Chemical and Pathway Proteomics
POWERFUL TOOLS FOR ONCOLOGY DRUG DISCOVERY AND PERSONALIZED HEALTH CARE*

Ulrich Kruse‡, Marcus Bantscheff‡, Gerard Drewes‡, and Carsten Hopf‡§¶

In recent years mass spectrometry-based proteomics has moved beyond a mere quantitative description of protein expression levels and their possible correlation with disease or drug action. Impressive progress in LC-MS instrumentation together with the availability of new enabling tools and methods for quantitative proteome analysis and for identification of posttranslational modifications has triggered a surge of chemical and functional proteomics studies dissecting mechanisms of action of cancer drugs and molecular mechanisms that modulate signal transduction pathways. Despite the tremendous progress that has been made in the field, major challenges, relating to sensitivity, dynamic range, and throughput of the described methods, remain. In this review we summarize recent advances in LC-MS-based approaches and their application to cancer drug discovery and to studies of cancer-related pathways in cell culture models with particular emphasis on mechanistic studies of drug action in these systems. Moreover we highlight the emerging utility of pathway and chemical proteomics techniques for translational research in patient tissue. Molecular & Cellular Proteomics 7:1887–1901, 2008.

LC-MS-BASED INVESTIGATION OF CANCER PATHWAYS AND ONCOLOGY DRUG ACTION

Cancers share a restricted set of capabilities crucial for the development of the tumor phenotype: proliferation in the absence of growth factors, insensitivity to growth-suppressive mechanisms, avoidance of apoptosis, limitless replication, angiogenesis, tissue invasion, and metastasis. These characteristics have been referred to as the hallmarks of cancer (1). They are acquired by cancer cells over time and are caused by the accumulation of mostly somatic gene mutations and by overexpression of key causative “driver” proteins. These pivotal proteins, which are differentially expressed between cancer patients and healthy individuals, are being sought after as possible drug targets or biomarkers for early diagnosis of disease, for clinical trial management, and for personalized health care. Consequently in the classical expression proteomics paradigm these signature proteins are identified by LC-MS profiling methods in increasingly sophisticated ways that have recently been reviewed elsewhere (2).

In addition to cellular changes in protein expression, it has been hypothesized that different peptidomes might be present in serum of cancer patients and healthy individuals as a result of cancer-associated changes in proteolytic processing. Based on this assumption proteomic peptide patterns (as opposed to identified peptide sequences), recorded by high resolution mass spectrometers and interpreted by suitable pattern analysis software, could serve as diagnostic tools (3). For example, proteomic MS patterns can distinguish between masked sets of serum samples from (known) ovarian cancer patients and those from healthy controls (4). Since this initial report, proteomics methods for exploiting the information content of the blood peptidome have evolved significantly (5). Recently proteomic pattern analysis has been used in a multicohort cross-institutional clinical trial analyzing serum samples obtained from non-small cell lung cancer (NSCLC)1 patients prior to treatment with the epidermal growth factor receptor (EGFR) inhibitors erlotinib (Tarceva) or gefitinib (Iressa). The study provided evidence for the utility of pattern-based approaches in classifying NSCLC patients for good or poor outcomes after treatment with EGFR inhibitors (6). However, as valuable as expression proteomics approaches in general and MS pattern-based methods in particular may be for cancer diagnosis, they provide little if any mechanistic insight into molecular processes governing the etiology of neoplasia. They also do not elucidate the modes of action by which small molecule drugs change cellular and tissue homeostasis. To address questions relating to molecular mechanisms underlying the pathogenesis of cancer and the therapeutic actions and side effects of oncology drugs, a different suite of techniques is necessary that will be reviewed here: pathway and chemical proteomics.

---

1 The abbreviations used are: NSCLC, non-small cell lung cancer; DDA, data-dependent acquisition; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FGFR, fibroblast growth factor receptor; iTRAQ, isobaric tags for relative and absolute quantification; MRM, multiple reaction monitoring; PDGFR, platelet-derived growth factor receptor; PTM, posttranslational modification; RPA, reverse phase protein array; RTK, receptor tyrosine kinase; SILAC, stable isotope labeling by amino acids in cell culture; TMT, tandem mass tags; BCR, breakpoint cluster region gene.
Chemical proteomics methods measure the interaction between small molecule compounds and protein targets. Most proteins overexpressed in cancer and identified by expression proteomics approaches are not suitable as drug targets (i.e. not “druggable”) even if they are key components of signal transduction pathways. However, other proteins of the same signal transduction pathways may represent alternative druggable targets, especially kinases. Although target-directed, many oncology drugs or drug candidates are not selective for a single target but can bind to several proteins. This is especially true for kinase inhibitors as their targets belong to a large family of closely related enzymes. Therefore, a careful assessment of drug selectivity is desirable even at early stages of drug discovery. The recent introduction of quantitative LC-MS methods has triggered a new wave of proteomics studies in target and drug discovery, especially in selectivity profiling of kinase inhibitors against entire kinomes in cell culture models and patient cells.

Most hallmarks of cancer are associated with the deregulation of signal transduction pathways controlling homeostasis (7, 8). Pathway proteomics approaches do not only elucidate the components of pathways and their interactions but also detect quantitative changes in posttranslational modifications (PTMs), traces left behind by protein-protein interactions too short lived to be captured by conventional biochemical purification approaches (9). Moreover they allow quantitative monitoring of the multilayered effects of oncology drugs on their direct targets and on downstream signaling events.

In an LC-MS-independent approach, reverse phase protein arrays (RPAs) have successfully been used in select cases. Invariably these RPA studies utilized specific antibodies directed against phosphorylation sites that had been associated with disease before (10). In an RPA format, each position in the array represents a complex protein test sample, such as a serum sample or a tissue lysate, and hundreds of patient samples can be combined on a single array slide. Multiple identical arrays can easily be prepared, each of which can be probed with a different detection tool, typically a (phosphorylation site-directed) antibody allowing medium to high throughput analysis. The sensitivity of RPA methods is usually high; as few as 20,000–100,000 patient cells are sufficient for one experiment. These cells can routinely be obtained from blood, by laser capture microdissection of a tissue specimen, or by standard biopsy procedures such as core needle biopsy in breast cancer diagnosis. Multiplex analysis of RPAs (combined with unsupervised hierarchical clustering analysis) with large sets of available phosphorylation site-specific antibodies is a promising tool in diagnostic analysis of molecular signaling networks deregulated in cancer (11). The ambitious goal of the phosphorylation site RPA technology is to enable personalized health care through individualized therapeutic selection based on a comprehensive proteomics-driven understanding of drug effects on each patient’s deregulated signaling network. A recent feasibility study tested biopsy samples from eight patients with metastatic breast cancer before and after treatment with 150 mg/day erlotinib. Six of eight pretreatment samples and five of eight posttreatment samples were classified correctly by unsupervised hierarchical clustering analysis of RPA data obtained with 18 phosphorylation site-specific and four other antibodies. Interestingly normalized relative intensity values generated with only two of the 18 phosphorylation site-specific antibody readouts (Ser-473 phospho-AKT and Tyr-1148 phospho-EGFR) were found to correctly segregate 14 of 16 samples suggesting an even higher degree of specificity with these two antibodies than with a panel of 22 (12). However, whereas multiplex phosphorylation site analysis of RPAs may provide the throughput, sensitivity, and specificity required for diagnostic applications in personalized health care, its performance and competitive edge will largely depend on the knowledge of cancer-related and oncology drug-modulated phosphorylation sites and on availability of phosphorylation site-directed antibodies. Low abundance phosphorylation sites on regulatory proteins will likely be particularly informative. Here LC-MS-based quantitative phosphoproteomics approaches offer the unparalleled capability of identifying novel functionally relevant phosphorylation sites in an unbiased fashion. We expect quantitative phosphoproteomics to continue to contribute significantly to discovery of phosphorylation site biomarkers and diagnostics.

In this review of LC-MS-based approaches applied to oncology drug discovery and to the identification of phosphorylation site-derived diagnostics we will therefore (i) discuss the most important current quantitative mass spectrometry and phosphorylation site enrichment techniques used in quantitative phosphoproteomics, (ii) summarize recent progress in combining affinity enrichment methods and quantitative mass spectrometry in chemical proteomics for target identification and selectivity profiling of oncology compounds in cells and animal tissues, (iii) review quantitative LC-MS methods enabling the characterization of signal transduction pathways in cellular models and of the impact of drug treatment, and (iv) comment on recent translational research efforts using LC-MS to demonstrate that new drugs show the expected effects in primary human tumor cells.

**ADVANCES IN LC-MS TECHNOLOGIES: QUANTIFICATION AND ANALYSIS OF POSTTRANSLATIONAL MODIFICATIONS**

Cancer has many faces and so does oncology pharmacotherapy. As trivial as it may seem the beauty and accuracy of a facial snapshot largely depends on the availability of suitable capturing devices and methodologies. That is as true for photography as it is for proteomics: a number of different proteomics technologies have been devised over the past decade, all aiming at capturing the biological and clinical essence of cancer. Recent advancements in mass
spectrometric technologies have strongly impacted the robustness and significance of clinical proteomics experiments (Table I). Most importantly, the development of quantitative MS methods enabled detailed studies on how proteins and their posttranslational modifications are regulated under defined physiological conditions (e.g. in response to a stimulus). Currently a variety of quantitative MS methods are available to proteomics researchers that use metabolic, chemical, or enzymatic labeling of proteins and peptides using stable isotopes (stable isotope labeling). Alternatively quantitative information, e.g. ion intensity or number of spectra identified per protein, can be extracted directly from the response of the mass spectrometer (label-free methods (13, 14)). A variety of commonly used quantitative proteomics methods is summarized in Table II. With respect to the number of publications two quantitative mass spectrometric methods clearly stand out: stable isotope labeling by amino acids in cell culture (SILAC (15)) and chemical labeling of tryptic peptides with isobaric tags for relative and absolute quantitation (iTRAQ)² (16)). Both approaches have clear advantages and limitations.

In the SILAC approach, cells are grown in a medium containing stable isotope-labeled amino acids, e.g. [13C₆]arginine and/or [13C₆]lysine, and mixed with a control sample that remains either unlabeled or has been labeled with amino acids containing a lighter isotope. Following proteolysis, sample peptides are separated via liquid chromatography and analyzed in the mass spectrometer. Relative quantification is

² ICAT® and iTRAQ™ are trademarks of Applera Corp.
performed by comparing the intensities of isotope clusters of the intact peptide in the survey spectrum. A particular advantage of this approach as well as of other metabolic labeling strategies is that the samples to be compared can already be combined on the level of intact cells. Thus, quantification errors introduced by losses and modifications during sample handling are minimized. This advantage renders the SILAC method particularly suitable for detection of small changes and for quantification of posttranslational modifications (17, 18). However, the use of metabolic labeling techniques is limited to growing cells. Consequently with only a few exceptions (19), the majority of published applications deal with immortalized cells. So far, a maximum of three conditions could be compared in one experiment, complicating the analysis of time course and dose-response experiments (17). Harsha et al. (135) recently suggested a fiveplex SILAC approach using four different variants of stable isotope-substituted arginine. Although promising, this approach uses $^{15}\text{N}_2$- and $^{13}\text{C}_6$-labeled arginine, constituting only a mass difference of 2 atomic mass units for peptides containing one arginine, which only insufficiently allows separation of isotope clusters for quantification. Moreover $^{13}\text{C}_6$-, $^{14}\text{N}_4$-, $^1$d$_7$-enriched arginine is used; this may cause retention time shifts compared with amino acids not containing deuterium (20). Recently a number of variations of the SILAC methodology have been developed that enable, for example, quantification of intact proteins (21) and absolute quantification (22).

Quantification using the commercially available iTRAQ reagent is based on the principle of isobaric mass tags initially introduced by Thompson et al. (23). With this method, amino termini and lysine residues of peptides are labeled postproteolysis with a set of reagents that produce isobarically labeled peptides that precisely co-migrate in liquid chromatography separations. Upon peptide fragmentation, the different isobaric tags fragment as well and give rise to reporter ions that are detected in the low mass region of the tandem mass spectra (e.g. $m/z$ 114, 115, 116, and 117 for iTRAQ reporter ions). Quantification is performed via integration of the reporter ion signal intensities. Because the reactivity of lysine residues in proteins and peptides is relatively similar, isobaric tagging reagents can, in principle, also be used for labeling of intact proteins (24). Labeling of intact proteins instead of proteolytic fragments can be advantageous in some situations because it permits carrying out an additional protein separation step with the combined samples, thus facilitating the characterization of protein isoforms. Although the standard iTRAQ reagents afford monitoring of up to four different conditions in a single MS analysis, very recently novel reagents have become commercially available that enable multiplexed quantification of up to eight samples (25, 26). The possibility of simultaneous quantitative analysis of more data points is particularly useful for studies of biological systems over multiple time points or in response to multiple treatment conditions, such as in concentration-response experiments.

iTRAQ experiments have traditionally been analyzed on quadrupole-TOF (16) and MALDI-TOF/TOF (27) mass spectrometers. Because of their low mass cutoff the very popular ion trap instruments have been regarded as not suitable for this kind of experiment (28). Recently two strategies have emerged to circumvent this limitation. Modern ion trap and Orbitrap instruments can be equipped with an additional collision cell that permits fragmentation of ions outside the ion trap and therefore omits the low mass cutoff (29, 30). The pulsed Q dissociation method provides a cost-effective alternative to the use of an additional collision cell (31). In contrast to conventional CID in ion trap instruments, ions are activated for a very short period of time at high Q value. After a short delay, ions are then transferred to lower Q values where fragmentation occurs. Typically reporter ion intensities observed with this approach are relatively small compared with fragment spectra generated in a collision cell. Nevertheless Griffin and co-workers (32, 33) and others (13) have demonstrated that good quality iTRAQ quantification can be achieved with this approach.

Detection and Quantification of Protein Phosphorylation—

Recent technological improvements in MS have dramatically improved our ability to identify a variety of posttranslational modifications from cells or tissues. Despite these encouraging advances, the study of protein phosphorylation remains a particularly challenging task because of the highly dynamic nature of phosphorylation/dephosphorylation cascades and, in many cases, the very low abundance of phosphorylation sites. Consequently an arsenal of enrichment methods for phosphorylated peptides and proteins has been developed to facilitate further analysis by LC-MS (34–36). Several large scale studies have reported impressive numbers of phosphorylation sites identified from trypsin-digested cell lysates by either enriching for phosphorylated peptides via strong cation exchange chromatography, titanium dioxide (TiO$_2$), IMAC, or a combination of these methods (17, 37–41). Although efficient, none of these methods alone appears to comprehensively enrich all phosphorylated peptides from a given sample (42), suggesting that further improvements are required. Although thousands of phosphorylation sites can be identified in large scale studies, key regulatory phosphorylation sites might be missed because of sample complexity and low abundance of the phosphorylated peptide. Therefore, it is in many cases advisable to add an additional enrichment step (Fig. 1) that specifically targets the proteins of interest. Immunoprecipitation of phosphotyrosine-containing proteins has proven to be a very powerful tool for studying receptor-proximal signaling events (43–48). Recently this approach has been improved and applied to phosphotyrosine-containing peptides directly retrieved from digested cell lysates (49, 50). Moreover specific phosphotyrosine-binding protein domains can be used to profile the global tyrosine phosphorylation state of cells (51, 52). In recent reports, immobilized kinase inhibitors have been used to specifically enrich for protein...
kinases prior to tryptic digestion and phosphopeptide enrichment (13, 53).

With modern mass spectrometers, phosphorylated peptides can be detected with very low false discovery rates (54). Assigning the actual amino acid residue that is phosphorylated within a given peptide, however, remains a major challenge and often requires laborious manual inspection of individual spectra. Lately probabilistic scoring algorithms have been introduced to estimate the likelihood of a phosphorylation being correctly assigned to a specific amino acid residue within a peptide (17, 54). The recently introduced electron transfer dissociation and electron capture dissociation techniques have the potential to overcome these limitations because these methods prevent the neutral loss of labile modifications (55–58).

**Targeted Analysis and Multiple Reaction Monitoring (MRM)—** Although LC-MS enables the identification and quantification of hundreds and even thousands of proteins and their posttranslational modifications in parallel, monitoring a select set of proteins and peptides over a number of different treatment conditions or time points is difficult because of the data-dependent acquisition (DDA) mode in which the instruments are typically operated. In DDA mode, the instrument selects peptide ions for fragmentation in an automated manner, typically starting with the most abundant ion species in any given survey spectrum, resulting in a suboptimal reproducibility of identified proteins and peptides. In such cases, quantification techniques that allow for a high degree of multiplexing, e.g. eightplex iTRAQ or sixplex TMT, are beneficial because each identified peptide can be quantified in all six to eight conditions in parallel (25, 26). To circumvent limitations associated with automated data acquisition, targeted approaches have been developed. In its simplest incarnation targeted approaches use inclusion lists that force the instrument to selectively fragment peptide ions of interest (59, 60). MRM, a method long known in analysis of small molecules, has recently become very popular in proteomics studies as well. This is especially true for studies dealing with highly complex samples like serum or in phosphoproteomics because MRM is well suited for analysis and quantification of any given peptide (61–66): in MRM the presence of a given peptide is monitored via its predicted m/z value and the presence of one or several specific fragment ions in a triple quadrupole mass spectrometer. Because the instrument in MRM is only scanning for a small select set of peptide fragment ions rather than acquiring full MS/MS spectra, very high duty cycles and, hence, very high sensitivity can be achieved. In a particularly elegant study the MRM approach was combined with iTRAQ quantification for a time-resolved study of tyrosine phosphorylation in the EGFR signaling network. Using the MRM approach the number of reproducibly identified and quantified phosphorylation sites in all four separate experiments more than tripled compared with a normal DDA approach (66).

3 TMT® is a trademark of Proteome Sciences.
The recent advances in quantitative proteomics technologies described above have enabled more direct and unbiased studies of the mechanism of action of a drug, in some cases directly in the complex “native proteome” of the target cell or the tissue of interest. This presents an important advantage in the design and development of small molecule oncology therapeutics as many effective drugs are not selective for a single protein target but can bind to several targets especially if the target belongs to a family of structurally conserved proteins. At the early stages of drug discovery, the industry traditionally uses assays of drug action that assess activity on the isolated, purified target often in the form of recombinant, purified enzymes or protein fragments. Correlation of the data for a candidate drug compound obtained from such assays with the observed efficacy in cellular or animal models or even in human clinical trials is often not straightforward because an isolated recombinant protein fragment may not accurately reflect the conformation and activity of the target in its physiological environment. In addition the therapeutic effects may be mediated by more than one target (for reviews, see Refs. 6 and 68); see also the interesting cases presented by McDermott et al. (69)). As noted, polypharmacology (i.e. the action of therapeutics on multiple targets and off-targets) is the rule rather than the exception for oncology drugs as they often target members of large protein classes with a high degree of structural conservation around the druggable binding site such as protein and lipid kinases, histone deacetylases, or heat shock proteins (70–74). Compared with a monoselective drug, such a spectrum of targets is more likely to produce toxic side effects, but in oncology the increase in therapeutic potential may outweigh this disadvantage. As the rational design of promiscuous drug candidates with polypharmacology remains a challenge (75), the discovery of such compounds often relies on serendipitous findings arising from the profiling of hit compounds from traditional biochemical or cell-based screens in ever larger enzyme assay panels. Cell-based screening in tumor cell lines offers a straightforward route to the discovery of compounds with polypharmacology, but the evaluation of the mechanism of action of active compounds requires a substantial experimental effort (69, 76).

Whereas conventional strategies by and large rely on assay panels of 20–100 purified enzymes to address compound potency, selectivity, and potential off-target liabilities (77), the recent developments in affinity-based proteomics techniques have enabled the direct determination of protein binding profiles of small molecule drugs under more physiological conditions. These techniques are in principle based on affinity chromatography, typically using immobilized drugs or tool compounds (for reviews, see Refs. 9 and 78–80) or covalent active site-labeling probes (for reviews, see Refs. 81 and 82). In both methods, the small molecule affinity labels are designed to selectively enrich for a larger set of up to several hundreds of proteins defined by structurally related ligand binding sites. Hence these protein sets can be viewed as chemically tractable subproteomes (83). Both the non-covalent affinity matrix approach and the covalent active site labeling approach have been used successfully for purine-binding proteins (84) and protein kinases (53). The covalent active site labeling approach has also been applied to other target classes relevant in oncology, for instance serine, cysteine, and metalloproteases (Refs. 82 and 85 and references therein) and histone deacetylases (86). However, although the affinity profiles of immobilized compounds reveal novel target candidates, these methods originally also suffered from false positives because of background problems caused by nonspecific interactions of the affinity matrix with abundant proteins.

These experimental difficulties can be circumvented if these techniques are used for the target profiling of drugs or drug candidates by quantitative competition methods. This is typically achieved by adding the compound of interest in its free form in the tissue lysate together with the affinity matrix or the active site label, causing the free compound to bind to its targets in the lysate, thereby effectively competing with the matrix or the label. At the subsequent protein quantification step, the presence of the competing compound will lead to decreased amounts of its targets but not of the majority of non-target proteins at the subsequent protein analysis step (13, 87, 88). By applying the free compound to the lysate over a range of concentrations, dose-response binding curves can be generated in theory for as many proteins as can be captured and robustly quantified on the affinity matrix. In the case of the Kinobeads matrix described by Bantscheff et al. (13) that was generated by immobilization of several different tool compounds, more than 1000 proteins were found to bind to the matrix of which more than 400 were quantified in drug profiling experiments using a combination of isobaric mass tags and high resolution LC-MS/MS peptide sequencing. The Kinobeads-based profiling of the drugs imatinib, dasatinib, and SKI-606 in cell lysate and in living erythroleukemia cells in culture confirmed the known targets of these compounds, including ABL and SRC family kinases, but also identified new targets for all three drugs. In the case of imatinib, the receptor tyrosine kinase DDR1 and the oxidoreductase NQO2 were identified as novel targets of imatinib with potencies comparable to that against breakpoint cluster region gene (BCR)-ABL. These data are of potential interest in the clinical use of imatinib as mutations in DDR1 and alteration in NQO2 expression have been implicated in certain tumors (Ref. 13 and references therein).

### QUANTITATIVE CHEMICAL PROTEOMICS IN DRUG DISCOVERY:

#### TARGET IDENTIFICATION AND SELECTIVITY PROFILING OF ONCOLOGY COMPOUNDS IN CELLS AND ANIMAL TISSUES

The recent advances in quantitative proteomics technologies described above have enabled more direct and unbiased studies of the mechanism of action of a drug, in some cases directly in the complex “native proteome” of the target cell or the tissue of interest. This presents an important advantage in the design and development of small molecule oncology therapeutics as many effective drugs are not selective for a single protein target but can bind to several targets especially if the target belongs to a family of structurally conserved proteins. At the early stages of drug discovery, the industry traditionally uses assays of drug action that assess activity on the isolated, purified target often in the form of recombinant, purified enzymes or protein fragments. Correlation of the data for a candidate drug compound obtained from such assays with the observed efficacy in cellular or animal models or even in human clinical trials is often not straightforward because an isolated recombinant protein fragment may not accurately reflect the conformation and activity of the target in its physiological environment. In addition the therapeutic effects may be mediated by more than one target (for reviews, see Refs. 6 and 68); see also the interesting cases presented by McDermott et al. (69)). As noted, polypharmacology (i.e. the action of therapeutics on multiple targets and off-targets) is the rule rather than the exception for oncology drugs as they often target members of large protein classes with a high degree of structural conservation around the druggable binding site such as protein and lipid kinases, histone deacetylases, or heat shock proteins (70–74). Compared with a monoselective drug, such a spectrum of targets is more likely to produce toxic side effects, but in oncology the increase in therapeutic potential may outweigh this disadvantage. As the rational design of promiscuous drug candidates with polypharmacology remains a challenge (75), the discovery of such compounds often relies on serendipitous findings arising from the profiling of hit compounds from traditional biochemical or cell-based screens in ever larger enzyme assay panels. Cell-based screening in tumor cell lines offers a straightforward route to the discovery of compounds with polypharmacology, but the evaluation of the mechanism of action of active compounds requires a substantial experimental effort (69, 76).

Whereas conventional strategies by and large rely on assay panels of 20–100 purified enzymes to address compound potency, selectivity, and potential off-target liabilities (77), the recent developments in affinity-based proteomics techniques have enabled the direct determination of protein binding profiles of small molecule drugs under more physiological conditions. These techniques are in principle based on affinity chromatography, typically using immobilized drugs or tool compounds (for reviews, see Refs. 9 and 78–80) or covalent active site-labeling probes (for reviews, see Refs. 81 and 82). In both methods, the small molecule affinity labels are designed to selectively enrich for a larger set of up to several hundreds of proteins defined by structurally related ligand binding sites. Hence these protein sets can be viewed as chemically tractable subproteomes (83). Both the non-covalent affinity matrix approach and the covalent active site labeling approach have been used successfully for purine-binding proteins (84) and protein kinases (53). The covalent active site labeling approach has also been applied to other target classes relevant in oncology, for instance serine, cysteine, and metalloproteases (Refs. 82 and 85 and references therein) and histone deacetylases (86). However, although the affinity profiles of immobilized compounds reveal novel target candidates, these methods originally also suffered from false positives because of background problems caused by nonspecific interactions of the affinity matrix with abundant proteins.

These experimental difficulties can be circumvented if these techniques are used for the target profiling of drugs or drug candidates by quantitative competition methods. This is typically achieved by adding the compound of interest in its free form in the tissue lysate together with the affinity matrix or the active site label, causing the free compound to bind to its targets in the lysate, thereby effectively competing with the matrix or the label. At the subsequent protein quantification step, the presence of the competing compound will lead to decreased amounts of its targets but not of the majority of non-target proteins at the subsequent protein analysis step (13, 87, 88). By applying the free compound to the lysate over a range of concentrations, dose-response binding curves can be generated in theory for as many proteins as can be captured and robustly quantified on the affinity matrix. In the case of the Kinobeads matrix described by Bantscheff et al. (13) that was generated by immobilization of seven different tool compounds, more than 1000 proteins were found to bind to the matrix of which more than 400 were quantified in drug profiling experiments using a combination of isobaric mass tags and high resolution LC-MS/MS peptide sequencing. The Kinobeads-based profiling of the drugs imatinib, dasatinib, and SKI-606 in cell lysate and in living erythroleukemia cells in culture confirmed the known targets of these compounds, including ABL and SRC family kinases, but also identified new targets for all three drugs. In the case of imatinib, the receptor tyrosine kinase DDR1 and the oxidoreductase NQO2 were identified as novel targets of imatinib with potencies comparable to that against breakpoint cluster region gene (BCR)-ABL. These data are of potential interest in the clinical use of imatinib as mutations in DDR1 and alteration in NQO2 expression have been implicated in certain tumors (Ref. 13 and references therein).

#### SIGNALING PATHWAYS IN CELLULAR MODELS: MODULATION BY ONCOLOGY COMPOUNDS

The EGF receptor has been selected as a target for the development of anticancer drugs because it is frequently
overexpressed in cancers and associated with a poor prognosis. EGFR is a member of the HER family of receptor tyrosine kinases (HER1/EGFR, HER2/ERBB2/NEU, HER3/ERBB3, and HER4/ERBB4). The receptors consist of an extracellular ligand-binding domain, a single pass transmembrane domain, and an intracellular tyrosine kinase domain (the exception being HER3, which is kinase-inactive). Ligand binding results in homodimerization as well as heterodimerization with other members of the HER family. Dimerization induces kinase activity leading to autophosphorylation of the carboxyl-terminal tail and association with various adaptor proteins. Downstream signaling is mediated by two signaling pathways, the Ras-Raf mitogen-activated kinases (ERK1 and ERK2) and the phosphatidylinositol 3-kinase/AKT pathway. The AKT pathway plays an important role in cell survival, whereas the ERK pathway regulates cell proliferation, transformation, and the progression to metastasis (89). Consequently EGFR signaling leads to increased proliferation, angiogenesis, metastasis, and increased cell survival. In normal tissues ligand binding is necessary to activate EGFR signaling, whereas in tumors the overexpression of EGFR protein can cause ligand-independent dimerization and receptor activation. Approximately 40–80% of NSCLCs overexpress EGFR, thus making it a promising drug target.

Several strategies have been used to target the EGFR, for example monoclonal antibodies directed at the extracellular ligand-binding domain and small molecules targeting the intracellular kinase domain (90). Small molecule kinase inhibitors typically compete with ATP for binding to the ATP-binding pocket. These inhibitors can be classified according to their selectivity (for example EGFR/HER1-selective) and binding mode (reversible versus irreversible). Two inhibitors have been approved by several regulatory agencies for the treatment of NSCLC patients, gefitinib and erlotinib. Gefitinib (Iressa, ZD1839, AstraZeneca) represents a selective and reversible EGFR inhibitor that prevents receptor activation. Erlotinib (Tarceva, OSI-774, OSI Pharmaceuticals) is the first EGFR inhibitor that received regulatory approval on the basis of prolongation of survival.

The availability of highly specific physiological stimuli (EGF) and/or clinically validated, highly selective small molecule inhibitors (erlotinib and gefitinib for EGFR) and monoclonal antibodies (for example, cetuximab/Erbitux and panitumumab/Vectibix for EGFR and trastuzumab/Herceptin for HER2/ERBB2) have made the EGFR/ERBB1 and HER2/ERBB2 signaling pathways prominent model systems for the development and benchmarking of LC-MS-based quantitative phosphoproteomics methods (43, 91). Several years of intense research on the EGFR and HER2/ERBB2 signaling networks have demonstrated the power of quantitative functional proteomics approaches in delineating the precise composition of protein complexes involved in signal transduction (91), the nature of phosphorylation sites modulated in response to (patho-)physiological stimuli (43), and the effects of small molecule drugs on both composition of protein modules or down-regulation of phosphorylation sites (92) (Table III).

Blagoev et al. (43), using three types of stable isotope-labeled amino acids in two combined SILAC experiments, studied the time course of EGF stimulation in HeLa cells. Proteins phosphorylated on tyrosine residues were enriched with anti-phosphotyrosine (anti-Tyr(P)) antibodies and quantitatively analyzed by LC-MS. The authors detected not only a time course of phosphorylation events of known EGFR signaling effectors but also identified 31 putative novel effectors.
The study was later extended to include very early signaling events (44). A similar approach has been used to monitor and distinguish between EGFR and platelet-derived growth factor receptor (PDGFR) signaling in differentiating human mesenchymal stem cells (46). Results have been compiled in the Phosida database (17). In a related study using ICAT2 labeling (93) the effects of erlotinib treatment (1 μM for 2 h) on downstream signaling events in HN5 squamous carcinoma cells displaying a high basal level of EGFR activity have been investigated, and a number of downstream signaling targets of this tyrosine kinase inhibitor have been identified (92).

In most cases reported so far functional validation in animal models or human cancer tissue has been sparse or lacking. Chen et al. (94) studied pervanadate-induced tyrosine phosphorylation in the MCF10AT cell line model of breast cancer progression, a series of cell lines that can induce preneoplastic lesions or low to high grade cancers when grafted into nude mice. Following identification of novel phosphorylation sites in the MCF10AT cell culture model, a number of these sites were validated by immunoprecipitation/Western blotting demonstrating that gefitinib treatment down-regulates these phosphorylation sites (94). In another variation of the EGFR signaling theme, Zhang et al. (95) examined an EGF stimulation time course in 184A1 parental human mammary epithelial cells using iTRAQ labeling and LC-MS/MS analysis. The sensitivity of phosphorylation site identification was improved by combining two steps of phosphorylation site enrichment for phosphorylated proteins with anti-Tyr(P) antibodies and for phosphopeptides with IMAC. A similar double enrichment strategy was adopted to identify phosphorylation sites in the ovarian cancer cell line SK-OV-3 and the breast cancer cell line BT-474, both of which naturally overexpress ERBB2 (96). The number of phosphorylation sites that disappeared after 2 days of cell treatment with 8 μg/ml Herceptin, a marketed monoclonal antibody that inhibits ERBB2 activation, was counted, but no quantification was done in this study (96). These and similar studies have demonstrated that quantitative LC-MS-based pathway proteomics approaches, in particular those with dual phosphorylation site enrichment, have now matured to the point where they can routinely be used to identify novel phosphorylation sites in cell culture models that respond in a time- and concentration-dependent manner to physiological ligands and therapeutics (both small molecule drugs and biopharmaceuticals such as growth factors or monoclonal antibodies).

Several studies investigating different FGFR signaling pathways have demonstrated that LC-MS phosphoproteomics is not limited to the ERBB family of receptor tyrosine kinases. SILAC mass labeling in combination with anti-Tyr(P) immunoprecipitation was used to examine the FGFR1 pathway and to delineate which phosphorylation sites were up-regulated in response to cell stimulation with FG2 (97). Studies of the FGFR1 signaling network are of particular interest because translocations leading to fusions between unrelated proteins (such as FGFR1OP2) and the FGFR1 kinase domain cause constitutive tyrosine kinase activity that has been associated with the 8p11 myeloproliferative syndrome (98). In an attempt to identify an in vitro model of the disease, Gu et al. (99) identified the KG-1 cell line by Western blot screening for cell lines displaying constitutive STAT5 phosphorylation. In contrast to other studies reviewed here, phosphoproteomics was used to find upstream signaling elements (“bottom-up phosphorylation site profiling”) responsible for the constitutive activation of a downstream factor. The authors delineated a variant form of FGFR1 as the culprit that they identified as a FGFR1OP2-FGFR1 fusion by rapid amplification of 5’-complementary DNA end analysis (99). Conversely in a related “top-down phosphorylation site profiling” approach, interleukin-3-treated wild-type Ba/F3 cells were compared with Ba/F3 cells expressing a constitutively active TEL-FGFR3 fusion protein associated with acute myeloid leukemia (100). Phosphorytrosine proteomics profiling identified p80 ribosomal S6 kinase (RSK2) as a downstream effector of FGFR3. Targeting RSK2 by RNA interference or with a specific small molecule inhibitor, fluoromethyl ketone-based kinase inhibitor (101), attenuated FGFR3-induced cytokine-independent growth of Ba/F3 cells and triggered apoptosis in translocation-bearing human myeloma cell lines (100). These reports suggest that LC-MS-based proteomics approaches may offer advantages over nucleic acid analysis methods in identifying upstream causes of a deregulated phosphorylation site in a downstream signal transduction substrate. Similarly downstream effects of genetically altered signal transduction modules such as in the case of fusion kinases can be effectively analyzed using quantitative LC-MS techniques (Table III).

A second cancer-related signaling network in which a chromosomal translocation causes a severe deregulation of signaling networks and for which highly selective small molecule inhibitors are available is the BCR-ABL kinase-driven oncogenesis in chronic myeloid leukemia mentioned above (102). A fusion of the BCR with the ABL tyrosine kinase as the result of a chromosomal translocation leads to the expression of a constitutively active kinase that activates a multitude of downstream signaling proteins (103). Goss et al. (104) studied in a SILAC experiment six different lines representing three different BCR-ABL fusion types. Following phosphopeptide enrichment and LC-MS analysis, they identified phosphorylation sites in many proteins including a set of phosphotyrosine peptides that they termed a phosphotyrosine signature of BCR-ABL kinase as it was independent of cellular background and fusion type. Treatment of K562 cells with 10 μM imatinib (Gleevec), a selective BCR-ABL inhibitor, for 3 h resulted in a greater than 2-fold reduction in phosphorylation of 10 proteins, including BCR-ABL itself and a number of downstream signaling proteins. An analogous analysis in EoL-1 cells, another cellular model of leukemia, expressing a fusion between the FIP1L1 protein and PDGFR, led to identification of a different set of phosphopeptides, including...
some imatinib-responsive peptides. The study suggests that the effect of one drug on different downstream signaling pathways can be readily dissected using current “top-down” phosphoproteomics technology (104). Bantscheff et al. (13) went one step further and examined the dose-dependent (0.01, 0.03, 0.1, 0.3, 1, and 5 μM) response of K562 cells to 5-h treatment with either imatinib or the second generation inhibitors dasatinib (Sprycel) and bosutinib. The latter two drugs are less selective BCR-ABL inhibitors than imatinib but are effective against most imatinib-resistant ABL mutants. Utilizing Kinobeads protein enrichment and IMAC phosphopeptide enrichment, 379 phosphorylation sites on 136 different proteins were identified; 20 sites on 13 different proteins were responsive to at least one of the drugs. Moreover, use of an affinity matrix such as Kinobeads makes it possible to distinguish between proximal drug targets and downstream signaling effectors of a kinase inhibitor: whereas binding of a drug like imatinib to a target protein like BCR-ABL inside cells prevents this protein from being captured by the affinity matrix, binding of downstream effectors to the matrix is not affected by the small molecule compound. Because the drug, however, down-regulates specific phosphorylation sites on downstream signaling effectors, they can be identified by systematic analysis of posttranslational modifications following affinity matrix enrichment (Fig. 1). In the case of imatinib-treated K562 cells, RSK3 was identified as a downstream effector affected by the kinase inhibitor (13). In an intriguing variation of the top-down phosphorylation site profiling approach, Skaggs et al. (105) used LC-MS methods to study resistance to BCR-ABL inhibitors, in particular the T315I ABL mutant that confers resistance to both imatinib and dasatinib. Cell clones expressing this mutant have an oncogenic growth advantage compared with the wild-type allele even in the absence of drug treatment. This is surprising as the mutation does not confer increased kinase activity. To elucidate the reason for this unexpected competitive advantage of the mutant over the wild-type allele, Skaggs et al. (105) performed LC-MS phosphorylation site profiling in retrovirally transduced Ba/F3 cells and uncovered a mutant-specific phosphotyrosine signature suggesting a different mode of substrate recognition by the mutant leading to severely perturbed downstream signaling.

Whereas the above mentioned phosphorylation site mapping endeavors have largely focused on specific signaling pathways, most recently the same technology has been applied to the global mapping of phosphorylation sites in entire animal organs or human cancer tissues (39, 49). It should be mentioned that phosphorylation sites do not represent the only PTMs that can be modulated by drug treatment and are amenable to LC-MS analysis. For example, histone PTMs such as acetylation, methylation, ubiquitination, ADP-ribosylation, and sumoylation have been associated with activation or repression of transcription (106). Inhibitors of histone deacetylases such as PXD101 (belinostat) are currently in clinical trials to assess their potential to treat a wide range of solid tumors and hematological malignancies. LC-MS analysis of histones purified from OC-NYH cells treated with various concentrations of PDX101 revealed several novel PTM (acetylation, methylation, and ubiquitination) sites on histones, some of which were responsive to PXD101 treatment (107).

**TRANSITIONAL RESEARCH: APPLICATION OF QUANTITATIVE MASS SPECTROMETRY METHODS IN CANCER DRUG DISCOVERY, CLINICAL TRIAL MANAGEMENT, AND DISCOVERY OF COMPANION DIAGNOSTICS**

The goal of personalized treatment of cancer patients is to get “the right drug to the right patient at the right dose.” Compared with other indications cancer poses two additional complications. First, cancers evolve over time and progress from localized lesions to tissue invasion to metastasis by acquiring several of the “hallmarks of cancer.” This implies on the gene level the acquisition of multiple somatic mutations giving rise to changed proteins (mutational activation and fusion proteins) or overexpressed proteins (e.g. caused by gene amplification). Second, after prolonged drug treatment cancer cells can acquire drug resistance due to a variety of mechanisms. Drug resistance mechanisms comprise increased efflux and altered drug metabolism (not discussed here) or genetic changes of the gene encoding the drug target itself or in other genes, leading to compensatory effects. The identification of resistance mechanisms should provide the basis to develop “second generation” inhibitors and rational drug combinations that can overcome drug resistance. Typically these changes driving cancer growth or drug resistance are identified by DNA sequencing methods, mRNA expression analysis, fluorescence in situ hybridization, or immunohistochemistry. Here we discuss the current progress and future directions in addressing these challenges by advanced proteomics methods, taking the EGF receptor as an example.

**Variable Tumor Responses to Small Molecule EGFR Inhibitors**—Originally it was expected that EGFR inhibitors like gefitinib and erlotinib would be useful for all patients with NSCLC, but during their clinical development it became clear that only a subpopulation of patients responded to treatment. These initial observations were puzzling and provoked a number of studies using different technologies to address this surprising finding. Initial clinical data suggested that, in female patients, patients of Asian origin, those who had never smoked, and those with adenocarcinoma NSCLC were more likely to respond to the treatment with EGFR inhibitors. Recent reports revealed that mutations in the ATP-binding pocket of EGFR predict sensitivity to gefitinib. These somatic mutations in the kinase domain of the EGFR occur in a number of lung adenocarcinomas. Approximately 90% of these mutations represent short in-frame deletions in exon 19 that eliminate four amino acids (LREA). In addition, a single point mutation was identified that substitutes arginine for leucine at...
position 858 (L858R) (108). It is noteworthy that these somatic mutations are acquired during early stages of tumor development implying that they confer a growth advantage to tumor cells presumably by constitutively activating the kinase function of EGFR. Interestingly these mutations lead to an increased sensitivity to kinase inhibitors such as gefitinib and erlotinib. Prospective clinical trials have demonstrated a 75% response rate for patients whose tumors harbor these mutations (109). Conversely tumors with wild-type EGFR are less likely to respond to treatment with these drugs. It has to be kept in mind that the drug-sensing EGFR mutations are acquired before drug treatment and are different from mutations leading to drug resistance.

**Acquired Resistance to EGFR Inhibitors**—Unfortunately lung cancers with drug-sensitive EGFR mutations that initially respond to gefitinib or erlotinib eventually develop drug resistance by acquiring drug resistance mutations in the EGFR or changes in other proteins. Approximately 50% of the cases harbor a second site point mutation in the EGFR kinase domain that substitutes methionine for threonine at position 790 (T790M; Table IV) (108).

Recently mechanistic insight into the consequences of this single amino acid exchange was obtained. Threonine 790 is the “gatekeeper residue” in EGFR because of its location at the entrance to a hydrophobic pocket in the ATP binding cleft. It was anticipated that the bulky methionine residue at this position prevents binding of small molecule kinase inhibitors to the ATP pocket because of steric hindrance. Surprisingly the T790M mutant remains sensitive to irreversible inhibitors, ruling out a simple blockade of access to the binding pocket. This apparent paradox was resolved by a quantitative analysis of ATP and drug binding to the EGFR (110). A direct binding assay was used to measure the intrinsic fluorescence of EGFR that is quenched by titration with inhibitors. These binding studies were performed with the L858R EGFR “sensitivity” mutant, the T790M “resistance” mutant, and a L858R/T790M double mutant and compared with wild-type EGFR. The result demonstrated that the T790M mutant retained low nanomolar affinity for gefitinib but increased the ATP affinity of the oncogenic L858R mutant by more than an order of magnitude. It has been suggested that the increased ATP affinity is the primary mechanism by which the T790M mutation confers drug resistance.

This finding is surprising because in other cases drug resistance is caused by loss of drug binding to the mutated target (for example resistance of BCR-ABL mutants to imatinib). In addition, it underscores the necessity to analyze the drug-target interaction in a quantitative manner under close to physiological conditions preferably with the target as it occurs in the tumor cell.

**Amplification of c-MET**—Not all cases of drug resistance could be accounted for by the T790M mutant, a fact that prompted the search for alternative resistance mechanisms. High resolution genome-wide profiling of EGFR tumor samples before and after treatment identified the MET oncogene as an additional factor responsible for drug resistance (111). MET encodes a heterodimeric receptor tyrosine (RTK) consisting of an extracellular α-chain that is linked by disulfide bonds to the membrane-spanning β-chain. The MET gene was amplified in tumors from nine of 43 (21%) patients with acquired resistance but only in two tumors from 62 untreated patients (3%). Among 10 resistant tumors from the nine patients with MET amplification, four also harbored the T790M resistance mutation (Table IV).

The authors also identified an established lung adenocarcinoma cell line (NCI-H820) that contained all three changes: a drug-sensitive mutation (exon 19 deletion), the T790M resistance mutation, and MET amplification. Interestingly this cell line was resistant to treatment with erlotinib and an irreversible EGFR inhibitor but sensitive to a multikinase inhibitor that potently inhibits MET kinase (XL880, Exelixis). In summary, MET mutations occur independently of the EGFR T790M mutation, and MET may provide an alternative target in EGFR inhibitor-resistant tumors.

A similar conclusion was reached by a cell culture (in vitro resistance) modeling approach (112). An NSCLC cell line (HCC827) harboring a gefitinib sensitivity mutant (exon 19 deletion) was exposed to increasing concentrations of gefitinib for 6 months, and a gefitinib-resistant cell line was selected (HCC827 GR). A phospho-RTK array was used to compare the effect of gefitinib on 42 phosphorylated RTKs in both cell lines. In the parental cell line EGFR, ERBB2, ERBB3, and MET underwent phosphorylation that was markedly reduced after gefitinib treatment. By contrast, in the resistant cell line phosphorylation of MET, ERBB3, and EGFR remained at higher levels in the presence of gefitinib. Genome-wide

| Cancer                        | Gene | Mutation                                                                 | Effect                                      | References |
|-------------------------------|------|--------------------------------------------------------------------------|---------------------------------------------|------------|
| Lung (NSCLC)                  | EGFR | In-frame deletion of four amino acids (LREA) in exon 9; point mutation at position 858 (L858R) | Increased sensitivity to gefitinib and erlotinib | 108, 109   |
|                               | EGFR | Point mutation of gatekeeper residue at position 790 (T790M)             | Resistance to reversible inhibitors; sensitive to irreversible inhibitors | 110        |
|                               | MET  | Gene amplification                                                       | Resistance to gefitinib and erlotinib       | 111–113    |
| Brain (glioblastoma multiforme)| EGFR | Deletion of exons 2–7 (EGFRvIII) resulting in truncation of the extracellular domain | Resistance to cisplatin unless combined with EGFR kinase inhibitors | 114        |
copy number analyses identified amplification of the MET proto-oncogene. This initial observation was further corroborated by the analysis of lung cancer samples. MET amplification was observed in four of 18 (22%) lung cancer samples that had developed resistance to gefitinib or erlotinib. Interestingly one patient had two separate sites of resistant metastases that were both examined. In one metastasis MET was amplified, whereas the second harbored the T790M mutation. This observation underscores the heterogeneity of tumor samples even within individual patients and poses an additional challenge for tumor diagnostics. Moreover the occurrence of different resistance mechanisms in the same patient indicates that dual inhibition of EGFR and MET may be necessary for effective treatment. In the cell culture model, it was demonstrated that the inhibition of MET signaling restored the sensitivity to gefitinib. Furthermore it was suggested that the amplification of MET causes gefitinib resistance by driving ERBB3-dependent activation of phoshatidylinositol 3-kinase. However, this explanation is at variance with the study by Bean et al. (111) who observed that in H820 cells ERBB3 signaling depends on MET and not EGFR activity. These discrepancies may be explained by the different ways in which the cell lines were derived. Results obtained from cell lines need to be interpreted with great care as the presence of (often unknown) multiple mutations can lead to wrong conclusions. Therefore, the analysis of primary cells or tumor samples should be included in proteomics studies wherever possible.

**Evolving Kinase Signaling Networks**—The dependence of cancer cells on oncogenic tyrosine kinases such as BCR-ABL, EGFR, or MET is thought to result from adaptations of signal transduction pathways downstream of these kinases. However, the question how MET amplification can lead to resistance toward EGFR inhibitors still requires a mechanistic explanation. Several large scale proteomics studies have further investigated this in cell lines and tumor samples harboring EGFR mutations.

A proteomics analysis of phosphotyrosine signaling identified an extensive RTK signaling network in various NSCLC and gastric cancer cell lines expressing activated EGFR, mutated K-RAS, or amplified MET (113). The tyrosine phosphorylation pattern was analyzed with PhosphoScan, a recently established phosphoproteomics method using phosphopptide immunoprecipitation with an anti-phosphotyrosine antibody and subsequent analysis by LC-MS/MS (50). A semi-quantitative spectral counting approach was used to sum up all phosphotyrosine-containing peptides for a protein of interest and to compare with total protein phosphorylation between different NSCLC cell lines. Comparison of the signaling networks in EGFR- and MET-dependent cells identified a core network of ~50 proteins that play a role in mediating the response to drug exposure. However, only established cancer cell lines were considered, and it needs to be confirmed whether similar results can be obtained with primary tumor tissue. A second study included NSCLC tumor samples (49). The analytical approach consisted of phosphopeptide immunoprecipitation and LC-MS/MS.

EGFR signaling does not only play a crucial role in lung cancer but also in brain tumors. In glioblastoma multiforme, the most aggressive brain tumor in adults, the EGFRVIII mutant with a truncation of the extracellular domain, is commonly found. Huang et al. (114) examined the effect of EGFRVIII protein levels on phosphotyrosine-mediated signaling networks using transfected mU87MG glioblastoma cells expressing various amounts of EGFRVIII. Serum-starved cells were lysed and after sample preparation peptides were stable isotope-labeled (iTRAQ) and mixed, and phosphotyrosine containing peptides were immunoprecipitated with an anti-phosphotyrosine antibody. Phosphorylated peptides were further enriched by the IMAC method and analyzed by LC-MS/MS. A total of 99 phosphorylation sites on 69 proteins were identified and quantified including eight phosphorylation sites on EGFRVIII. The phosphorylation pattern on EGFRVIII may not be qualitatively different from wild-type EGFR, but quantitative differences at individual sites may have an impact on downstream signaling events. In addition, EGFRVIII-induced phosphorylation and activation of MET was observed. Furthermore co-treatment of cells with MET and EGFR kinase inhibitors demonstrated enhanced cytotoxicity.

**CONCLUSIONS AND PERSPECTIVES**

Quantitative mass spectrometric proteomics techniques have matured significantly in recent years. Together with an emerging tool box for systematic enrichment and analysis of posttranslational modifications they can provide functional context for mutations/translocations identified by genomics research. They can thereby significantly contribute to the delineation of molecular culprits responsible for deregulated downstream phosphorylation (bottom-up phosphoproteomics) and of molecular consequences of overexpressed or mutated driver proteins (top-down phosphoproteomics). In drug discovery, quantitative mass spectrometry methods enable new approaches in target identification for small molecule compounds including potential toxicity targets. The discovery of DDR1 and NQO2 as novel targets of imatinib demonstrates the value of the methodology for in-depth repfiling of oncology drugs. This case shows that a simple pulldown experiment with an immobilized compound followed by identification of all captured proteins is not sufficient to identify specific binding events. Therefore, a quantitative analysis of drug-target interactions in a competitive binding situation using the unmodified drug is preferred. Applications of mass spectrometry in drug discovery extend beyond target discovery because they also enable MS-based selectivity profiling early in the discovery process and contribute important information for understanding the mode of action of a drug. Toward the end of using proteomics techniques or markers for dose selection or assessment of drug combinations, analysis of...
transformed cell lines will not be sufficient. Application of the methodology reviewed here to translational studies in tumor tissue has just started. The methods outlined in this review show promise for the discovery of phosphorylation site-based diagnostics, in particular drug activity and surrogate markers, that could open up new avenues in personalized health care and in clinical trial management, respectively.

Acknowledgment—We thank Frank Weisbrot for excellent graphics art work.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Cellzome AG, Meyerhofstr. 1, D-69117 Heidelberg, Germany. Tel.: 49-6221-13757-377; Fax: 49-6221-13757-210; E-mail: Carsten.Hopf@cellzome.com.

REFERENCES

1. Hanahan, D., and Weinberg, R. A. (2000) The hallmarks of cancer. Cell 100, 57–70.
2. Qian, W. J., Jacobs, J. M., Liu, T., Camp, D. G., II, and Smith, R. D. (2006) Advances and challenges in liquid chromatography-mass spectrome-
try-based proteomics profiling for clinical applications. Mol. Cell. Proteo-
tics 5, 1727–1744.
3. Wulfkuhle, J. D., Liotta, L. A., and Petricoin, E. F. (2003) Proteomic applications for the early detection of cancer. Nat. Rev. Cancer 3, 267–275.
4. Petricoin, E. F., Ardekani, A. M., Hitt, B. A., Levine, P. J., Fuzzo, V. A., Steinberg, S. M., Mills, G. B., Simone, C., Fishman, D. A., Kohn, E. C., and Liotta, L. A. (2002) Use of proteomic patterns in serum to identify ovarian cancer. Lancet 359, 572–577.
5. Petricoin, E. F., Belluco, C., Araujo, R. P., and Liotta, L. A. (2006) The blood proteome: a higher dimension of information content for cancer biomarker discovery. Nat. Rev. Cancer 6, 961–967.
6. Taguchi, F., Solomon, B., Gregorc, V., Roder, H., Gray, R., Kasahara, K., Nishio, M., Brahmer, J., Spreafico, A., Ludwig, V., Massion, P. P., Dzialdzuusko, R., Schiller, J., Gregorieva, J., Tsypin, M., Hunsucker, S. W., Caprioli, R., Duncan, M. W., Hirsch, F. R., Bunn, P. A., Jr., and Carbone, D. P. (2007) Mass spectrometry to classify non-small-cell lung cancer patients for clinical outcome after treatment with epidermal growth factor receptor tyrosine kinase inhibitors: a multicohort cross-institutional study. J. Natl. Cancer Inst. 99, 838–846.
7. Bache, K. G., Slagsvold, T., and Stenmark, H. (2004) Defective downregula-
tion of receptor tyrosine kinases in cancer. EMBO J. 23, 2707–2712.
8. Reynolds, A. R., and Kyriianou, N. (2006) Growth factor signalling in prostatic growth: significance in tumour development and therapeutic targeting. Br. J. Pharmacol. 147, Suppl. 2, S144–S152.
9. Hopf, C., Bantschaff, M., and Drewes, G. (2007) Pathway proteomics and chemical proteomics team up in drug discovery. Neurodegener. Dis. 4, 270–280.
10. Azad, N. S., Rasool, N., Anunnziata, C. M., Minasian, L., Whiteley, G., and Kohn, E. C. (2006) Proteomics in clinical trials and practice: present uses and future promise. Mol. Cell. Proteomics 5, 1819–1829.
11. Choe, L., D’Saceno, M., Reinkin, N. R., Pappin, D., Ross, R., Williamson, B., Guertin, S., Pribil, P., and Lee, K. H. (2007) 8-plex quantitation of changes in cerebrospinal fluid protein expression in subjects undergo-
ing intravascular immunoglobulin treatment for Alzheimer’s disease. Proteomics 7, 3651–3660.
12. Dayon, L., Hainard, A., Vicker, V., Turk, N., Kuhn, K., Hochstrasser, D. F., Burkhard, P. R., and Sanchez, J. C. (2008) Relative quantification of proteins in human cerebrospinal fluids by MS/MS using 6-plex isobaric tags. Nat. Chem. 3, 5142–5149.
13. Wulffkuhle, J. D., Kratcmarova, I., and Kristensen, D. B., Steen, H., Pandey, A., and Mann, M. (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol. Cell. Proteomics 1, 376–386.
14. Park, K. S., Mohapatra, D. P., Bantscheff, M., and Trimmer, J. S. (2006) Graded regulation of the Kv2.1 potassium channel by variable phos-
phorylation. Science 313, 976–979.
15. Graumann, J., Hubner, N. C., Kim, J. B., Ko, K., Moser, M., Kumar, C., Cox, J., Scholer, H., and Mann, M. (2008) Stable isotope labeling by amino acids in cell culture (SILAC) and proteome quantitation of mouse embryonic stem cells to a depth of 5,111 proteins. Mol. Cell. Proteomics 7, 672–683.
16. Zhang, R., Sioma, C. S., Wang, S., and Regnier, F. E. (2001) Fractionation of isotypically labeled peptides in quantitative proteomics. Anal. Chem. 73, 5142–5149.
17. Waanders, L. F., Hanke, S., and Mann, M. (2007) Top-down quantitation and characterization of SILAC-labeled proteins. J. Am. Soc. Mass Spec-
trom. 18, 2058–2064.
18. Hanke, S., Besir, H., Cesterheilt, D., and Mann, M. (2008) Absolute SILAC for accurate quantitation of proteins in complex mixtures down to the attomole level. J. Proteome Res. 7, 1118–1130.
19. Thompson, A., Schafer, J., Kuhn, K., Kienle, S., Schwarz, J., Schmidt, G., Neumann, T., Johnstone, R., Mohammed, A. K., and Hamon, C. (2003) Tandem mass tags: a novel quantitation strategy for comparative analysis of complex protein mixtures by MS/MS. Anal. Chem. 75, 1895–1904.
20. Wiese, S., Reidegeld, K. A., Meyer, H. E., and Warscheid, B. (2007) Protein labeling by iTRAQ: a new tool for quantitative mass spectrometry in proteome research. Proteomics 7, 340–350.
21. Choe, L., D’Ascenzo, M., Reinkin, N. R., Pappin, D., Ross, R., Williamson, B., Guertin, S., Pribil, P., and Lee, K. H. (2007) 8-plex quantitation of changes in cerebrospinal fluid protein expression in subjects undergo-
ing intravascular immunoglobulin treatment for Alzheimer’s disease. Pro-
teomics 7, 3651–3660.
22. Le Blanc, J. C., Hager, J. W., Ilisiu, A. M., Hunter, C., Zhong, F., and Chu, I. (2003) Unique scanning capabilities of a new hybrid linear ion trap mass spectrometer (Q TRAP) used for high sensitivity proteomics ap-
plications. Proteomics 3, 859–869.
23. Olsen, J. V., Macek, B., Lange, O., Makarov, A., Horning, S., and Mann, M. (2007) Higher-energy C-trap dissociation for peptide modification analy-
sis. Nat. Methods 4, 709–712.

Molecular & Cellular Proteomics 7.10
31. Choi, H., Lee, H. S., and Park, Z. Y. (2008) Detection of multiphosphorylated peptides in LC-MS/MS analysis under low pH conditions. Anal. Chem. 80, 3007–3015
32. Griffin, T. J., Xie, H., Bandhakavi, S., Popko, J., Mohan, A., Carls, J. V., and Higgins, L. (2007) iTRAQ reagent-based quantitative proteomic analysis on a linear ion trap mass spectrometer. J. Proteome Res. 6, 2007–2015
33. Meany, D. L., Xie, H., Thompson, L. V., Arriaga, E. A., and Griffin, T. J. (2007) Identification of carbonylated proteins from enriched rat skeletal muscle mitochondria using affinity chromatography-stable isotope labeling and tandem mass spectrometry. Proteomics 7, 1150–1163
34. Mann, M., Ong, S. E., Gronborg, M., Steen, H., Jensen, O. N., and Pandey, A. (2002) Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. J. Proteome Res. 1, 1809–1818
35. Temporini, C., Calleri, E., Massolini, G., and Caccialanza, G. (2008) Integrated analytical strategies for the study of phosphorylation and glycosylation in proteins. Mass Spectrom. Rev. 27, 207–236
36. Ballif, B. A., Villen, J., Beausoleil, S. A., Schwartz, D., and Gygi, S. P. (2004) Phosphoproteomic analysis of the developing mouse brain. Mol. Cell. Proteomics 3, 1093–1101
37. Beausoleil, S. A., Jedrychowski, M., Schwartz, D., Elias, J. E., Villen, J., Li, J., Cohn, M. A., Cantley, L. C., and Gygi, S. P. (2004) Large-scale characterization of HeLa cell nuclear phosphoproteins. Proc. Natl. Acad. Sci. U. S. A. 101, 12130–12135
38. Villen, J., Beausoleil, S. A., Gerber, S. A., and Gygi, S. P. (2007) Large-scale phosphorylation analysis of mouse liver. Proc. Natl. Acad. Sci. U. S. A. 104, 1488–1493
39. Zhai, B., Villen, J., Beausoleil, S. A., Mintseris, J., and Gygi, S. P. (2008) Phosphoproteome analysis of Drosophila melanogaster embryos. J. Proteome Res. 7, 1675–1682
40. Pinkse, M. W., Mohammed, S., Gouw, J. W., van Breukelen, B., Vos, H. R., and Heck, A. J. (2008) Highly robust, automated, and sensitive online TiO₂-based phosphoproteomics applied to study endogenous phosphoproteins of D. melanogaster. J. Proteome Res. 7, 667–667
41. Bodenmiller, B., Mueller, L. N., Mueller, M., Domon, B., and Aebersold, R. (2007) Reproducible isolation of distinct, overlapping segments of the phosphoproteome. Nat. Methods 4, 231–237
42. Blagoev, B., Ong, S. E., Kratchmarova, I., and Mann, M. (2004) Temporal analysis of phosphotyrosine-dependent signaling networks by quantitative proteomics. Nat. Biotechnol. 22, 1139–1145
43. Dengjel, J., Akimov, V., Olsen, J. V., Bunkenborg, J., Mann, M., Blagoev, B., and Andersen, J. S. (2007) Quantitative proteomic assessment of very early cellular signaling events. Nat. Biotechnol. 25, 566–568
44. Dengjel, J., Akimov, V., Blagoev, B., and Andersen, J. S. (2007) Signal transduction by growth factor receptors: signaling in an instant. Cell Cycle 6, 2913–2916
45. Krutchevski, I., Blagoev, B., Hacq-Sorensen, M., Kassem, M., and Mann, M. (2005) Mechanism of divergent growth factor effects in mesenchymal stem cell differentiation. Science 306, 1472–1477
46. Krug, M., Krutchevski, I., Blagoev, B., Tseng, Y. H., Kahn, C. R., and Mann, M. (2008) Dissection of the insulin signaling pathway via quantitative phosphoproteomics. Proc. Natl. Acad. Sci. U. S. A. 105, 2451–2456
47. Pandey, A., Podtelejnikov, A. V., Blagoev, B., Bustelo, X. R., Mann, M., and Lodish, H. F. (2000) Analysis of receptor signaling pathways by multiple reaction monitoring mass spectrometry. Mol. Cell. Proteomics 1, 899–915
48. Miranda, E., de la Torre, J. M., and Whetton, A. D. (2005) Multiple reaction monitoring to identify sites of protein phosphorylation with high sensitivity. J. Proteome Res. 4, 3681–3692
49. Cox, D. M., Zhong, F., Du, M., Duschov, E., Sakuma, T., and McDermott, J. C. (2005) Multiple reaction monitoring as a method for identifying protein posttranslational modifications. J. Biomol. Tech. 16, 83–90
50. Unwin, R. D., Griffiths, J. R., Leverentz, M. K., Grallert, A., Hagan, I. M., and Rankovic, Z. (2004) From magic bullets to designed multiple ligands. Drug Discov. Today 9, 64–651
51. Hall, S. E. (2006) Chemoproteomics-driven drug discovery: addressing high attrition rates. Drug Discov. Today 11, 495–502
52. Murphy, R., Kay, C., and Rankovic, Z. (2004) From magic bullets to designed multiple ligands. Drug Discov. Today 9, 64–651
Chemical and Pathway Proteomics in Oncology Drug Discovery

104, 19936–19941
70. Stephens, L., Williams, R., and Hawkins, P. (2005) Phosphoinositide 3-kinases as drug targets in cancer. Curr. Opin. Pharmacol. 5, 357–365
71. Dancey, J., and Sausville, E. A. (2003) Issues and progress with protein kinase inhibitors for cancer treatment. Nat. Rev. Drug Discov. 2, 296–313
72. Mohassel, M. J., and Kelly, W. K. (2007) Histone deacetylase inhibitors: biology and mechanism of action. Cancer J. 13, 23–29
73. Budillon, A., Bruzzone, F., Di Gennaro, E., and Caraglia, M. (2005) Multiple-target drugs: inhibitors of heat shock protein 90 and of histone deacetylase. Curr. Drug Targets 6, 337–351
74. Neckers, L., and Neckers, K. (2005) Heat-shock protein 90 inhibitors as novel cancer chemotherapy—an update. Expert Opin. Emerg. Drugs 10, 137–150
75. Hopkins, A. L., Mason, J. S., and Overington, J. P. (2006) Can we rationally design promiscuous drugs? Curr. Opin. Struct. Biol. 16, 127–136
76. Caldwell, J. S. (2007) Cancer cell-based genomic and small molecule screens. Adv. Cancer Res. 96, 145–173
77. Flii, A. F., Loging, W. T., Thadieo, P., and Volkman, R. A. (2005) Analysis of drug-induced effect patterns to link structure and side effects of medicines. Nat. Chem. Biol. 1, 389–397
78. Daub, H. (2005) Characterisation of kinase-selective inhibitors by chemical proteomics. Biochim. Biophys. Acta 1754, 183–190
79. White, F. M. (2007) On the iTRAQ of kinase inhibitors. Nat. Biotechnol. 25, 994–996
80. Peters, E. C., and Gray, N. S. (2007) Chemical proteomics identifies unanticipated targets of clinical kinase inhibitors. ACS Chem. Biol. 2, 681–684
81. Sadagiani, A. M., Verhelst, S. H., and Bogoy, M. (2007) Tagging and detection strategies for activity-based proteomics. Curr. Opin. Chem. Biol. 11, 20–28
82. Cravatt, B. F., Wright, A. T., and Kozarich, J. W. (2008) Activity-based probes that target diverse cysteine protease families. Nat. Chem. Biol. 4, 33–38
83. Salisbury, C. M., and Cravatt, B. F. (2007) Activity-based probes for proteomic profiling of histone deacetylase complexes. Proc. Natl. Acad. Sci. U. S. A. 104, 1171–1176
84. Leung, D., Hardouin, C., Boger, D. L., and Cravatt, B. F. (2003) Discovering potent and selective reversible inhibitors of enzymes in complex proteomes. Nat. Biotechnol. 21, 687–690
85. Patriccii, M. P., Szardening, A. K., Liyanage, M., Nomanbhoy, T. K., Wu, M., Weissig, H., Alman, A., Chun, D., Tanner, S., and Kozarich, J. W. (2007) Functional interrogation of the kinome using nucleotide acyl phosphates. Biochemistry 46, 350–358
86. Olaiyewo, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000) The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J. 19, 3159–3167
87. Hickey, M. (2008) Targeted Therapies in Cancer: Myth or Reality?
88. Patricelli, M. P., Szardenings, A. K., Liyanage, M., Nomanbhoy, T. K., Wu, M., Weissig, H., Alman, A., Chun, D., Tanner, S., and Kozarich, J. W. (2007) Functional interrogation of the kinome using nucleotide acyl phosphates. Biochemistry 46, 350–358
89. Olaiyewo, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000) The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J. 19, 3159–3167
90. Beck, H. C., Nielsen, E. C., Matthiesen, R., Jensen, L. H., Sehested, M., Finn, P., Grauslund, M., Hansen, A. M., and Jensen, O. N. (2006) Quantitative proteomic analysis of post-translational modifications of human histones. Mol. Cell. Proteomics 5, 1314–1325
91. Sharma, S. V., Bell, D. W., Settlemier, J., and Haber, D. A. (2007) Epidermal growth factor receptor mutations in lung cancer. Nat. Rev. Cancer 7, 169–181
92. Riely, G. J., Politi, K. A., Miller, V. A., and Pao, W. (2008) Update on epidermal growth factor receptor mutations in non-small cell lung cancer. Clin. Cancer Res. 14, 7232–7241
93. Yun, C. H., Mengwasser, K. E., Toms, A. V., Woo, M. S., Greulich, H., Wong, K. K., Meyerson, M., and Le QT (2003) The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. Proc. Natl. Acad. Sci. U. S. A. 100, 19466–19471
94. Engelman, A. J., Ziejnajula, K., Matsuoka, T., Song, Y., Hyland, C., Park, J. O., Lindemann, N., Gale, C. M., Zhao, X., Christensen, J., Kosaka, T., Holmes, A. J., Rogers, A. M., Cappuzzo, F., Mok, T., Lee, C., Johnson, B. E., Canfield, L. L., and Janne, P. A. (2003) MET amplification leads to
last names