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Revealing promiscuous drug–target interactions by chemical proteomics

Marcus Bantscheff[1], Arjen Scholten[2,3] and Albert J.R. Heck[2,3]

[1] Cellzome AG, Meyerhofstrasse 1, 69117 Heidelberg, Germany
[2] Biomolecular Mass Spectrometry and Proteomics Group, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Sorbonnnelaan 16, 3584 CA Utrecht, The Netherlands
[3] Netherlands Proteomics Centre, Sorbonnnelaan 16, 3584 CA Utrecht, The Netherlands

The (poly-)pharmacological activities of a drug can only be understood if its interactions with cellular components are comprehensively characterized. Mass spectrometry-based chemical proteomics approaches have recently emerged as powerful tools for the characterization of drug–target interactions in samples from cell lines and tissues. At the same time, off-target activities can be identified. This information can contribute toward optimization of candidate drug molecules and reduction of side effects. In this review, we describe recent advances in chemical proteomics and outline potential applications in drug discovery.

Introduction

At present, considerable effort in the pharmaceutical industry is directed at the generation of drugs specifically inhibiting individual members of protein families, such as protein and lipid kinases, histone deacetylases, proteases and heat shock proteins. These efforts are hampered by structural conservation within these enzyme families, which makes the design of mono-selective inhibitors challenging. While for some disease indications, for example cancer, multitarget activities of inhibitors can be tolerated or are even desired [1,2], identification of inhibitors with optimal selectivity profiles is a challenging task. In this context it is important to point out that, off-target activities of therapeutics are not necessarily restricted to the target protein class because inhibitors are often designed as mimetics of ligands commonly used by several different enzyme families, for example ATP. In conventional drug discovery strategies, compound selectivity and off-target liabilities are typically addressed by assay panels comprising a subset of the target protein family as purified recombinant enzymes [3–5]. However, correlating assay results with efficacy in cellular or animal models is often difficult, which can be at least partially explained by the biased nature and the limited physiological relevance of recombinant enzyme assays.

Corresponding author: Heck, Albert J.R. (a.j.r.heck@uu.nl)

Recent developments in chemical proteomics, a multidisciplinary research area integrating biochemistry and cell biology with organic synthesis and mass spectrometry, have enabled a more direct and unbiased analysis of a drug's mechanism of action in the context of 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. Moreover, affinity enrichment strategies using immobilized drugs or tool compounds now allow to characterize the expressed 'interactome' of a drug directly from cell extracts.

In this review, we will outline typical chemical proteomics workflows focusing on affinity-based proteomics technologies using immobilized capture ligands for proteome-wide detection of drug targets and off-target effects. Potential applications of chemical proteomics in the drug discovery process will also be discussed.

Chemical proteomics approaches

Chemical proteomics approaches employ a variety of different experimental procedures. Among the most popular ones, three major experiment types can be distinguished: (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), which employs small-molecular probes to covalently capture a distinct class of proteins and finally

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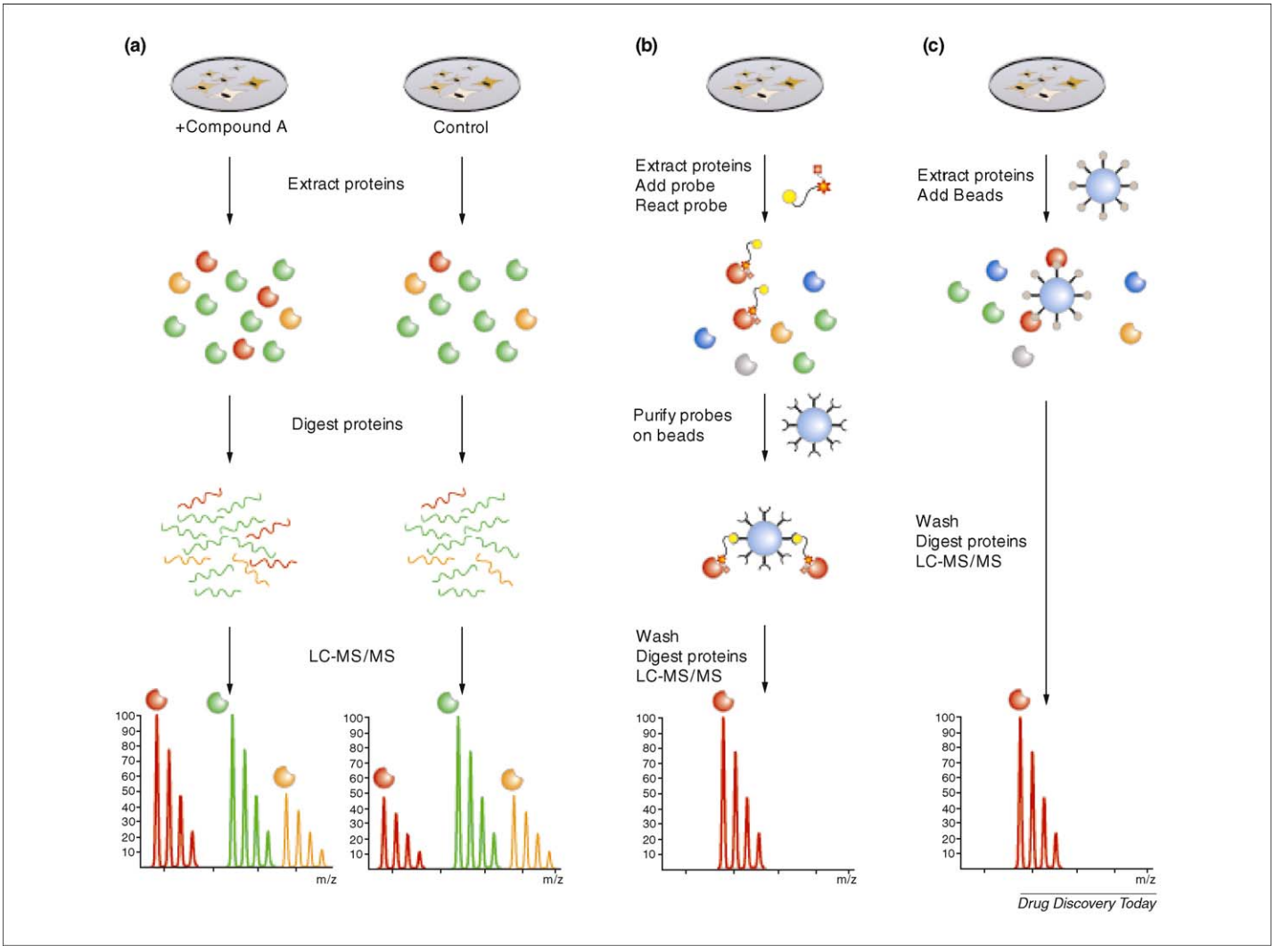

FIGURE 1

Experimental workflows in chemical proteomics. The term chemical proteomics typically encompasses three different workflows. **(a)** Global proteomics approaches at the comprehensive analysis of protein expression or post-translational modifications of a drug treated versus a control cell, tissue or animal sample. After treatment, proteins are extracted from the sample, digested to peptides and analyzed by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). Quantitation is achieved by either stable isotope labeling or spectral counting. **(b)** Affinity- or 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.

(iii) immobilized inhibitors that can be used for affinity enrichment of interacting proteins (Fig. 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 we will briefly describe in this section.

Several studies have recently applied global proteomics approaches to evaluate the cellular response to drug treatment [6–9]. In these approaches, cells or animals are treated with a drug before system-wide proteome analysis to evaluate the cellular response in a global and unbiased fashion. A particular advantage of this strategy is that it is unbiased as the unmodified drug interacts with its endogenous targets. However, because typically no target enrichment is used, the changes that can be observed are limited by the analytical depth of the analysis, and are often restricted to the most abundant proteins. The analysis is further complicated by the fact that proteins found to be changed in abundance are not necessarily part of inhibited signaling pathways but often represent highly abundant proteins involved in stress response and/or housekeeping [9,10]. Hence, identification of direct drug–protein interactions is rarely accomplished by global proteome profiling approaches. In examples illustrating the advantages and limitations of global proteomics approaches, Chen and coworkers [9,11,12] 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. The authors found 27 and 13 proteins to be differentially expressed, most of which can be classified as highly abundant [10]. Yamanaka *et al.* applied a global proteomics approach for toxicological studies in animals [13]. The authors studied the effects of 63 chemical compounds on protein

expression in rat liver after 28 daily dosings and employed statistical methods to detect proteins characteristic for carcinogenicity. Several proteins including lamin A, enzymes like methyltransferases and dehydrogenases, albumin and HSP60 were suggested as biomarkers for prediction systems to detect genotoxic and nongenotoxic carcinogens in short-term bioassays.

In addition to protein abundance, global changes in post-translational modifications, such as phosphorylation, can be evaluated upon drug treatment as reported for key players in the BCR-ABL kinase pathway [8]. In a targeted variation of this approach, Lee and coauthors monitored histone modifications in response to treatment with histone deacetylase (HDAC) inhibitors [7]. In this study, human colon cancer cells were treated with HDAC inhibitors of varying degrees of selectivity followed by a simple pre-fractionation method to enrich for histone proteins. By employing a quantitative mass spectrometry approach, the authors were able to identify HDAC-1 specific histone acetylation patterns and quantify these in response to inhibitor treatment.

A second strategy in chemical proteomics is ABPP. In this approach, soluble small-molecular probes are used to capture a distinct class of proteins to which they can covalently attach through a reactive group. Typically, the reactive probe is fused to an affinity tag, such as biotin, via a spacer. In a first step, the small-molecule probe is incubated with the biological sample and allowed to covalently attach to proteins it has affinity for. Subsequently, the formed probe–protein adducts are captured on a solid support using the affinity tag. For some enzyme classes, for example cytochrome P450 proteins, the reactive group can be tailored in such a way that it only reacts with the protein in its active conformation [14]. Designing such an activity-based probe requires detailed structural information to ensure that the reactive group is in close proximity to 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 the activity of specific target class members in human pathologies [15]. The main advantage of the ABPP approach is its ability to directly assess enzyme activity [14]. The optimization of the specificity of the probe, however, requires a significant amount of time and resources. ABPP is a very powerful means to specifically enrich and characterize drug target classes and could be a valuable tool in drug discovery. For example, fluorescent activity-based probes were reported that enable substrate-free identification of inhibitors of uncharacterized enzymes. By using fluorescence polarization as a read-out, the approach is compatible to high-throughput screening with recombinant enzymes [16]. Similar probes can then be used in a secondary proteomic assay to rapidly determine the specificity of screening hits. Detailed ABPP requirements and target classes have recently been reviewed [17,18] and will therefore not be further discussed in this review.

Using immobilized drugs as affinity chromatography material before mass spectrometric analysis is a third strategy often applied in chemical proteomics. In contrast to ABPP, the compound of interest is first immobilized on a solid support and then incubated with a protein lysate to identify interacting proteins. No reactive group is required to covalently attach to proteins. Although, this approach is as generic as ABPP, the number of target classes addressed with this method is still limited at the moment. The majority of publications in this area have focused on protein kinase inhibitors (see below). In other reports, endogenous signaling molecules such as ATP/ADP [19], phosphatidylinositols [20,21] and cyclic nucleotides [22] have been used for the affinity enrichment of interacting proteins. The use of specific peptide sequences as baits has also been reported [23,24].

In the remainder of this article we focus on the main experimental aspects and recent developments of the last approach, affinity-chromatography-based chemical proteomics.

Experimental considerations in affinity-chromatography-based chemical proteomics

In general, not only the affinity-chromatography-based strategy, but also ABPP, 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 crucially in decreasing the complexity of the 'drug-interactome' before mass spectrometric analysis, thereby allowing the identification of low-abundant proteins, which are generally missed in global proteome analyses. The efficiency of this approach, however, depends 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. Drugs and signaling molecules do not necessarily contain such groups, which makes the synthetic design of the drug–support linker moiety an essential part of chemical proteomics. Special care must be taken that modifications to the molecule do not impede its bioactivity. High-resolution structural data of protein–drug complexes can be helpful to identify which part of the drug molecule is available for chemical modification. The small-molecule derivative made for the linkage to the beads should be tested for its bioactivity in a validated enzymatic assay. Effects of 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 [22]. These immobilized analogs revealed a distinct protein profile in affinity pull-downs. Tanaka and coworkers noted that the linker between the resin and the compound can also affect the binding of affinity-enriched proteins significantly [25].

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.* [26] were able to identify more than a dozen of PKA–AKAP complexes in their affinity pull-down using immobilized cAMP, they did not detect several well-known membrane associated PKA–AKAP complexes.

However, recent reports using immobilized kinase inhibitors showed good coverage of receptor tyrosine kinases [27,28] and Winkler *et al.* reported on affinity capturing experiments of the active γ-secretase complex with a biotinylated transition-state analog inhibitor and optimized detergent conditions [29].

Abundance of the target protein

Sufficient expression of target and potential off-target proteins in the cell line or tissue under investigation is a prerequisite for any successful chemical proteomics experiment. Typically, chemical proteomics experiments are performed from cells expressing the target protein. However, side effects caused by off-target activities (e.g. liver toxicity) might occur in other cell types or tissues expressing a different set of off-target proteins. Hence, comprehensive analysis of off-target activities by chemical proteomics requires profiling of different cell types.

Further, any target proteins of successful drugs are low-abundant proteins that have high affinity (in the nM range) for the compound. However, in affinity pull-down experiments there is competition for binding to the immobilized compound between low-abundant high-affinity binders and high-abundant proteins of lower affinity (in the μM range). In particular, high-abundant proteins such as albumin and hemoglobin are known to have medium affinity for 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, the amounts of individual proteins captured do not represent the affinities of these proteins to the immobilized compound and additional experiments are required to distinguish low-abundant high-affinity interactors from low-affinity abundant ones (see below) [30–34].

Background binding of proteins to the affinity matrix

In addition to genuine target proteins, affinity chromatography also enriches for a large number of 'contaminant' proteins, including those that bind nonspecifically, or with low affinity to the matrix or the linker molecule used (background proteins). To a certain degree, the level of these background proteins can be reduced by using more stringent purification methods. However, this can also remove lower abundant specific proteins and their secondary interactors that is proteins interacting with the drug target. Recently, Trinkle-Mulcahy *et al.* [35] identified a large set of proteins that bind nonspecifically to several commonly used affinity matrices, revealing important differences that might 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 for example inactive analogs of the compound of interest [36]. 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 too expensive to have inactive structural analogs, hence other strategies are required to limit, or identify, background binding. To reduce background binding, beads can be precleared with specific washing steps [37]. Yamamota and coworkers [38] introduced an elegant serial affinity chromatography strategy for distinguishing specific from nonspecific binding. First the lysate was incubated with the immobilized ligand beads, which were then removed. Freshly immobilized beads were then incubated with the 'left-over' lysate. Both resins captured the same amount of nonspecific high-abundant background proteins, while the first resin was enriched for the more specific target(s) [39]. 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, for example using quantitative mass spectrometry, target proteins can be identified by their reduced abundance on the affinity matrix in the presence of the free compound [19,27,40]. 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 nonspecific binders.

Target identification of protein kinase inhibitors by chemical proteomics

Protein kinases are well-known drug targets in oncology but their relevance is also increasingly recognized for a variety of nononcology diseases, such as inflammation, autoimmune disease [41–43], metabolic disorders [44] and central nervous system disorders [45,46]. It is estimated that the human genome encodes 518 protein kinases and more than 2000 other ATP- and purine-binding proteins [47,48]. The ATP-binding pocket of kinases is formed by a conserved arrangement of secondary structure elements and is readily accessible to small-molecule inhibitors. Most kinase inhibitors discovered to date are ATP competitive [49]. However, the structural conservation of the ATP-binding site makes synthesis of selective small-molecule ATP-competitive kinase inhibitors 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 enrichment of target proteins using immobilized inhibitors

Affinity chromatography using immobilized inhibitor molecules has been demonstrated to be a powerful tool for the detection of off-targets of kinase inhibitors [30–32,36,50]. As an example, Godl and coworkers used an immobilized analog of the PDGFRβ inhibitor SU6668 to detect kinase off-targets. Several protein kinases were found to bind to the immobilized SU6668 derivative 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 the inhibition of AurA and that SU6668 potently suppressed antiviral and inflammatory responses by interfering with TBK1-mediated signal transmission, thereby indicating the relevance of their findings [31]. In a recent study, a linkable version of imatinib, a block buster drug for the treatment of chronic myeloid leukemia (CML), and two second-generation drugs nilotinib and dasatinib were analyzed for their target spectrum in affinity precipitation experiments [34]. Originally developed for the inhibition of the fusion oncoprotein BCR-ABL, the biochemical hallmark of CML, imatinib is known for its remarkable selectivity for ABL, KIT and PDGFR [51,52]. In the study, strong binding of immobilized imatinib to the quinone oxidoreductase NQO2

was observed, which was recently confirmed by a crystal structure of the imatinib–NQO2 complex [53]. For the close analog nilotinib DDR1 and ARG were identified as additional target proteins. Further, dasatinib developed as a dual-specificity ABL- and SRC-family kinase inhibitor [54] was found to bind a total of 24 protein kinases in experiments done with K562 cells.

Competition-binding approaches

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 [30–33]. 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 cofactors [19]. Proteins interacting with several antimalarial compounds were 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 (Fig. 2A). In a variation of this approach, Patricelli *et al.* [40] 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 (Fig. 2B).

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 [55]. 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 [27]. These mixed kinase inhibitor beads have been used to specifically enrich for a large fraction of the kinome expressed in various cell types; for example using LC–MS/MS analysis, a total of 173 and 179 protein kinases from HeLa and K562 cells could be identified from single experiments [27]. Daub *et al.* used a similar approach purification to analyze the cell-cycle regulation of protein kinases [28]. A total of 219 protein kinases were identified and quantified from S and M phase-arrested human cancer cells. It should be noted that in this study approximately 1000 kinase phosphorylation sites were identified of which many were differentially regulated in mitosis. This indicates that target class selective enrichment strategies can be an important tool for signal transduction research and disease biology in general [27,28,55].

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 [27,56,57]). 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 (Fig. 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 CML; the phase II compound SKI-606 and the marketed drugs imatinib and dasatinib [27]. 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). By 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 biochemical assays. 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 [58]. When combined with phosphorylation specific enrichment strategies, this approach further allows to distinguish 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 as a result of the reduced signaling by the target kinase. In the case of imatinib-treated K562 cells, RSK3 was identified as one of the nine proteins exhibiting a significant downregulated phosphorylation state [27]. Hence, the combination of target class specific enrichment and competition-binding experiments can be a valuable approach to assess target specificity of inhibitor molecules and, at the same time, to gain insights about downstream signaling events affected by these inhibitors.

Cyclic nucleotide regulated signaling studied by chemical proteomics

As an alternative to ATP mimetics, kinase inhibitors can also be designed to bind to allosteric and cofactor binding sites. Hence, chemical proteomics approaches to specifically enrich, for example cofactor binding proteins could be valuable for assessing target selectivity of non-ATP competitive inhibitors.

In a recent study, Scholten and coworkers investigated the specific interactome of four differently immobilized cyclic nucleotides (2AHA-cAMP, 8AEA-cAMP, 2-AH-cGMP and 8AET-cGMP, Fig. 3a) to assess cAMP-dependent protein kinase (PKA) targeting efficiency of the beads, as well as to evaluate cross-reactivity.

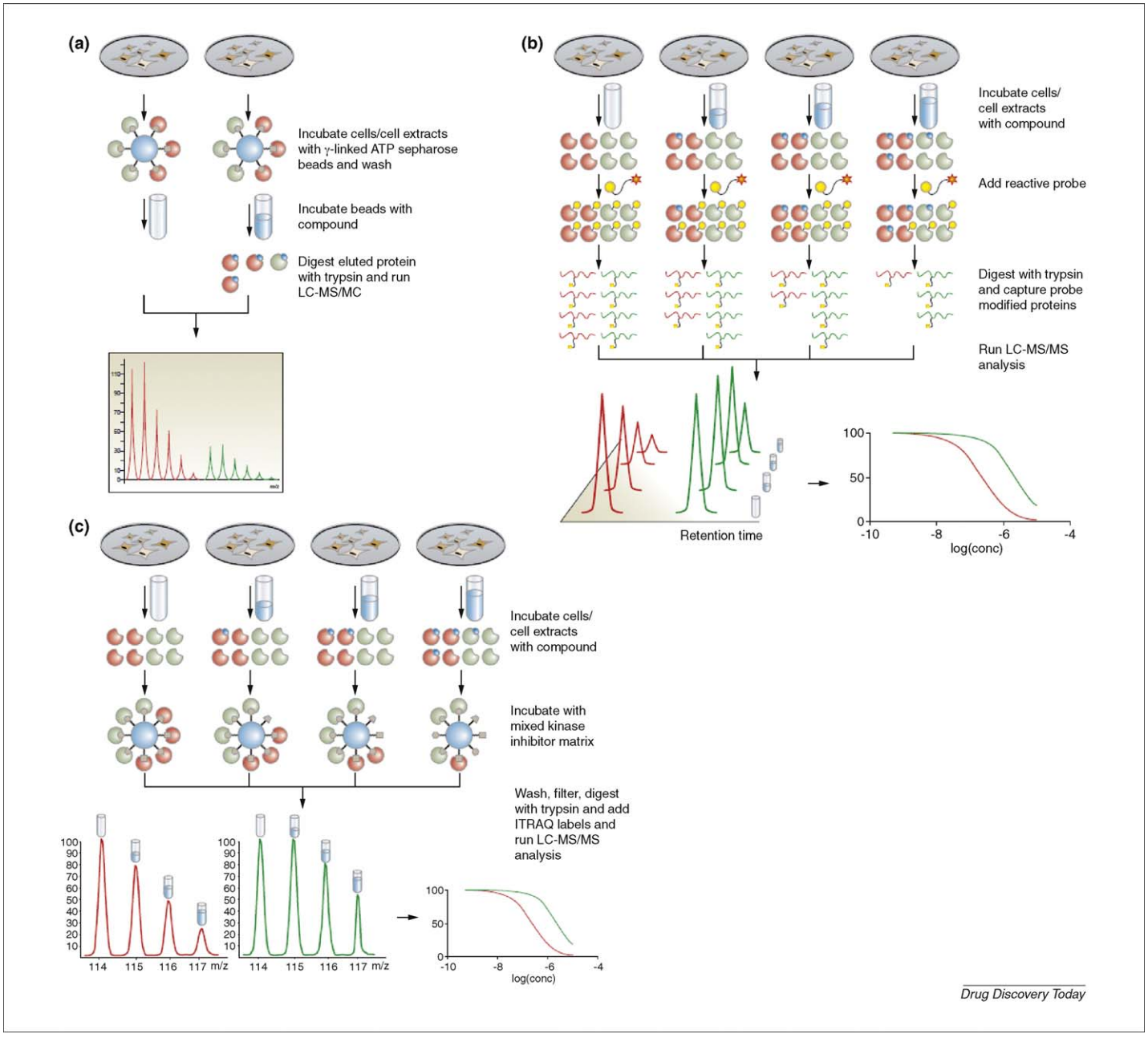

FIGURE 2

Competition-binding approaches employed in chemical proteomics. **(a)** γ-ATP approach [19]. Cell extracts are incubated with γ-ATP-linked sepharose beads. Target proteins are identified upon incubation of the beads with the compound of interest and subsequent analysis of eluted proteins by mass spectrometry. **(b)** affinity- or activity-based protein profiling approach using acyl phosphate containing nucleotides [40]. Cell extracts are first incubated with increasing amounts of inhibitor compound and subsequently 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 the detection of target potencies of inhibitor to identified ATP-binding proteins. **(c)** Mixed kinase inhibitor matrix [27]. 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 (e.g. iTRAQ, TMT) enabling relative quantification in the subsequent mass spectrometric analysis. Target kinases of the tested inhibitor compound will display a dose-dependent reduction in binding.

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 [37]. These off-targets do not specifically bind to cAMP, but to NADP(H) and with lower potency to all diphosphate nucleotides. By incubating the beads with ADP, less specific binders could be separated from the lower abundant, cAMP interactome [37]. 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

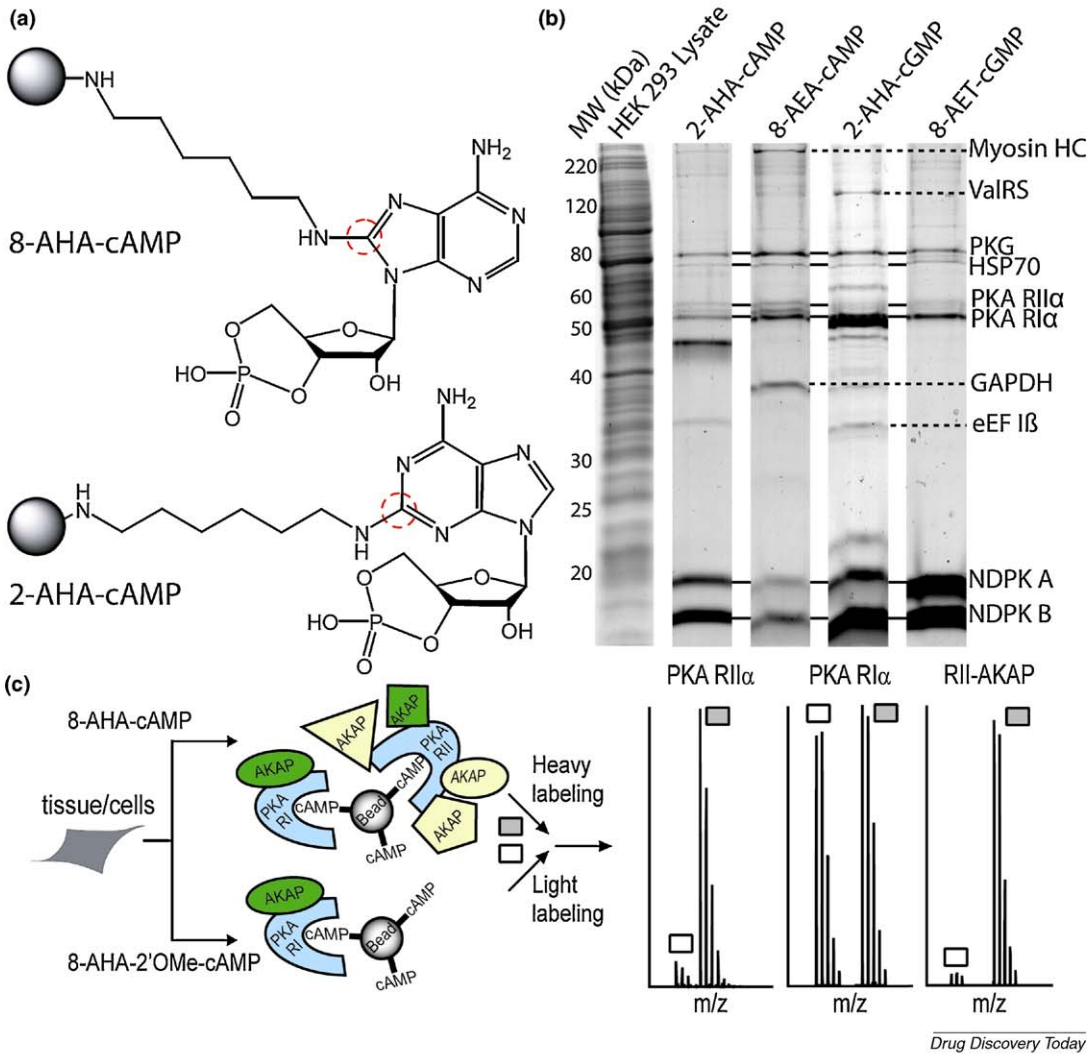

FIGURE 3

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 binding of each bead type is different as indicated by the band pattern [22]. **(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 that is 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 are shown.

specificity of the beads [22]. 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 [26]. 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 toward the modifications of cyclic nucleotides in the 8-position, hence they were only captured with the 2-position type beads [22]. Aye *et al.* discovered that a substituted cAMP analog (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 [26].

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 multiprotein signaling complexes [59]. Using the immobilized cyclic nucleotide approach, the association of PKA with many different A-kinase anchoring proteins (AKAPs) could be evaluated in different tissues [22,26,37]. This approach was combined with quantitative mass spectrometry to screen a selection of AKAPs for their PKA-R specificity (Fig. 3c) [26]. Because of 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 [60].

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 inhibitors as reviewed above, but the methodology is not limited to this class of compounds. Phosphoinositide-binding proteins have also been the subject of several studies [20,21]. Phosphoinositide-binding proteins influence many crucial processes in eukaryotic cells, including signaling by cell-surface receptors and vesicle trafficking. Krugmann *et al.* [21] used affinity matrices carrying analogs of phosphoinositides to capture phosphoinositide-binding proteins from cell and tissue lysates and were able to identify several phosphoinositide-binding proteins. More recently, Pasquali *et al.* [61] 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 interactions might be preserved during affinity purifications. Related to these studies Gharbi *et al.* [20] used an immobilized analog of a known PI3K inhibitor (LY294002), and revealed that this inhibitor was promiscuous and interacted with several other kinases such as CK2, Pim-1 and DNA-PK.

Future outlook: chemical proteomics and drug discovery

Notwithstanding the promising results reported in the past decade, chemical proteomics approaches are not yet regarded as a standard element of assay cascades generally employed in drug discovery. This might be in part because of the very specialized and expensive mass spectrometric equipment required and the relatively high costs per data point compared to conventional enzyme assays. For example, in kinase drug discovery, researchers can choose from a large variety of biochemical assays to assess the selectivity of kinase inhibitors in development. Nowadays, cost-effective selectivity profiling of compounds can be performed against panels exceeding 300 protein kinases (companies offering such services include Invitrogen, Upstate, Ambit). Apart from traditional biochemical kinase assays employing recombinant proteins and substrate peptides, a variety of new methods have emerged including competition-binding assays using phage tagged kinases [4,62,63]. However, the application of recombinantly expressed kinases bears significant limitations. Recombinant kinases typically employed in screening panels almost always include just the kinase domain, rather than the full-length protein. Further, proteins overexpressed in bacteria and insect cells are unlikely to exhibit the proper state of post-translational modifications, and might even be partially mis-folded or denatured. *In vivo* kinase activity is regulated by post-translational modifications, cofactor 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. This is for example illustrated by the controversy about potential inhibition of the Bcr-Abl T315 mutant by the p38 inhibitor BIRB-796 that could not be confirmed in cellular assays [62,64]. The recent advances in chemical proteomic technologies described allow researchers to determine the binding of inhibitors 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 because of 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. Because of 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, for example to study drug resistance in patient cells caused by mutations of the target protein.

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