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Review

Mass Spectrometry-Based Proteomics in Preclinical Drug Discovery

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Preclinical stages in the drug discovery process require a multitude of biochemical and genetic assays in order to characterize the effects of drug candidates on cellular systems and model organisms. Early attempts to apply unbiased proteomic techniques to the identification of protein targets and off-targets as well as to elucidate the mode of action of candidate drug molecules suffered from a striking discrepancy between scientific expectations and what the technology was able to deliver at the time. Dramatic technological improvements in mass spectrometry-based proteomic and chemoproteomic strategies have radically changed this situation. This review, therefore, highlights proteomic approaches suitable for preclinical drug discovery illustrated by recent success stories.

Introduction

The majority of small molecule drugs and biologics act on protein targets. These proteins do not act in isolation but are embedded in cellular pathways and networks and are thus tightly interconnected both physically and functionally with many other proteins and cellular components. In addition, the several hundred different cell types that make up the organs of a human being constitute different physical and functional contexts in which proteins exist and on which drugs may therefore act with desirable or undesirable consequences. Given this complexity, it seems natural to apply proteomics in the drug discovery process in order to understand the effects of drug candidates on their protein targets and shed light on the cellular mechanisms resulting in the observed phenotype. The success of proteomics in basic biology research has been striking. However, similar to the early genomic promises, expectations toward proteomics in drug discovery were often higher than what the technology was able to deliver at the time. Early applications in this area were largely confined to measuring global effects of drugs on protein expression with little direct information on the mechanisms by which the observed effects were generated. At the same time, the technology was underdeveloped because it suffered from limited analytical depth and quantification capability. Over the last 15 years, proteomic technology has made dramatic progress in several areas (Mallick and Kuster, 2010). The introduction of separation and analytical strategies including multidimensional liquid chromatography of peptides coupled to high-performance tandem mass spectrometry (Graumann et al., 2008; Olsen et al., 2005, 2009; Washburn et al., 2001) led to a dramatic increase in the depth of sampling of a given proteome. Furthermore, a robust quantitative dimension was added to mass spectrometric measurements by stable isotope labeling (Ong et al., 2002; Ross et al., 2004; Thompson et al., 2003) or so-called label-free techniques (Bantscheff et al., 2007b). In addition, tremendous improvements have been made in the large-scale analysis of low molecular weight posttranslational modifications (PTMs). Examples include phosphorylation (Beausoleil et al., 2004; Olsen et al., 2006) and acetylation (Choudhary et al., 2009), which both play a crucial role in regulation of protein activity, stability, as well as protein interactions.

The ability of state-of-the-art proteomics to measure the changes of proteins and their various isoforms quantitatively to a depth of 5–10,000 proteins and across 4–6 logs of dynamic range in abundance makes it, in our eyes, an important tool at various stages during small molecule drug discovery (Beck et al., 2011; Nagaraj et al., 2011). Currently, drug discovery efforts typically follow one of two strategies that differ in the way they lead to compound selection and optimization (Figure 1). Target-based approaches start with the selection of a protein target based on its presumed or validated role in the relevant disease. Biochemical or biophysical assays, typically using purified protein, are developed to monitor modulation of target activity and to identify hits in high-throughput screens (HTSs) using large libraries of small molecules. After hit validation, lead compounds are selected and further optimized with regard to potency, selectivity, pharmacodynamic and pharmacokinetic properties, and tested for in vivo efficacy in the respective disease model. Recently, phenotypic screening regimen in general and genetically designed pathway-centric approaches in particular have (re)gained popularity in drug discovery because the conditions of such screens resemble more closely the physiological situation compared to assaying a target in isolation (Fishman and Porter, 2005). Here, a cellular assay is used for screening a small molecule library using a cellular response readout such as cytokine release, cell death, or pathway activity (e.g., the phosphorylation status of a signaling protein or reporter gene activity). The targets of hits generated in this way are initially unknown. Therefore, a phenotypic screen is typically followed by target deconvolution (for a definition see below and Box 1), target validation, and elucidation of the mode of action by which the small molecule hit exerts its pharmacological effect.

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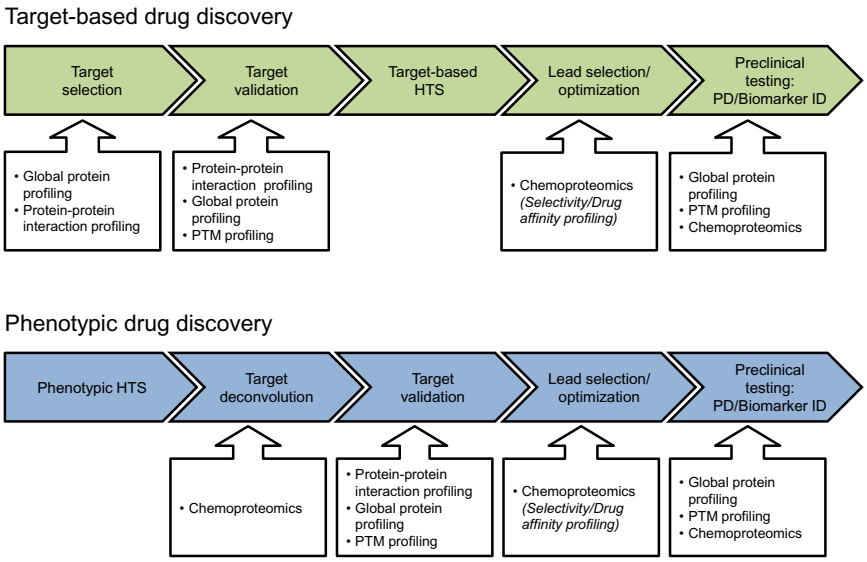

Figure 1. Applications of Proteomics at Different Stages in the Drug Discovery Process

Overall goals of these applications are similar for both traditional, target-based drug discovery (upper panel) and phenotypic, chemical genetics-based drug discovery (lower panel), but there are differences in when they are applied during the process. ID, identification.

For both target-based and phenotype-based workflows, proteomics enables a multitude of investigations relevant to the different steps and addressing different questions in the process (Figures 1 and 2). These applications can be roughly grouped into (1) those characterizing direct or indirect drug-target interactions for target deconvolution and selectivity profiling, (2) those aimed at elucidating the mechanism of action (MoA) by which a drug exerts its pharmacological effect, target characterization, and validation, and (3) those aimed at the identification of biomarkers that can be used for monitoring the effect of target modulation in an in vivo setting. Due to space constraints, we focus in this review on the contributions of mass spectrometry-based proteomics to the preclinical stages of small molecule drug discovery. For clinical applications of proteomics including biomarker discovery, we refer the interested reader to a number of reviews that have been published on the topic (Rifai et al., 2006; Ioannidis, 2011).

Characterization of Drug-Protein Interactions

Target Deconvolution and Selectivity Profiling

We use the term target deconvolution to describe proteomic experiments aiming at identifying the full spectrum of (protein) targets associated with a bioactive molecule and the cellular phenotype it induces. This level of compound characterization has not traditionally been done in drug discovery in part because it was not technically possible. The value of this information is, however, quite obvious. Small molecule drugs should be expected to bind to more than one protein, and there is evidence that polypharmacology is indeed more often the case than not. In a physiological context, binding to or activity modulation of more than one protein may have desirable or undesirable consequences. Knowledge about the spectrum of proteins interacting with a small molecule could inform early on about drug safety (by the identification of potential toxicity targets), help in the decision making along the development process of a small molecule (e.g., which lead series to prioritize), and even lead to the repositioning of existing drugs (e.g., by the identification of additional targets). It may, therefore, be argued that detailed target deconvolution ought to be part of every drug discovery project.

For target-based drug discovery the protein target is nominated before screening (e.g., based on a known mechanistic role in disease or on the correlation of factors such as gene copy number, mutational status, or levels of RNA or protein with disease status). Because the target proteins are usually expressed in recombinant form and screened against a large/focused library of test compounds using their enzymatic activities, target deconvolution primarily means establishing target selectivity (i.e., which other proteins of the same or different protein classes are bound/inhibited/activated by the small molecule?). In part this can be achieved using panels of enzyme assays (e.g., for kinases, proteases, GPCRs, ion channels, P450 enzymes, etc.), but these assays cannot identify unexpected drug-target interactions. Target deconvolution in the context of phenotypic screens is quite different. These screens are unbiased in the sense that they do not focus on traditional target classes and assay types. Instead, the screens focus on the desired cellular phenotype that, mechanistically, may be due to modulation of any protein within the probed signaling pathways. In a nutshell, the protein target responsible for the observed effect is unknown, which, not surprisingly, complicates or even precludes the chemical optimization of screening hits (Terstappen et al., 2007). Target deconvolution in this context, therefore, primarily needs to establish the efficacy target. In addition to the enzyme panels mentioned above, a variety of unbiased techniques can be employed for this purpose including in silico target prediction and genetic and transcriptional profiling (e.g., haploinsufficiency profiling; Giaever et al., 1999, 2004; for a recent review see Cong et al., 2011). Alternatively, affinity-based techniques can be employed as a strategy for measuring drug-target interactions directly. Commonly used methods here include yeast or mammalian three-hybrid systems, phage display, and chemoproteomics (Rix and Superti-Furga, 2009; Terstappen et al., 2007). Chemoproteomic target deconvolution is based on classical drug affinity chromatography pioneered by Schreiber and colleagues in their seminal work on the identification of molecular targets of immunosuppressants (Brown et al., 1994; Harding et al., 1989) and inhibitors of histone deacetylation (Taunton et al., 1996). Nowadays, the affinity purification is typically followed by mass spectrometry for protein identification and quantification. The main applications of chemoproteomic target deconvolution can be grouped into two categories: (1) drug-centric profiling, and (2) binding mode-centric profiling (selectivity profiling).

Box 1. Glossary of Terms	
ADME	Appropriate properties in absorption, distribution, metabolism, and excretion (ADME) are required to make a bioactive compound an effective drug.
Drug development	Preclinical and clinical studies required to establish drug safety and efficacy.
Drug discovery	Process by which drugs are designed or discovered.
Drug target deconvolution	Process of identifying target molecules of bioactive (small) molecules, e.g., for compounds active in a phenotypic screen.
Mechanism of action (MoA)	Mechanism by which a drug exerts its pharmacological effect.
Off-target	Additional targets whose modulation is not or not necessarily related to the desired phenotypic response.
Pharmacodynamic biomarker	Marker of a pharmacological response monitored in dose optimization studies, e.g., substrate phosphorylation state of kinase targets.
Pharmacodynamics	Study of the concentration dependence of biochemical or physiological effects of a drug on an organism.
Pharmacokinetics	Study of ADME properties of a drug as a function of time, in particular the rate at which a drug action begins and the duration of the effect.
Phenotypic drug discovery	Drug discovery based on phenotypic screens in which libraries of small molecules are tested for their ability to exert a desired phenotypic response in a cellular assay, such as cytokine release, cell death, or transcriptional activity of engineered reporter genes.
Target	Molecular entity expressed in a cell or organism whose structure or function is directly modulated by drug binding, thus leading to a desired phenotypic/therapeutic response.
Target-based drug discovery	Design of therapeutics specifically modulating the function of a distinct validated target.
Target validation	Process aiming to demonstrate that modulation of the target will have the desired therapeutic effect (in vivo).
Therapeutic index	Comparison of the drug concentration required to achieve the desired therapeutic effect to the drug concentration that causes toxicity or death.

Drug-Centric Chemoproteomic Profiling

In this approach the bioactive molecule of interest is chemically conjugated to a suitable affinity moiety (e.g., biotin) or immobilized directly on a resin such as Sepharose beads. In both cases, chemical synthesis of a suitable functionalized analog of the compound will generally be required (typically bearing an amine, carboxyl, hydroxyl, or sulfhydryl group). Detailed information on the structure activity relationship (SAR) of a compound is necessary to ensure that the functionalized molecule retains similar target binding and biological activity properties. The resulting affinity probe is then incubated with cell extracts, and bound proteins are identified using mass spectrometry (Bantscheff et al., 2009; Lolli et al., 2003; Oda et al., 2003; Rix and Superti-Furga, 2009). Successful applications of this approach cover a diverse set of target classes such as protein kinases (Bantscheff et al., 2007a; Brehmer et al., 2004, 2005; Daub et al., 2008; Godl et al., 2005; Li et al., 2010; Rix et al., 2007), proteins binding to ATP/ADP (Graves et al., 2002), phosphatidylinositols (Gharbi et al., 2007; Krugmann et al., 2002), cyclic nucleotides (Hanke et al., 2011; Scholten et al., 2006), histone deacetylases (HDACs) (Bantscheff et al., 2011), and tankyrases (Huang et al., 2009). The same idea is, in principle, also applicable to protein therapeutics and other biologics (Geuijen et al., 2005). The interpretation of chemoproteomic experiments is often complicated by the fact that dozens to hundreds of proteins are identified in a typical experiment. Hence, additional evidence is required to distinguish genuine high-affinity target proteins from low-affinity but highly abundant proteins. For example, albumin and hemoglobin are known to have low affinity for a range of small molecules, and many NADH/NADPH binding proteins also bind to immobilized ATP mimetics (Brehmer et al., 2004, 2005; Godl et al., 2005; Remsing Rix et al., 2009; Rix et al., 2007). In addition, proteins might bind to the resin itself or to additional groups introduced to a compound for probe generation (e.g., linkers, reactive groups, biotin, etc.). Such proteins are detected frequently in independent experiments using different probe matrices and are often simply neglected in the further analysis (Trinkle-Mulcahy et al., 2008). A more elaborate strategy to avoid false-positive target deconvolution results is to design active and inactive analogs of the affinity probe (Oda et al., 2003). Experiments with both matrices are then performed in parallel, and candidate target proteins can be short-listed on the basis of differential purification. However, inactive analogs of candidate molecules are often not available, and synthesizing additional such probes is laborious. Competition binding experiments provide a simple but very efficient alternative to address false-positive target deconvolution. Here, the affinity probe is incubated with the cell extract in the presence or absence of the original (i.e., unmodified) bioactive compound. Genuine target proteins show significantly reduced binding to the resin in this experiment compared to vehicle control, whereas nonspecific binders do not. Technically, the reliability of the results of such competition binding assays hinges on a number of parameters. The abundance of the target protein and its affinity to the small molecule are the two most important biochemical determinants of the observable competition. In particular the rate at which proteins dissociate from the immobilized compound (k_{off}) determines how much of a protein can be recovered given the time required to perform the affinity purification. For a high-abundance target, interactions with dissociation constant (K_D) values

of up to low 40 μM have been characterized successfully (Ong et al., 2009). More generally though, de novo target deconvolution experiments typically require compounds with cellular potencies in the submicromolar range because most signaling proteins tend to be of rather low abundance. The precision and accuracy of the mass spectrometric protein quantification are other important factors in these experiments. Here, the use of stable isotope labeling such as SILAC (Ong et al., 2009) or isobaric labeling tags (Bantscheff et al., 2007a, 2011; Borawski et al., 2009; Burgett et al., 2011; Huang et al., 2009) has improved the data quality significantly in recent years and now enables measurements within 20% CV (coefficient of variation).

As an alternative to the use of reversible binders, reactive probes can be employed that covalently attach to a target protein active site (commonly known as ''activity-based protein profiling'' [ABPP], reviewed by Cravatt and coworkers in this issue of *Chemistry & Biology*) (Cravatt et al., 2008; Nomura et al., 2010; Sadaghiani et al., 2007). Similarly to the aforementioned methods, the bioactive molecule of interested needs to be chemically modified to enable the reaction of the active site probe with suitable amino acid residues within or close to the enzyme catalytic site. This is attractive because the covalent nature of the binding has the potential to overcome the affinity issues often encountered with reversible binders. In addition, chemical probes for particular target classes can be designed. However, significant chemical effort is required in defining a chemical probe that is sufficiently similar to the bioactive molecule under investigation. This is likely a main reason why reactive probes have been primarily reported for binding mode-centric profiling rather than compound-centric chemoproteomics (see section below). A variation on this theme that aims at streamlining probe development employs trifunctional probe designs that consist of (i) a selectivity group that can reversibly interact with a particular target protein or target class, (ii) a common reactive group that stabilizes the interaction, and (iii) a common sorting function (e.g., biotin) that allows purification of the drug-target complex (Köster et al., 2007). Despite synthetic challenges, reactive probes for photolabeling (using, for example, diazirine or benzophenone moieties) are of particular interest for target proteins that are less amenable to simple affinity enrichment such as G protein-coupled receptors and other integral membrane proteins (Dubinsky et al., 2011; Tantama et al., 2008).

For all affinity/activity-based methods described thus far, chemical synthesis of suitably functionalized analogs is the rate-limiting step. To overcome this, recent reports suggest that structural changes in target proteins induced by binding of the free (unmodified) bioactive compound may be directly interrogated by mass spectrometry. The method of ''drug affinity responsive target stability'' (DARTS) introduced by Lomenick et al. (2009) takes advantage of a local or global reduction in the protease susceptibility of a target protein upon drug binding. For example, thermolysin digestion of resveratrol-treated yeast and human cell lysates revealed stabilization of wild-type eIF4A, but not the A64Q eIF4A mutant protein, suggesting that the protein translation machinery may be a molecular target of resveratrol in life span extension. Another method in this group is ''Stability of Proteins from Rates of Oxidation'' (SPROX), which measures the thermodynamic stability of proteins and protein-ligand complexes by measuring hydrogen peroxide-induced protein oxidation as a function of denaturant concentration (e.g., guanidinium hydrochloride) (West et al., 2008) or heat denaturation (West et al., 2010a). Measurements in the absence or presence of ligand thus enable evaluation of protein-ligand affinities. The potential of such methods has been highlighted recently by the simultaneous assaying of the protein-folding and ligand-binding properties of 327 proteins in a yeast cell lysate using the immunosuppressive drug cyclosporin A (West et al., 2010b).

The impact of drug-centric chemoproteomic profiling for target deconvolution has been rapidly increasing lately. For example Fleischer et al. used affinity-based proteomics to delineate nicotinamide phosphoribosyltransferase as the target of the potent and selective cytotoxic agent CB30865. This enzyme is a member of the NAD biosynthetic pathway that helps cancer cells to sustain their increased energy metabolism (Fleischer et al., 2010). Following a HTS using a Wnt-responsive reporter assay, Huang et al. used chemoproteomics to identify tankyrases as the targets of the small molecule hit XAV939. This experiment validated tankyrases as tractable targets in the Wnt signaling pathway, which plays an important role in the development of colorectal cancer (Huang et al., 2009). Raj and colleagues used a SILAC-based chemoproteomic approach to identify several enzymes of the cellular stress response to reactive oxygen species (ROS), including glutathione S-transferase pi 1 (GSTP1) and carbonyl reductase 1 (CBR1), as interactors of the natural product piperlongumine. In contrast to other compounds increasing ROS levels such as paclitaxel, the authors found that the increase in ROS levels and apoptotic cell death caused by piperlongumine is restricted to cancer cells, whereas no effect was observed in normal cells. Biochemical and cellular data indicate that this effect is at least partially mediated by the putative targets GSTP1 and CBR1. These results suggest an approach to cancer therapy exploiting the increased dependence of cancer cells on the ROS stress response pathway (Raj et al., 2011). Nicodeme et al. performed cell-based screening for inhibitors 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 MoA 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 (Nicodeme et al., 2010). Bradner and coworkers recently demonstrated that the small molecule compound JQ1 displaces BET proteins from the chromatin and that this compound is efficacious in patient-derived xenograft models of squamous carcinoma carrying a recurrent translocation of BRD4 (Filippakopoulos et al., 2010). Dawson et al. applied a multitier proteomic strategy to characterize BET-dependent histone binding of various protein complexes including the super elongation complex (SEC) and the polymerase-associated factor complex. These data provided the basis for therapeutic intervention in MLL-fusion leukemia via the displacement of the BET family of proteins from chromatin, and in vivo studies with the lead compound I-BET151 demonstrated a marked survival benefit in two distinct mouse models of murine MLL-AF9 and human MLL-AF4 leukemia (Dawson et al., 2011).

Taken together, these recent success stories demonstrate that chemoproteomic approaches not only enable the

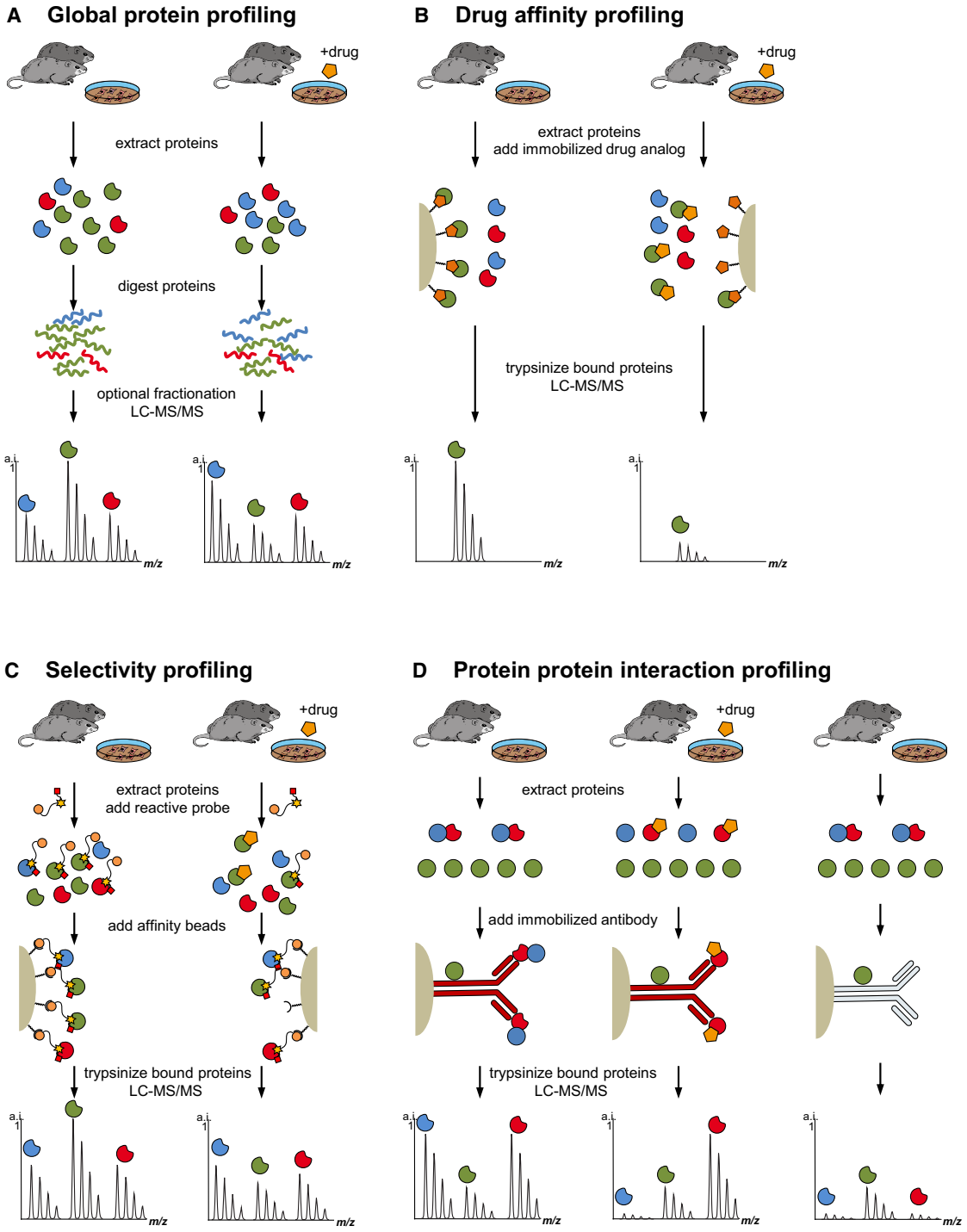

Figure 2. Schematic Representations of Exemplary Proteomic Workflows Addressing Different Questions Related to Drug Discovery
In all cases, relative protein quantification is achieved by quantitative mass spectrometry using either stable isotope labeling or label-free methods.
(A) Global protein profiling and its variant PTM profiling aim at the comprehensive analysis of protein abundance and levels of PTMs of proteins in a drug-treated sample versus a control cell (tissue) sample or test animal. After treatment, proteins are typically extracted from the sample, digested into peptides, and analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). For PTM profiling, a PTM-specific enrichment step is typically included.
(B) Drug affinity profiling for target deconvolution using *Drug-centric chemoproteomic profiling* based on an immobilized bioactive molecule. The specificity of protein binding to the drug matrix is probed by competition with the free drug. The abundance of target proteins of the drug (green) is reduced in experiments performed in the presence of excess concentrations of the free drug.
(C) Selectivity profiling using *Binding mode-centric chemoproteomic profiling*, here exemplified by a reactive probe-based strategy. Probes are designed to enable binding and subsequent purification of a whole class of protein targets (e.g., kinases) from cell extracts. In a second step, probes and bound targets are

identification of direct targets of bioactive molecules but, at the same time, also provide insight into regulatory mechanisms depending on protein-protein interaction rather than single proteins. This extends the classical target definition to protein complexes. Indeed, selective targeting of protein complexes was recently demonstrated in a chemoproteomic study on HDAC inhibitors (Bantscheff et al., 2011). Here, a competition binding approach was applied to the profiling of inhibitors binding to native megadalton HDAC complexes in cell extracts. 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, benzamide inhibitors did not bind to the SIN3 repressor complex, but both benzamide and hydroxamate-type compounds efficiently inhibited the NuRD and Co-Rest complexes.

It should be noted that, strictly speaking, chemoproteomic experiments generate target *hypotheses*, in particular when using noncovalent chemical probes. These putative targets may often be ranked by affinity or correlation with potency within an SAR series of molecules of the same chemical scaffold. However, formal proof for direct compound-protein binding still needs to be provided, for example using purified protein and biophysical methods such as surface plasmon resonance or isothermal titration calorimetry. Moreover, direct interaction still does not necessarily prove involvement of a target in the desired phenotype. It may also represent an undesired (or no) effect or indicate involvement of this protein, e.g., in compound metabolism. In order to establish a functional relationship, bioinformatic analysis for a connection of the putative target to the observed cellular response is often used as a first-pass approach to prioritize a list of potential protein targets for follow-up. For many (but by far not all) enzymes, biochemical assays with purified protein can be established to verify modulation of activity by the compound. The most important step is, however, the functional validation in vivo. This typically includes experiments that aim to phenocopy the compound treatment by modulating the protein genetically (e.g., by RNA interference or cDNA overexpression) and by using known inhibitors (if available) of the putative target. If genetic and pharmacological intervention leads to the same phenotype, the target hypothesis is generally valid. It should, however, be noted that phenotypic screens often identify compounds that work via multiple targets (related or not), which all need to be targeted together to achieve the desired effect. The downstream validation experiments can, therefore, easily lead to false-negative results. For example, co-knockdown of both the TNKS1 and TNKS2 enzymes was required to recapitulate the effect of the tankyrase inhibitor XAV939 on Wnt signaling mentioned above (Huang et al., 2009).

Binding Mode-Centric Profiling (Selectivity Profiling)

In contrast to completely unbiased drug-centric chemoproteomic profiling (see above section), binding mode-centric profiling focuses on the binding/activity of small molecules against proteins of a particular protein target class to establish the selectivity of a compound. Like for the drug-centric approaches, current techniques typically comprise competition binding assays based on affinity proteomics using noncovalent (Bantscheff et al., 2009; Hall, 2006; Rix and Superti-Furga, 2009) or covalent chemical probes (Cravatt et al., 2008; Sadaghiani et al., 2007; Nomura et al., 2010) and quantitative mass spectrometry. The main differences to the drug-centric mode of operation are that (1) the proteins that constitute the target class need to contain a molecularly conserved and druggable binding site (such as a cofactor binding site), and (2) the chemical probe employed ideally binds to all members of the target class. For selectivity assessment the compound of interest is typically used as a competitor over a range of concentrations in a lysate (or on cells) of a disease-relevant cell line or tissue. The affinity of the compound to all members of the target class is determined by quantifying the (reduced) amount of proteins captured by the affinity matrix or activity probe. More specifically, inhibition of binding curves is obtained from which apparent K_D values can be calculated (Patricelli et al., 2011; Bantscheff et al., 2011; Sharma et al., 2009). This is a very powerful approach because proteins are assayed under close-to physiological conditions (e.g., use of relevant cell line or tissue; proteins at endogenous expression levels and with natural modification status). In addition the multiplexing capability of mass spectrometry for protein identification and quantification provides ranked affinities of a compound against all members of the target class in one experiment.

For protein kinases the conserved ATP-binding site has been used by several groups to generate nonselective ATP-competitive affinity matrices that provide selectivity assessments for up to 150 kinase targets in a single experiment (Bantscheff et al., 2007a; Sharma et al., 2009; Schirle et al., 2012). Such matrices have been successfully applied to the selectivity profiling of clinical BCR-ABL inhibitors in the chronic myeloid leukemia cell line K562 (Bantscheff et al., 2007a), EGFR inhibitors in HeLa cells (Sharma et al., 2009), and a range of 13 investigational and clinical multi-kinase inhibitors in patient-derived primary chronic lymphocytic leukemia cells (Kruse et al., 2011). Immobilized kinase inhibitors have also been used to identify targets in head and neck cancer by analyzing the kinase complement across 34 squamous cell carcinoma lines established from patients (Wu et al., 2011). Other examples for a pan-target family affinity matrix are the hydroxamates suberoylanilide hydroxamic acid (SAHA) and givinostat, used for selectivity profiling of class I and IIb HDACs in the context of different physiological protein complexes (Bantscheff et al., 2011).

As mentioned earlier, ABPP typically uses active site-reactive chemical probes to probe for selectivity across a given enzyme family (reviewed in Cravatt et al., 2008 and in this issue). These probes react either in a truly activity-based mode with the

purified using affinity chromatography before trypsin digestion and LC-MS/MS analysis. Drug selectivity within the probed target class is revealed by experiments performed in the presence or absence of excess free drug (green protein is a drug target, whereas red and blue proteins are not).
(D) Protein-protein interaction profiling, here exemplified by an antibody-based strategy. Cell extracts are incubated with an immobilized antibody directed against the protein target (red) or an isotype-matched unspecific IgG preparation ("mock IP," light-blue antibody, right panel). Only the target protein and endogenous functional interactors of the target protein (blue) are enriched in experiments with bait-directed antibodies versus mock IPs (green protein, background). In some cases, drug binding affects protein complex composition (middle panel shows loss of the blue protein) either by direct binding to the protein-protein interaction site or via induced conformational changes.

catalytic center or with a reactive amino acid in the vicinity of the binding site. Examples for the former include the fluorophosphonate probes targeting serine hydrolases (SHs) (Liu et al., 1999) and (acyloxy)methyl ketone probes targeting cysteine proteases (Krantz et al., 1991). Examples for the latter are the acylphosphate probe for kinases that reacts with a conserved lysine residue (Patricelli et al., 2007) and the photoreactive SAHA-based probe for HDACs (Salisbury and Cravatt, 2007). In addition to the examples above, probes have also been developed for a variety of other enzyme classes including phosphatases (Kumar et al., 2004) and glycosidases (Vocadlo and Bertozzi, 2004). A recent report demonstrated how ABPP can be used to screen compound libraries against an entire target class (Bachovchin et al., 2010). The authors first synthesized a probe to capture 80% of all mammalian SHs, then screened 70 SHs against 140 structurally diverse carbamates and assessed the selectivity of hits using the very same approach. Importantly, activity-based probes can be adapted for in situ and in vivo labeling by introducing a bio-orthogonal chemical handle, such as an alkyne. Probe-labeled enzymes can then be captured by click chemistry conjugation to azide-containing reporter tags (Wright and Cravatt, 2007; Speers et al., 2003; Speers and Cravatt, 2004). The trifunctional probe design mentioned in the Drug-Centric Chemoproteomic Profiling section has also been applied to whole-enzyme classes by using a suitable pan-target class selectivity group, e.g., for cAMP-binding proteins (Luo et al., 2009), kinases (Fischer et al., 2010), and methyltransferases (Dalhoff et al., 2010).

Drug Mode of Action and Target Validation

Unbiased approaches to target deconvolution of hits and leads, including the aforementioned proteomic strategies but also genetic and bioinformatic techniques do not necessarily identify well-annotated and -characterized target proteins. Hence, a frequent initial challenge is to link these proteins to disease biology and to elucidate the mode of action of how the drug molecule generates the observed phenotype. One might argue that this area of drug discovery is most closely related to basic research because the focus is on the mechanistic understanding of how modulation of a protein target can lead to an observed or desired phenotype. A variety of proteomic experimental strategies have been applied successfully in this area, both from a basic research and a more targeted, drug discovery angle. It should be noted that, in many cases, proof-of-concept studies are done in model organisms with less-complex proteomes, and the transfer to more complex mammalian systems is not always straightforward. This is why in this section we will focus on strategies that have either been applied successfully to mammalian systems or where the complexity of the proteome does not pose a conceptual challenge.

Protein-Protein Interactions

The generation of protein-protein interaction networks using affinity proteomic approaches can help to characterize the functional environment of the protein under investigation. Using the "guilt by association concept," this often works even for proteins without any prior functional annotation. In an ideal scenario, placing a protein into an interaction network identifies a protein directly as a player in the disease process under investigation (Ruffner et al., 2007; Kruse et al., 2008; Oeljeklaus et al., 2009). Technically, a protein of interest can be purified along with associated interactors either using specific antibodies or one of a variety of available tandem or single epitope tag systems (Bauer and Kuster, 2003; Brizzard, 2008). Although immunoprecipitation (IP) allows for the purification of endogenous protein complexes, tagged proteins offer greater experimental flexibility (say to investigate wild-type versus mutant or enzymatically active versus inactive forms of a protein). For both systems, differential bait protein expression often combined with quantitative mass spectrometry can be useful to discriminate between bona fide interactors and high-abundance, low-affinity false positives. This may be achieved for example by combining protein knockdown by siRNA with IP (Selbach and Mann, 2006) or by using an inducible system for expression of the bait protein (Medina et al., 2000). Alternatively, mock IPs using isotype-matched IgG mixtures have been used successfully to create background data sets for specificity assessment (Bantscheff et al., 2011). Moreover, for tagged proteins the knockdown of the untagged endogenous protein by RNA interference can help to increase enrichment efficiency of interactors that are otherwise distributed between tagged and untagged versions of the protein (Forler et al., 2003).

In the case of enzymes as targets, the experimental strategy can be tailored to the identification of substrates. For example, substrate-trapping mutants of phosphatases have been generated where the transient enzyme-substrate interaction is stabilized by mutating residues involved in the catalytic mechanisms (Flint et al., 1997). Combining this with an unbiased mass spectrometry readout has allowed the identification of substrates for several phosphatases, including PTPN22 (Wu et al., 2006). Another described example is the use of a tagged poly(ADP-ribosyl) (PAR)-binding WWE domain and quantitative proteomics. This experiment identified proteins that are PARsylated by tankyrase, the target of the Wnt pathway inhibitor XAV939, and whose stability is regulated by the PAR-recognizing E3 ligase RNF146 (Zhang et al., 2011). Moreover, affinity matrices consisting of small molecules and modified peptides have been used to capture and characterize endogenous, biologically relevant complexes. In the aforementioned study of BET inhibitors, an in-depth characterization of functional BET protein complexes was achieved by the combination of an immobilized BET family inhibitor, acetylated histone H4 peptides (endogenous binding partners of the BET family), and BET IP (Dawson et al., 2011).

In addition, protein-protein interaction studies can be used to shed light on mechanisms other than direct inhibition or activation by which a drug can modulate target activity. Differential protein complexes observed with and without compound treatment, either on cell, in lysate or during the purification procedure, allow the identification of compound-sensitive protein-protein interactions. Using tagged subunits of the SIN3A HDAC complex, Smith and colleagues found that SAHA, the first FDA-approved HDAC inhibitor for the treatment of cancer, causes dissociation of the ING2 subunit from this complex. Absence of ING2 leads to loss of binding of the SIN3A complex to the *p21* promoter and, thus, directly contributes to the growth inhibitory effect of SAHA (Smith et al., 2010). Another example is the identification of the interaction of tagged cAMP-dependent protein kinase (PKA) subunit Cβ1 and CAP1, which was shown to be sensitive to an ATP-competitive PKA inhibitor (Erlbruch

et al., 2010). Finally, the generation of large-scale protein interaction maps, e.g., of complete disease-related signaling pathways, can also enable the identification of druggable components that might even have a more favorable target profile than others. An early focused example was the interaction mapping around 32 members of the pro-inflammatory TNF-α-induced NKκB pathway. This study resulted in 80 novel protein interactions, and genetic validation of a modulatory role in TNF-α signaling could be shown for 10 of these proteins (Bouwmeester et al., 2004). More recently, an interesting study has been published by Moulick et al. in which the authors identified the selective binding of the HSP90 inhibitor PU-H71 to cancer-specific oncoprotein-HSP90 complexes using an immobilized PU-H71 matrix (Moulick et al., 2011). Malovannaya et al. recently published a massive global IP study that characterized the endogenous human complexome and its organization (Malovannaya et al., 2011). The authors amassed data from >3,000 affinity purifications identifying >10,000 proteins, which may turn out to be a valuable future resource for drug discovery strategies targeting protein complexes.

Global Proteomic Profiling

On a global proteomic scale, advances in mass spectrometry instrumentation, sample preparation, and data analysis are now allowing for the identification of some 5–10,000 proteins in a single-cell line (Beck et al., 2011; Nagaraj et al., 2011). Although still fairly time consuming (1–10 days per experiment), global proteome profiling is reaching a level of depth at which it becomes a viable complement to gene expression profiling for mode of action studies. Monitoring (co)regulation of proteins implicated in a particular cellular process upon drug treatment can functionally link a drug and/or target to direct or indirect induction or inhibition of these mechanisms. This approach should prove particularly useful when investigating drugs that directly affect the stability of proteins such as inhibitors of proteases or other enzymes involved in proteolytic pathways, such as the ubiquitin system.

Proteomic Profiling of PTMs

Proteomic approaches are becoming an important tool to characterize the mode of action of compounds modulating enzymes involved in PTM of substrate proteins such as phosphorylation, acetylation, and ubiquitination. For example, differential phosphoproteomic analyses using selective small molecule inhibitors of particular kinases have been used to identify substrates in human cell lines and characterize the effect of inhibition on signaling events. Examples include the MAPK inhibitors U0126 and SB202190, the clinical BCR-ABL inhibitor Dasatinib (Pan et al., 2009), and, more recently, inhibitors of Aurora and Polo-like kinases (Kettenbach et al., 2011). Because such global studies do not distinguish between changes in phosphorylation status of direct substrates and downstream members of the signaling cascade, extensive bioinformatic analysis and in vitro validation are required. In contrast the chemogenetic kinase substrate-trapping approach originally developed by the Shokat group (Shah et al., 1997) allows for direct and unequivocal identification of kinase substrates. The system essentially uses a genetically engineered kinase ATP-binding pocket that can bind an unnatural bulky ATP analog (which cannot be bound by the wild-type kinase) and transfer its phosphate group to substrate proteins. Using thio-ATP followed by a covalent capture/release enrichment step and identification of modified peptides by mass spectrometry, this strategy has also been used successfully for the characterization of CDK1 and CDK2 targets in human cells (Blethrow et al., 2008; Chi et al., 2008).

Another current focus of drug discovery efforts is epigenetic targets that modulate the posttranslational modification state of histones. Quantitative proteomics has been used successfully in many cases to study the effect of small molecule inhibitors by monitoring protein acetylation and methylation. In an early example, Garcia et al. described the quantitative effects of the HDAC inhibitor trichostatin A on the histone modification state in a murine model of systemic lupus erythematosus (Garcia et al., 2005). Lee and colleagues used label-free mass spectrometry to quantify the effect of HDAC inhibitors of varying degrees of selectivity on histone acetylation (Lee et al., 2008). More recently, proteomic approaches have been used to study the effect of inhibition of the histone demethylase JMJD2A by pyridine-2,4-dicarboxylic acid derivatives (Mackeen et al., 2010) as well as the histone methyltransferases G9A and GLP by the small molecule inhibitor UNC0638 (Vedadi et al., 2011). Finally, the recent development of antibodies recognizing the diglycyl modification of lysine side chains resulting from trypsinolysis of ubiquitin conjugates allows monitoring the effect of inhibitors of components of the ubiquitin/proteasome system. These long-awaited reagents have been used in two recent studies that describe quantitative changes in the ubiquitin-modified proteome upon treatment with the proteasome inhibitors MG132 and Bortezomib (Wagner et al., 2011; Kim et al., 2011). These antibodies will also turn out to be useful for substrate identification for other, upstream, targets in the ubiquitin system. Obviously, application of the proteomic strategies described above is not restricted to the identification of the mode of action of a drug but can also be applied to the identification of cellular mechanisms that result in drug resistance. Upregulation of compensatory pathways can be detected by changes in protein abundance or in proxies of pathway activity such as its phosphorylation state. In a recent example, Gioia et al. used quantitative phosphoproteomics to identify an oncogenic signaling cascade mediated by LYN and SYK that can bypass BCR-ABL in chronic lymphoid myeloma cells, thus conferring resistance to chemotherapy targeting BCR-ABL (Gioia et al., 2011).

Biomarker Discovery and Preclinical Drug Discovery

The area of proteomic biomarker discovery and verification has acquired a lot of attention and spurred a great deal of research activity during recent years (reviewed in Brennan et al., 2010; Diamandis, 2004; Makawita and Diamandis, 2010; Rifai et al., 2006; Schiess et al., 2009; Zolg and Langen, 2004; Colburn, 2003; Sinha et al., 2007). Within the scope of this review, we must, however, confine ourselves to presenting just a few aspects of the identification of pharmacodynamic biomarkers using proteomics. Pharmacodynamics (PD) can pragmatically be summarized as the study of what a drug does to the body (or a relevant model system for that matter) and in our context, therefore, mainly relates to questions about drug efficacy, drug toxicity, and the therapeutic index of a drug. The application of proteomics to the identification of such PD biomarkers can take many forms but ideally closely follows and complements the panel of in vitro and in vivo assays established for a particular

drug discovery project. Broadly speaking, a molecular PD biomarker should be able to monitor the (successful) intervention in a disease-relevant process or highlight toxic effects in response to the treatment.

Proteomic Biomarkers for Evaluating Drug Efficacy

In 2005, Fishman and Porter proposed "a new grammar for drug discovery" (Fishman and Porter, 2005) that argued that "to realize the potential of the genome for identifying candidate drugs we must move beyond individual genes and proteins. The signaling pathways in cells provide the right level for such analyses." Much about what was said about pathways and the identification of drug targets in this publication also applies to the identification of molecular protein biomarkers that aim to measure the efficacy of a drug. Because many drugs target enzymes, a viable proteomic biomarker approach is to identify substrates that are directly or indirectly affected by the treatment. This in fact is often tightly connected to MoA studies of a drug (see previous section). Therefore, the identification of a (mechanistic) biomarker of drug efficacy can be achieved for example via monitoring the levels of PTMs such as protein phosphorylation for kinase substrates, protein acetylation for monitoring (de)acetylase activity, or the identification of protein fragments indicative of protease activity. The power of mass spectrometry-based proteomics here is its ability to discover these modifications at a large scale (hundreds to thousands) and to monitor their response to drug treatment or other system perturbations quantitatively (Choudhary et al., 2009; Gevaert et al., 2003; Olsen et al., 2006). This is an important conceptual and technical advantage over traditional antibody-based methods such as PTM-specific western blotting. Immunological methods are of course very valuable in the verification phase and for monitoring large numbers of samples, but the appropriate reagents can also often be difficult and time consuming to generate. An alternative to using the output of an enzymatic activity as a molecular PD biomarker as described above is to monitor global protein levels as a surrogate for the effect of the applied treatment. This is for example relevant for therapeutic strategies targeting the proteasome (Kraus et al., 2007) or the protein-folding machinery (Schumacher et al., 2007) but can also apply to cases where the pharmacological loss of function of one protein causes the cell to express increased levels of another protein with redundant function (Guo et al., 2008).

Proteomic Biomarkers for Evaluating Drug Toxicity

A large proportion of failures in drug discovery and development projects are not due to limited efficacy but result from toxicity. This is also true for drugs in clinical trials, and indeed, many approved drugs are later withdrawn from the market because of issues of toxicity. Drug toxicity can of course have many reasons, but for the sake of argument, these can broadly be divided into the two categories of on-target toxicity and off-target toxicity. On-target toxicity refers to the situation in which a drug targets the desired protein, but the induced loss or gain of function exhibits undesirable biological effects limiting the usefulness of the treatment. Well-known examples for this category include drugs targeting the p38 MAP kinase in inflammatory diseases (Hammaker and Firestein, 2010). Conversely, off-target toxicity refers to an unintended interaction of a drug with one or more proteins that may lead to adverse effects on the function of organs such as the liver, kidney, and others. The prime example for this category is the inhibition of drug-metabolizing enzymes of the P450 family, but also for example kinase inhibitors with limited selectivity can fall into this class. Proteomic studies on drug selectivity and MoA can, therefore, often highlight potential toxicity issues early on and, thus, provide a valuable source for appropriate molecular (toxicity) biomarkers (see also the above sections) (Amacher, 2010; Kennedy, 2002). Liver toxicity is a particularly problematic issue and is indeed frequently observed. Here, global proteome profiling of human hepatocytes or rodent livers exposed to a drug can be employed to obtain an appreciation of the effects the treatment may impose. Proteins identified to change in abundance in response to drugs may thus be useful surrogate PD biomarkers (Yamanaka et al., 2007; Ortiz et al., 2010; Ge et al., 2007). Much of the aforementioned PD biomarker discovery work is carried out in model cell lines. It is, however, important to translate such findings into relevant animal models of disease and, eventually, the human situation. Samples from drug-treated rodents for proteomic analysis can often quite easily be obtained, but translation to humans is often complicated by a number of points. First, the molecular mechanism by which a drug works may not be the same in both species (which would indeed question the validity of the animal model). Second, the pharmacokinetics and PD of a particular drug treatment are clearly different between cellular model systems, whole organisms and species, so it may not be very clear how an effective dose determined from work in cell lines can be extrapolated to the animal situation. Third, the disease often only affects a small number of cells in the rodent or human subject, and these cells are surrounded by stroma or other cell types. Direct proteomic analysis on these systems by mass spectrometry can be difficult because the biomarker signal is diluted out by the presence of the other tissue components. Therefore, molecular monitoring of treatment efficacy in animal models (and humans) mostly relies on methods with good spatial resolution such as immunohistochemistry. In this regard the Human Protein Atlas project (Uhlen et al., 2010) may become a valuable resource of reagents and information for the clinical validation of biomarkers identified by proteomics because the project has already generated antibodies to >10,000 human proteins along with immunohistochemistry profiles in several disease tissue types. It should be noted though that this panel of antibody reagents is not yet fully validated in terms of protein detection specificity.

Conclusion and Outlook

In light of the wide range of successful applications of proteomics to small molecule drug discovery described in this review, one may ask the general question what the overall role of proteomics in drug discovery is. Quite obviously, proteomics (and genomics for that matter) constitutes a rather small piece in the very complex drug discovery puzzle, and proteomic information may sometimes be decisive and sometimes not. From a bibliographic analysis, one notes that 1.4% of all publications in PubMed mentioning *drug discovery* in addressable fields of the database (~92,000 at the time of writing) also mention *proteomics* (2.8% for genomics). Interestingly, of the 1,300 PubMed entries on "Proteomics and Drug Discovery," more than half are classified as reviews, and the overall number of publications and the ratio of reviews to all publications are only changing slowly (Figure 3).

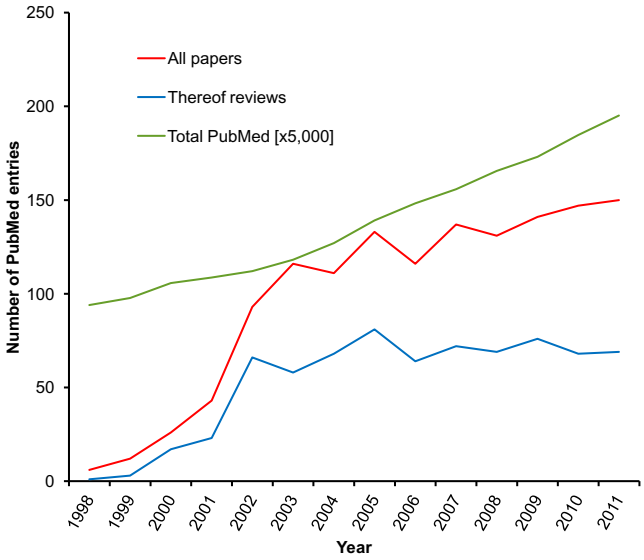

Figure 3. Bibliographical Analysis of Proteomics in Drug Discovery
Searching PubMed in all addressable fields for "proteomics and drug discovery" reveals a slow but steady increase in the number of publications over the past 12 years, which is broadly in line with the overall growth of publications in PubMed. Interestingly, the gap between reviews and all publications on the subject has only begun to widen recently, indicating that proteomics is beginning to make more significant contributions to the field.

This can of course have many reasons, one of which likely is the aforementioned fact that proteomics actually constitutes only a small aspect of drug discovery research. A second reason that is much more relevant for drug discovery than for other basic science research may be that intellectual property considerations often preclude the disclosure of results. Therefore, a mere survey of the published literature may not be a fair representation of the actual situation. We believe that after an early first wave at the end of the last century, proteomics in drug discovery is now experiencing a second and much more significant wave of focused activities that will undoubtedly have increasing impact on the way we discover and assess drugs. In particular for the rapidly growing area of chemogenetics and the renaissance of phenotypic screening, proteomics is among the most promising approaches to target deconvolution and mechanism-based biomarker discovery. An unbiased assessment of the full spectrum of drug-target interactions and their molecular mode of action is now technically within reach. This should not only lead to a better understanding of what a small molecule actually does to a biological system but also to a better appreciation of how this information may be exploited therapeutically.

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