,39]. Because of the structural conservation of the ATP binding site, synthesis of selective small molecule ATP-competitive kinase inhibitors is a challenging task. Consequently, when tested against a large enough panel of kinases, more often than not, additional targets are discovered for kinase inhibitors presumed to be selective, thus revealing their generally promiscuous nature.

Affinity chromatography using immobilized inhibitor molecules has been demonstrated as a powerful tool for detection of off-targets of kinase inhibitors [25,26,28,40]. As an example, Godl et al. used an immobilized analog of the PDGFRb inhibitor SU6668 to detect kinase off-targets. Several protein kinases were found to bind to the matrix including Aurora kinase A (AurA) and TBK1. In follow-up experiments the authors could show that a cell cycle block induced by SU6668 was caused by inhibition of AurA and that SU6668 potently suppressed antiviral and inflammatory responses by interfering with TBK1-mediated signal transmission indicating the relevance of their findings [26]. In a recent study, a linkable version of imatinib, a block buster drug for the treatment of chronic myeloid leukemia (CML), and the two second generation drugs nilotinib and dasatinib were analysed for their target spectrum in affinity precipitation experiments [41]. Originally developed for inhibition of the fusion oncoprotein BCR-ABL the biochemical hallmark of CML, imatinib is known for its remarkable selectivity for ABL,

KIT and PDGFR [42,43]. In the study, strong binding of immobilized imatinib to the quinone oxidoreductase NQO2 was observed. For the close analogue nilotinib DDR1 and ARG were identified as additional target proteins. Further, dasatinib developed as a dual-specificity ABL- and SRC-family kinase inhibitor [44] was found to bind a total of 24 protein kinases in experiments done with K562 cells.

As discussed previously, the qualitative binding profiles obtained in simple affinity enrichment studies using immobilized compounds give only limited information about binding potencies of targets and off-targets detected. Consequently, the relevance of any detected off-target protein has to be validated using the standard repertoire of activity based assays (e.g. recombinant kinase assays) [25,26]. These limitations were partially addressed by linking ATP to sepharose-beads through the gamma phosphate group, thus generating an affinity matrix suitable for capturing ATP-binding proteins including protein kinases as well as a variety of other proteins utilizing purine co-factors [14]. Proteins interacting with a certain compound under investigation where then determined using a competitive binding assay. In this approach, the ATP-affinity matrix is first used to enrich for ATP-binding proteins and then this matrix is incubated with increasing amounts of the compound of interest. Hence, target proteins will be eluted from the matrix in a dose dependent manner (figure 2A). In a variation of this approach Patricelli and coworkers [32] used acyl phosphate-containing nucleotides, prepared from a biotin derivative and ATP or ADP to covalently modify ATP-binding proteins. Biotinylated peptide fragments from labeled proteomes were subsequently captured and identified by mass spectrometry. Using a competition binding assay between acyl phosphate probes and inhibitors, target proteins against protein kinases and hundreds of other ATP binding proteins could be determined (figure 2B).

In order to further increase the specificity of affinity enrichment approaches for protein kinases, resins containing immobilized unspecific kinase inhibitors have been suggested, rather than using ATP/ADP. Wissing et al. used sequential multistep prefractionation via several potent inhibitor resins to identify a total of 140 different members of the protein kinase family [45]. Similarly, a set of tool compounds that displayed little selectivity and interacted with protein kinases located on different branches of the phylogenetic tree was used to create mixed inhibitor resins with up to seven different capturing ligands [22].

These mixed kinase inhibitor beads have been used to specifically enrich for a large fraction of the kinome expressed in various cell types; e.g. using LC/MS-MS analysis, a total of 173 and 179 protein kinases from HeLa and K562 cells could be identified from single experiments [22]. When combined with phosphopeptide prefractionation, such resins enable to study phosphorylation dependent activation and deactivation of kinases. Thus, target class selective enrichment strategies can be an important tool for signal transduction research and disease biology in general [22,23,45]. In a recent example, Daub et al. used kinase selective affinity purification to analyze the cell-cycle regulation of protein kinases [23]. Therein, a total of 219 protein kinases were identified and quantified from S and M phase-arrested human cancer cells. Out of the approximately 1000 identified phosphorylation sites on human kinases many were found up-regulated in mitosis. The data further indicated potential phosphorylation networks involving protein kinases that had not been established in mitotic progression, before.

The relatively generic binding of mixed kinase inhibitor matrices makes them particularly attractive for application in competition binding experiments with ATP competitive kinase inhibitor compounds. Identification of target proteins and their binding potencies largely depends on the ability to quantify differences between vehicle control samples and samples incubated with different amounts of inhibitor. In chemical proteomics experiments this is typically accomplished by quantitative mass spectrometric technologies (for recent reviews see [22,46,47]). Pretreatment of cells, or cell lysates with inhibitor compounds at varying concentrations before incubation with the mixed kinase inhibitor beads will lead to reduced binding of target and off-target proteins to the beads. Using quantitative mass spectrometry, cellular targets of inhibitor molecules and their associated binding potencies ($IC_{50}$ values) can be determined (figure 2C). Such a quantitative affinity profiling experiment was recently described for the study of three inhibitors of the tyrosine kinase ABL developed for the treatment of chronic myelogenous leukemia; the phase II compound SKI-606 and the marketed drugs imatinib and dasatinib [22]. For the three drugs, dose-response binding profiles for >500 proteins in each sample were generated, including ~150 kinases. For imatinib, 13 proteins exhibited more than 50% binding reduction on the beads at 1 μM drug in the lysate. Among the competed proteins were ABL/BCR-ABL, ARG, and two novel target

candidates, the receptor tyrosine kinase DDR1 (90 nM), and the quinone oxidoreductase NQO2 (43 nM). In contrast, Dasatinib and SKI-606 revealed very broad target profiles (46 and 42 proteins respectively showed >50% competition at 1 μM), including the three imatinib targets ABL/BCR-ABL, ARG, and DDR1. Inhibition of several of the novel targets has further been validated using target specific biochemical assays and inhibition of DDR1 by imatinib and dasatinib has recently been followed-up and could give guidance for the development of specific DDR1/2 inhibitors for a variety of therapeutic areas including inflammatory, fibrotic and neoplastic diseases [48]. When combined with phosphorylation specific enrichment strategies, this approach further enables distinguishing between proximal drug targets and downstream signaling effectors of a kinase inhibitor. When cells are treated with an inhibitor, direct targets will 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 signaling by the target kinase. In the case of imatinib-treated K562 cells, RSK3 was identified as one of 9 proteins exhibiting a significant downregulated phosphorylation state [22].

**Cyclic nucleotide regulated signaling studied by chemical proteomics**

When applied to kinases, most chemical proteomics approaches generally target the ATP-binding site to achieve broad kinase coverage [22,23]. A different strategy in chemical proteomics introduced by Scholten et al. [17] is to use allosteric/co-factor sites for specific enrichment of a smaller subset of kinases. In this particular example, the target of interest was cAMP-dependent protein kinase (PKA) and the specific interactome of four differently immobilized cyclic nucleotides (2AHA-cAMP, 8AEA-cAMP, 2-AH-cGMP, 8AET-cGMP, figure 3A) was investigated to assess PKA-targeting efficiency of the beads, as well as evaluate cross reactivity. Figure 3B shows that each type is suited to bind both PKA and the closely related PKG (cGMP-dependent protein kinase). Mass spectrometric interrogation of the 8AEA-cAMP interactome obtained from mouse ventricular tissue revealed the abundant presence of the proteins GAPDH and different NDPK-type proteins [29]. These off-targets do not specifically bind to cAMP, but to NADP(H) and with lower potency to all diphosphate nucleotides, respectively. By incubating the beads with ADP, less specific binders could be separated from the lower abundant, cAMP interactome [29]. By applying cGMP to the beads, a large portion of PKG could be separated from PKA. For the 2AH-cGMP an even more diverse off-target profile was observed with ADP, GDP, NADP(H) and CoA binding proteins being purified. Here, a similar elution strategy with consecutive elution by ADP and GDP proved very valuable for increasing specificity of the beads [17]. In later applications of this strategy, a competitive treatment of the lysate with ADP and GDP was shown to prevent binding of these off-targets altogether [21]. Figure 3B shows that the coupling position and the nature of the cyclic nucleotide are important for target and off-target profiles of chemical proteomics resins. For instance, the class of phosphodiesterases (PDEs) is very sensitive towards modifications of cyclic nucleotides in the 8-position, hence they were only captured with the 2-position type beads [17] . Aye et al. discovered that a substituted cAMP analogue (8-AHA-2’-OMe-cAMP) can distinguish between PKA-R type I and II and does not bind to PKG at all, allowing an even more focused specific interactome [21].

Besides probing PKA and PKG presence and phosphorylation state in different samples, the cyclic nucleotide beads were also used to investigate the involvement of these kinases

in multi-protein signaling complexes [49]. PKA is expressed in a variety of tissues and cell types. Even within a single cell type a multitude of functions and targets are ascribed to PKA. Hence, specific regulation of individual activities is essential. PKA achieves this specificity through interaction with the functionally related, although structurally highly diverse, protein class of A-kinase interacting proteins (AKAPs). All AKAPs contain a specific localization sequence as well as a PKA anchoring sequence to tether PKA activity to specific sites within the cell. Using the immobilized cyclic nucleotide approach, the association of PKA with many different AKAPs could be evaluated in different tissues in a single experiment [17,21,29]. When combined with quantitative mass spectrometry this approach allowed it to screen a selection of AKAPs for their PKA-R specificity, directly in tissue (figure 3C) [21]. Due to its omnipotent nature, PKA is not considered as a drug target. However with solving the exact function of specific PKA-AKAP complexes a new set of promising drug targets is developing [50].

**Targets in Lipid Kinase Signaling**

Chemical proteomics approaches utilizing immobilized compounds for affinity enrichment of interacting proteins have thus far been most widely applied to profile protein kinase inbibitors as reviewed above, but the methodology is not limited to this class of compounds. Several studies have targeted phosphoinositide binding proteins [15,16]. Phosphoinositides are membrane phospholipids that dictate the localization and function of many intracellular proteins. These phosphoinositide binding proteins influence many critical processes in eukaryotic cells, including signaling by cell-surface receptors, vesicle trafficking, and cytoskeletal (dis)assembly. Krugmann et al. [16] used affinity matrices carrying analogs of natural phosphoinositides to capture phosphoinositide binding proteins from cell and tissue lysates and were able to enrich for and identify several known and a few novel phosphoinositide binding proteins. More recently, Pasquali et al. [51] reported on the use of cleavable immobilized phosphoinositide lipid baits, which improved the sensitivity and selectivity of the approach significantly. They were able to validate some of the detected putative novel phosphoinositide interacting proteins in lipid protein kinase assays and control pull-down

experiments using the recombinant proteins. These studies clearly indicate that protein-lipid interaction may be preserved during affinity purifications as performed in chemical proteomics. Related to these studies Gharbi *et al.* [15] used an immobilized analog of a known PI3K inhibitor (LY294002), and revealed that this inhibitor was quite promiscuous and interacted with several other kinases such as CK2, Pim-1 and DNA-PK.

**Peptide baits in chemical proteomics**

Another class of compounds that has been used extensively in chemical proteomics approaches has been bioactive peptides. For instance the group of Mann [18,19] used a SILAC approach with tyrosine phosphorylated *versus* non-phosphorylated peptides immobilized to beads. They found in this manner many phosphorylation-specific interaction partners of the ErbB-receptor family [18] and in another study those present in the insulin signaling pathway [19]. Several groups have interrogated the interactome of histones, targeting the specific protein interactions regulated by post-translational modifications [52-54] . Vermeulen et al. [54] prepared peptides containing the N-terminal 17 amino acids of the histone H3, trimethylated at lysine 4 (H3K4me3), which is regarded as a hallmark of promoters, and immobilized them on magnetic beads. Subsequent incubation with nuclear extracts revealed that H3K4me3 is largely required for TFIID binding and therefore plays an important role in regulation of RNA polymerase II-mediated transcription in eukaryotes.

**Future Outlook; chemical proteomics and drug discovery**

Notwithstanding the promising results reported in the last decade, chemical proteomics approaches are not yet regarded as a standard element of assay cascades generally employed in drug discovery. Partially, this might be due to the very specialized and expensive mass spectrometric equipment required and the relatively high costs per data point compared to more conventional enzyme assays. For example in kinase drug discovery, researchers can choose from a large variety of biochemical assays to assess selectivity of kinase inhibitors in development. Nowadays, cost effective selectivity profiling of compounds can be performed against panels exceeding 300 protein kinases. Apart from traditional biochemical kinase assays employing recombinant proteins and substrate peptides a variety of new methods have emerged [2,55]. In a particularly interesting approach, protein kinases were expressed as fusion proteins on the surface of a phage [55]. Similarly to proteomics approaches outlined above, binding affinities of compounds are determined by measuring the dose dependent reduction of binding of phage-tagged kinases to an affinity matrix containing immobilized unspecific kinase inhibitors. Very high sensitivities for detection and quantification of kinases can be achieved by amplification of the phage signal. In a recent study, interaction maps for 38 kinase inhibitors across a panel of 317 protein kinases were reported, constituting one of the most comprehensive studies of kinase inhibitor selectivity to date [56]. However, the application of recombinantly expressed kinases bears significant limitations. Commercial recombinant proteins kinases almost always include just the kinase domain, rather than the full-length protein. Further, proteins over-expressed in bacteria and insect cells, likely not exhibit the proper state of post-translational modifications, and might even be partially miss-folded or denatured. *In vivo* kinase activity is regulated by post-translational modifications, co-factor binding and higher order structures (homodimerization, binding of regulatory subunits and other interacting proteins). Hence, the lack of cellular context limits the predictive power of such *in vitro* kinase assays for *in vivo* efficacy. The recent advances in chemical proteomic technologies described above have enabled to determine the binding of small molecule compounds to their targets directly in cells or cell extracts of relevant tissues. Competition binding assays in combination with quantitative mass spectrometry provide versatile tools to map a drug's

direct and indirect targets in a single set of experiments. We anticipate that due to their inherent general applicability affinity-based proteomic approaches will prove valuable at various stages of drug discovery including validation and selectivity assessment of screening hits and of molecules developed during lead optimization phase. Moreover, in later stage preclinical development, compounds are typically tested in animal disease models. Due to insufficient availability of animal specific recombinant enzyme panels it is often difficult to establish compound potencies and specificities in different species. With the growing availability of fully sequenced genomes, mass spectrometry based chemical proteomics approaches can help to overcome this limitation. Naturally, chemical proteomics technologies would be equally well suited for translational studies of drug action in patient tissues.

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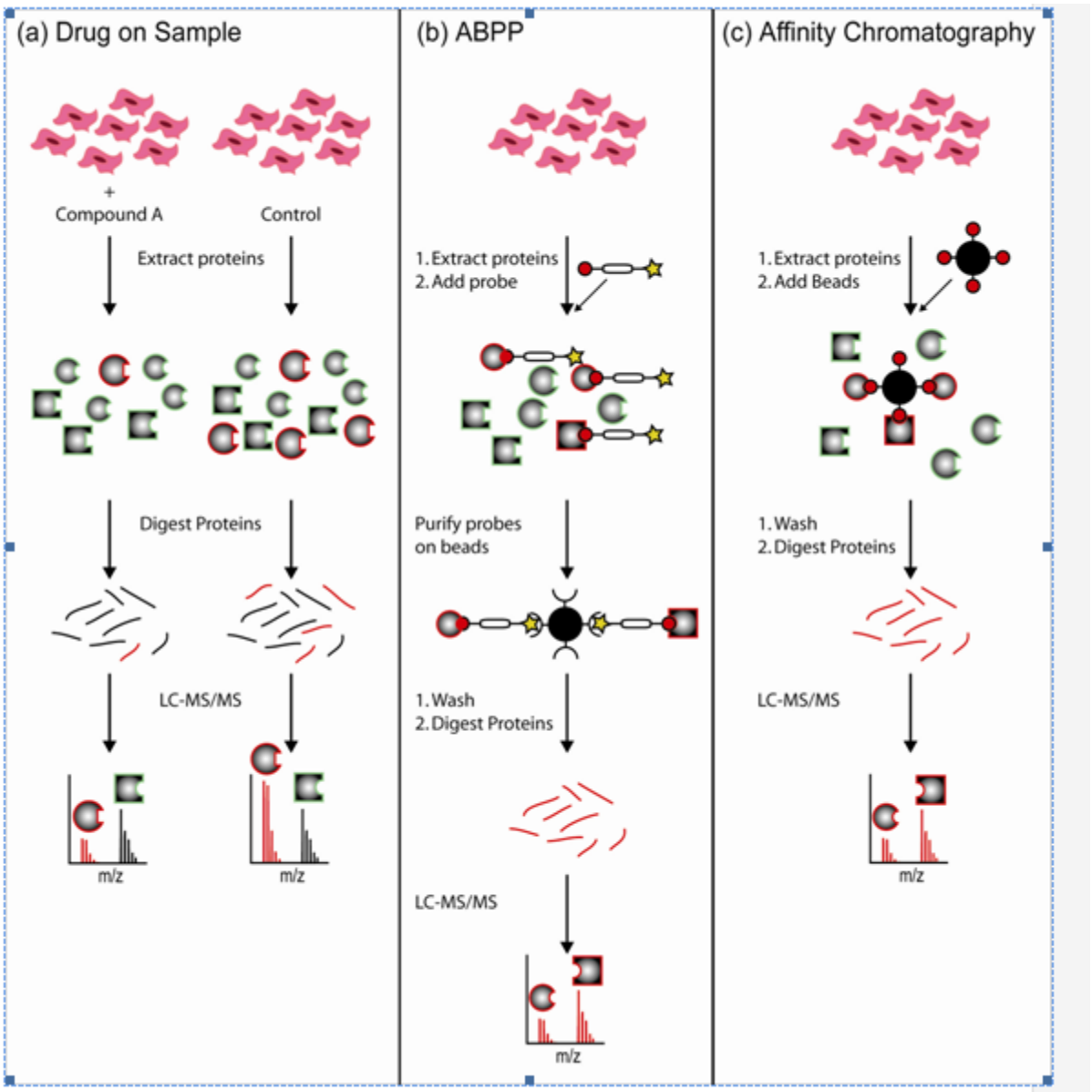
## Figures

**Figure 1: Experimental workflows in chemical proteomics.** Three different workflows are typically denominated under the term chemical proteomics**. (a)** The drug on sample strategy involves the global proteome comparison of a drug treated vs. a control cell, tissue or animal sample. After treatment, proteins are extracted from the sample, digested to peptides and analyzed by LC-MS/MS. Quantitation is achieved by either stable isotope labeling or spectral counting. **(b)** Affinity/Activity Based Protein Profiling (ABPP) involves the specific targeting and subsequent purification of a drug target protein class with small molecular probes. After protein extraction, the lysate 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. **(c)** For affinity chromatography, the compounds of interest are modified and immobilized on a solid support. The immobilized drug is subsequently incubated with a protein sample of choice to specifically purify target proteins that are then analyzed by LC-MS/MS again.


(a) Drug on Sample
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(b) ABPP
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on beads
1. Wash
2. Digest Proteins
LC-MS/MS
m/z
(c) Affinity Chromatography
1. Extract proteins
2. Add Beads
1. Wash
2. Digest Proteins
LC-MS/MS
m/z

**Figure 2: Competition binding approaches employed in chemical proteomics.**

A) **γ-ATP approach[14]:** Cell extracts are incubated with γ-ATP-linked sepharose beads. Identification of target proteins is achieved upon incubation of the beads with the compound of interest and subsequent identification of eluted proteins by mass spectrometry.

B) **ABPP approach using acyl phosphate containing nucleotides [32].** Cell extracts are incubated with increasing amounts of inhibitor compound and subsequently reacted with the reactive probe. After proteolytic degradation, peptides containing the probe are enriched via their biotin affinity tag and subsequently detected via mass spectrometry. Label-free quantification allows for detection of target potencies of inhibitor to identified ATP-binding proteins.

C) **Mixed kinase inhibitor matrix[22].** Cells or cell extracts are incubated with increasing amounts of inhibitor compound. Protein kinases and other nucleotide binding proteins are captured upon incubation with mixed kinase inhibitor resin. Bound proteins are digested with trypsin and labeled with isobaric mass tags enabling relative quantification in the subsequent mass spectrometric analysis. Target kinases of the tested inhibitor compound will display a dose-dependent reduction in binding

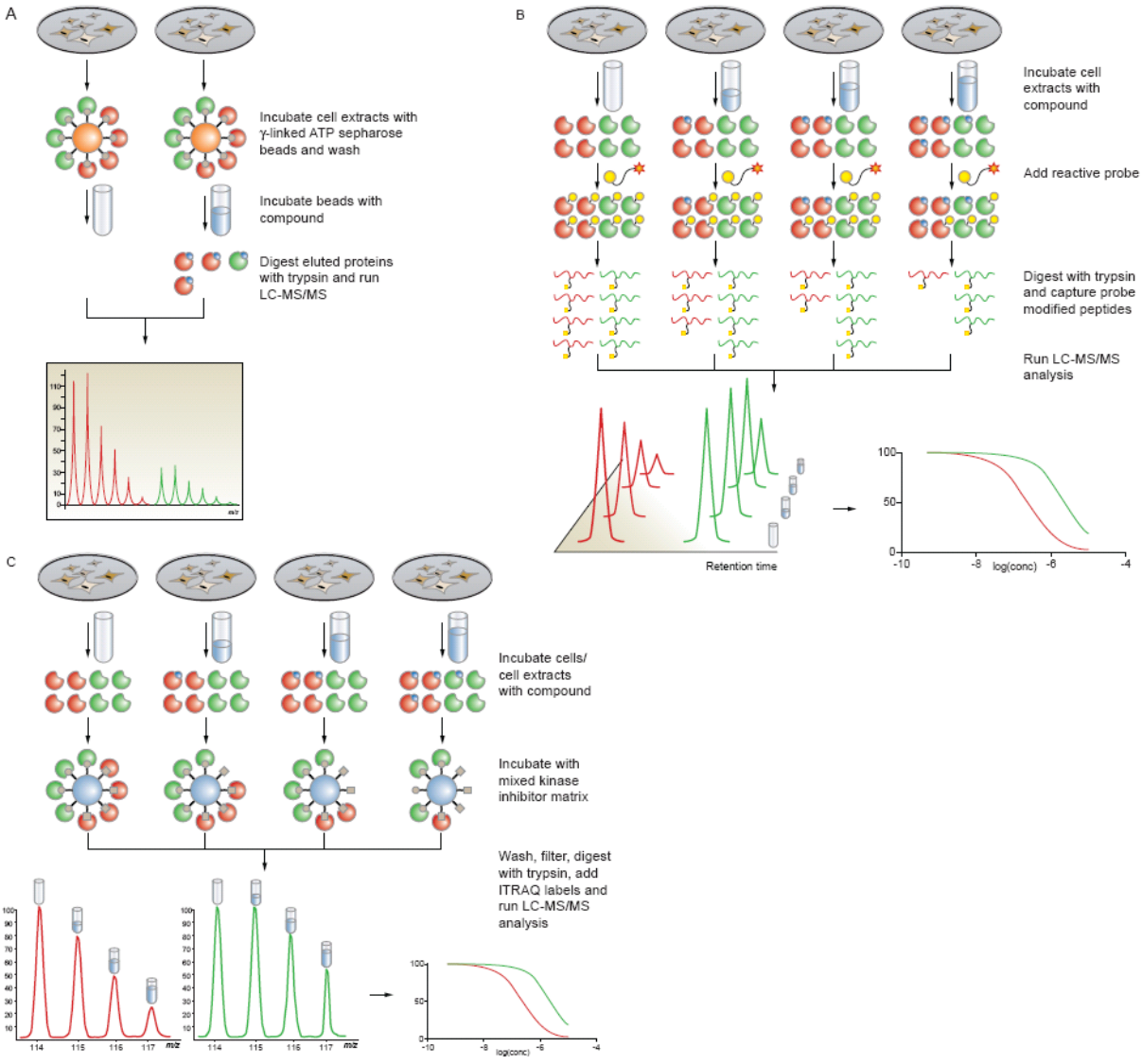
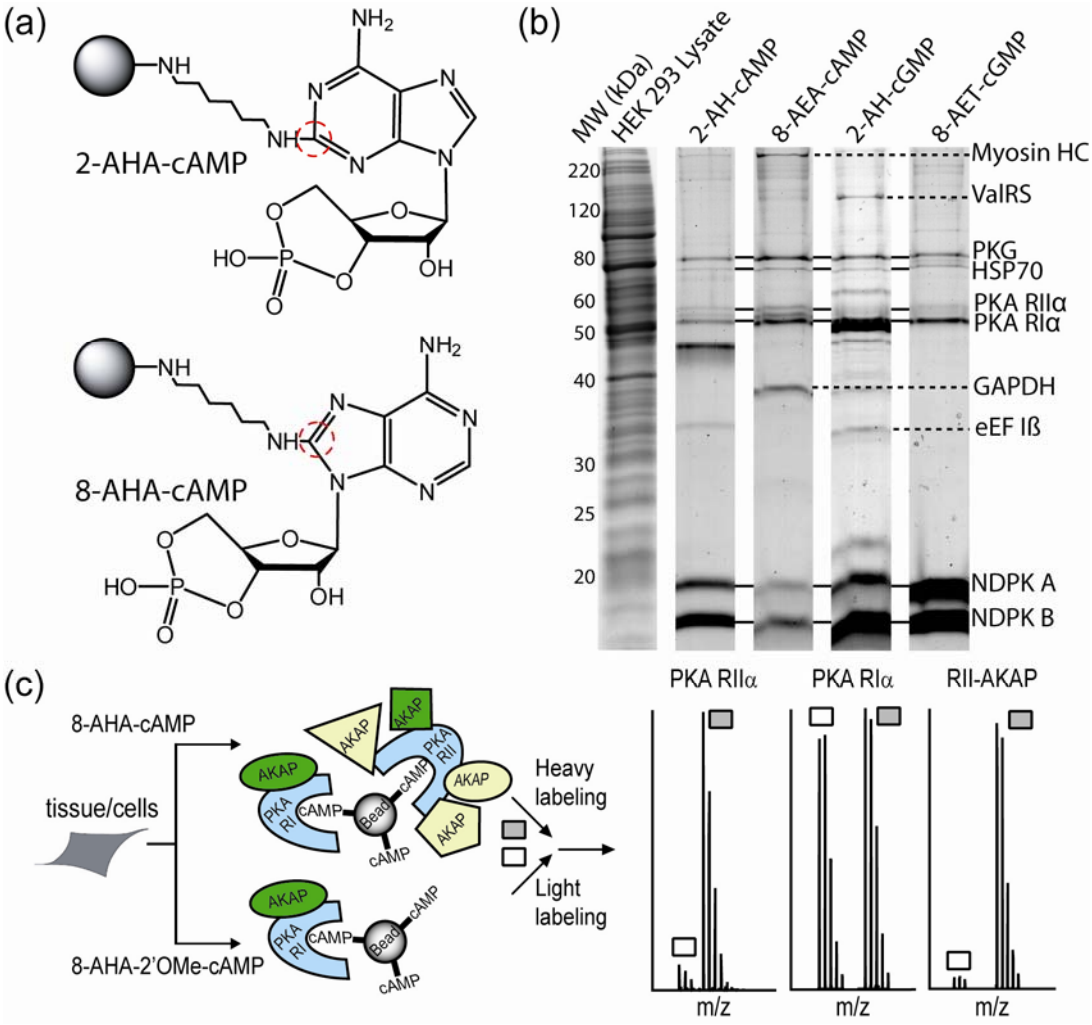

A
Incubate cell extracts with γ-linked ATP sepharose beads and wash
Incubate beads with compound
Digest eluted proteins with trypsin and run LC-MS/MS
B
Incubate cell extracts with compound
Add reactive probe
Digest with trypsin and capture probe modified peptides
Run LC-MS/MS analysis
Retention time
log(conc)
C
Incubate cells/ cell extracts with compound
Incubate with mixed kinase inhibitor matrix
Wash, filter, digest with trypsin, add ITRAQ labels and run LC-MS/MS analysis
m/z
log(conc)

**Figure 3: Revealing signaling modules of cAMP by chemical proteomics.**

**(a)** Coupling of the second messenger cAMP to beads can be attained at different coupling positions using the 1,6-diaminohexyl spacer moiety. **(b)** SDS-PAGE analysis of a pull-down experiment in HEK293 cells shows that different coupling positions and different nucleotides all yield binding of PKA and PKG, however the low-affinity background of each bead type is different as judged from the band pattern [17]. **(c)** Multiplexing 8-AHA-cAMP, which binds PKA RI and RII equally well, and 8-AHA-2'-OMe-cAMP which binds with higher affinity to PKA RI, in a quantitative proteomics experiment. The differential enrichment ratio of PKA RI and RII on both beads is matched by their secondary binders, i.e. A-kinase anchoring proteins (AKAPs), of which the PKA-R subtype specificity could be elucidated. Typical mass spectra from which the enrichment ratios could be calculated.



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