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Review

# Methods for investigation of targeted kinase inhibitor therapy using chemical proteomics and phosphorylation profiling

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ABSTRACT

Phosphorylation acts as a molecular switch for many regulatory events in signaling pathways that drive cell division, proliferation, and apoptosis. Because of the critical nature of these protein post-translational modifications in cancer, drug development programs often focus on inhibitors for kinases and phosphatases, which control protein phosphorylation. Numerous kinase inhibitors have entered clinical use, but prediction of their efficacy and a molecular basis for patient response remain uncertain. Chemical proteomics, the combination of drug affinity chromatography with mass spectrometry, identifies potential target proteins that bind to the drugs. Phosphorylation profiling can complement chemical proteomics by cataloging modifications in the target kinases and their downstream substrates using phosphopeptide enrichment and quantitative mass spectrometry. These experiments shed light on the mechanism of disease development and illuminate candidate biomarkers to guide personalized therapeutic strategies. In this review, commonly applied technologies and workflows are discussed to illustrate the role of proteomics in examining tumor biology and therapeutic intervention using kinase inhibitors.

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**Contents**



## 1. Introduction

Tumor formation and progression can be driven by signaling pathways controlled by reversible protein phosphorylation events [1–4]. Of the kinases and phosphatases that cause tumors, approximately half are protein tyrosine kinases [5] (*e.g.* the

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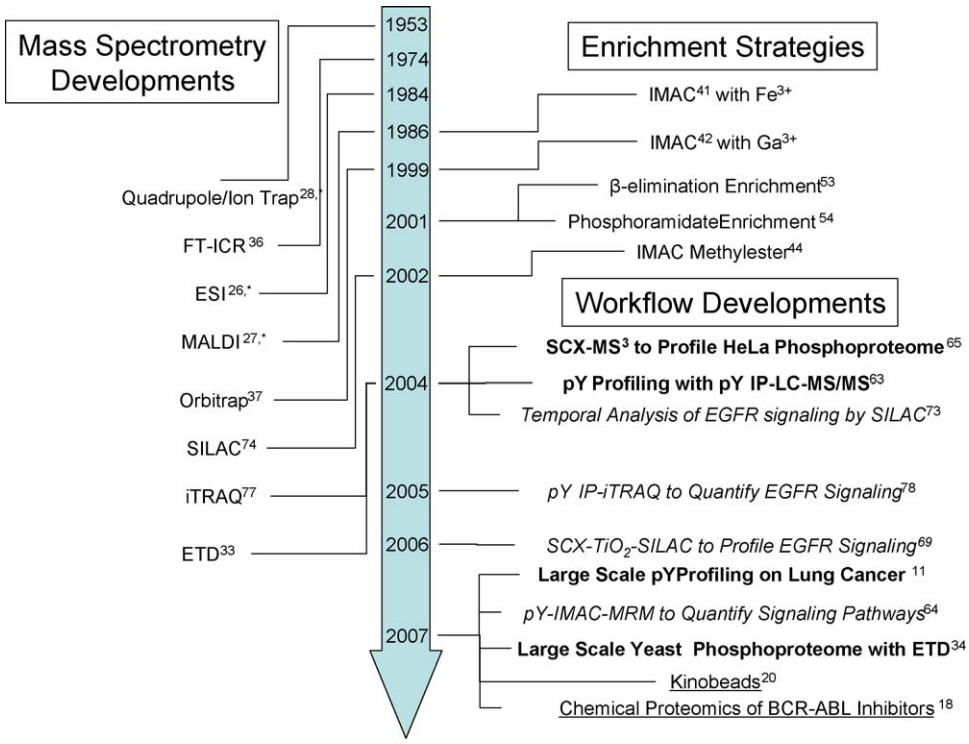


**Fig. 1.** Selected milestones in proteomics. Events in mass spectrometry required for the development of current LC–MS/MS capabilities are shown on the left. On the right, different enrichment and separations strategies are listed with fully developed workflows for phosphorylation analysis. The asterisks denote later award of Nobel Prize (Paul, Physics 1989; Fenn and Tanaka, Chemistry 2002) Profiling experiments are presented in bold; italics are used for quantitative protocols and examples of chemical proteomics are underlined.

epidermal growth factor receptor (EGFR), which drives various tumors, including lung cancer [6]); the remainder are serine/threonine kinases (*e.g.* constitutively active mutant B-Raf V600E that causes melanoma). Because of their importance in the development of cancer, protein kinases are approximately 20% of current druggable targets [7]. Monoclonal antibodies that target protein tyrosine kinases and small molecule protein tyrosine kinase inhibitors (TKIs) have already been used to treat patients with various types of cancer [8]. For example, Imatinib (Gleevec, STI571; Novartis, Basel, Switzerland), a BCR/ABL and Src family tyrosine kinase inhibitor, has been approved to treat patients with chronic myelogenous leukemia (CML) [9]. Trastuzumab (Herceptin; Genentech, South San Francisco, CA), an antibody against HER2/Neu/Erb2 receptor-type tyrosine kinase that causes its inactivation, has shown promising effect in combination with other anticancer agents in treating HER2-overexpressing breast cancers [10]. Although these treatments can improve patient survival, most tyrosine kinase driven oncogenic pathways remain unclear due to the complexity of the network. Furthermore, multiple activated tyrosine kinases can work together in tumors [11]. In addition, patients often acquire drug resistance and biomarkers to accurately predict patients' response to therapy are not currently available. These challenges remind us that comprehensive identification of drug target proteins and examination of downstream signaling pathways are necessary to assist in the development of personalized medicine.

Chemical proteomics and phosphorylation profiling technologies based on liquid chromatography coupled to tandem mass spectrometry peptide sequencing (LC–MS/MS) have emerged as the tool of choice to identify the full spectrum of drug-binding proteins and to characterize phosphorylation events. The discoveries from these studies shed light on the mechanism of tumor development, help to develop rational drug targets, and illuminate candidate biomarkers for targeted therapeutic strategies [12]. A brief historical perspective of developments in biological methods, separations and mass spectrometry is presented (see Fig. 1). Each method will be described as it was applied to identification of phosphorylation sites in a single protein and in proteome-wide experiments. All of these techniques and technologies contribute to our current capabilities in chemical proteomics and phosphorylation analysis and profiling. Current benchmark experiments established in the primary literature are discussed, and example protocols are included as supplementary material.

## 2. Chemical proteomics

Drug affinity chromatography is a well established method for protein purification, taking advantage of the highly specific and reversible binding between enzymes and their specific inhibitors. A protein mixture containing the enzyme of interest is passed through a column containing immobilized inhibitor. The proteins that bind to the inhibitor are retained on the column. Elution of bound proteins is performed by addition of a competitive inhibitor in solution [13] or protein denaturation.

Chemical proteomics, which combines drug affinity chromatography and mass spectrometry, can provide a comprehensive profile of drug-binding proteins. Identification of all potential drug targets also helps us to understand the mechanisms of side effects and drug resistance; tailored therapeutic strategies can be derived to maximize drug efficacy and minimize the side effects or toxicity [14]. These techniques have been implemented to study kinases and kinase inhibitors [15,16]. The study on a widely used p38 MAP kinase inhibitor, SB 203580, revealed several previously unknown protein kinase targets, including cyclin G-associated kinase (GAK), casein kinase 1 (CK1), Rip-like interacting caspase-like apoptosis-regulatory protein (RICK) and kinase/Rip2/CARDIAK (CLARP). While chemical proteomics only indicates binding between the inhibitor and the kinase, subsequent *in vitro* kinase assays verify

specific inhibition by the drug, SB 203580. The identification of these other kinases as drug targets will significantly influence the development of p38 inhibitors as anti-inflammatory drugs [17]. Chemical proteomics techniques were also applied to determine protein binding partners of BCR-ABL tyrosine kinase inhibitors (*i.e.* imatinib, nilotinib and dasatinib) that are currently used to treat chronic myeloid leukemia [18]. The comprehensive drug–protein interaction profiles revealed different interaction profiles for each drug. Dasatinib bound a significantly larger number of kinases, including Src, Abl and major regulators of the immune system. The receptor tyrosine kinase, DDR1, was identified as an additional major target of nilotinib. Non-kinase targets can also be identified; *e.g.* the oxidoreductase, NQO2, was bound to and inhibited by imatinib and nilotinib.

Large scale kinase identification can also be achieved using inhibitor affinity selection coupled with LC–MS/MS protein identification. Panels of inhibitors have been used to isolate protein kinases from cell lysates; additional phosphorylation analysis was also used to examine their modifications [19]. Kinobeads have been formulated with seven immobilized inhibitors, including Bis-(III) indolyl-maleimide, purvalanol B, staurosporine, CZC8004, analogs of PD173955, sunitinib, as well as vandetanib; following protein target recovery and LC–MS/MS, several hundred phosphorylation events were identified on ~200 proteins [20]. A pyrido[2,3-d]pyrimidine-based molecule, PP58, exhibited high potency and non-selectivity when applied to capture protein kinases; approximately 25% of the human kinome could be recovered [21]. Based on structure–activity relationship data previously described for Cdk4 inhibition by pyrido(2,3-d]pyrimidine kinase antagonists [22]), a novel affinity matrix containing the immobilized compound, VI16832, was developed as an enrichment tool, which enabled detection 1200 phosphorylation sites on more than 200 distinct protein kinases when implemented in leukemia cells [23]. These broad scale experiments with panels of kinase inhibitors or individual molecules with less specific interaction profiles can enable detection of numerous kinases in biological samples, opening the door for systems biology approaches to phosphoproteomics.

## 3. Phosphorylation analysis of individual proteins

Mass spectrometry-based phosphoproteomics is a powerful tool to catalog and quantify phosphorylation events, because of existing enrichment techniques, multidimensional peptide separations, peptide sequencing, and available bioinformatics tools [24]. The 'bottom-up' proteomics approach involves the proteolytic digestion and peptide sequencing with LC–MS/MS. During experiment design, *in silico* sequence analysis can be used to maximize protein sequence coverage and detection of potentially modified amino acids. For example, the web-based *in silico* Proteomics Experiment Planner (iPEP) [25] can compare the effectiveness of different proteolytic digestions for detecting specific amino acid residues or sequences using different types of mass spectrometers, resulting in informed selection of proteolytic reagents and maximum detection of the residues of interest for an individual protein.

When characterizing the phosphorylation events on a single protein, the initial step is to prepare the target protein from the biological samples, ideally in a purified form. Immunoprecipitation is the method of choice when antibodies against the target protein are available. Other methods include affinity purification techniques using fusion proteins that include tags like glutathione *S*-transferase, which can be recovered by specific binding to in columns containing immobilized glutathione. Regardless of purification strategy, SDS-PAGE is often used to separate co-eluted protein components and remove certain buffer components that are not compatible with mass spectrometry. After denaturation, reduction, and alkylation, the eluted proteins can be digested either directly in-solution or in-gel.

After proteolytic digestions, peptides are recovered and ready for mass spectrometry analysis. The development of soft ionization techniques (electrospray ionization [26] and matrix assisted laser desorption/ionization [27]) and modern mass analyzers (from quadrupole and ion trap [28] to high performance FT-ICR and orbital ion trap instruments) have all made essential contributions to LC–MS/MS peptide sequencing, protein identification, and post-translational modification (PTM) analysis. As Fig. 2 illustrates, LC–MS/MS is a powerful tool to detect and sequencing phosphorylated peptides: when phosphorylation occurs, one phosphate group will be added onto a serine, threonine or tyrosine residue, which will exhibit a +80 Da mass shift. LC–MS/MS measures the mass difference between intact unmodified and phosphorylated peptides (Fig. 2A and C) and also provides peptide sequence information that can lead to assignment of the specific phosphorylation site even when there are multiple potential phosphorylation sites in the sequence, as shown in Fig. 2B and D.

During MS/MS fragmentation caused by low-energy collision-induced dissociation (CID), phosphoserine and phosphothreonine residues in phosphopeptides will often lose phosphate ($HPO_3$, 80 Da) or undergo β-elimination of phosphoric acid ($H_3PO_4$, 98 Da), because the phosphate bonds are the weakest in the molecule [29]. If the peptide has neutral loss of 80 Da, the phosphorylation site cannot be assigned from the fragments because no mass tag remains. In the case of β-elimination, phosphoserine becomes dehydroalanine and phosphothreonine becomes dehydroaminobutyric acid; these modified amino acids can be used to localize the site of post-translational modification. In either case, peptide backbone fragmentation and subsequent sequence assignments are poor, when the neutral loss reaction is predominant. As a result, less information can be extracted from LC–MS/MS analysis. One strategy is to isolate the neutral loss fragment in MS/MS spectra to generate MS/MS/MS ($MS^3$) spectra, to obtain more informative peptide backbone fragments [30,31]. Beausoleil et al. were able to identify 2002 phosphorylation sites from 2D-LC–MS/MS of tryptically digested HeLa cell nuclear fractions by applying data-dependent $MS^3$ scans [32].

In addition to CID, a complementary peptide fragmentation strategy using electron transfer dissociation (ETD) was introduced [33]. In this process, electrons are transferred to peptides from fluoranthene radical-anions generated in a chemical ionization source. This reaction triggers fragmentation of the peptide backbone between the nitrogen of the amide bond and the chiral carbon to produce c and z fragment ions. Because ETD is not directed at the weakest bonds, phosphorylation is retained on the modified amino acid, facilitating the assignment of the phosphorylation sites. In example studies using ETD, 1252 phosphorylation sites were identified from 30 μg of yeast protein [34], and over ten thousand phosphorylation sites were identified in human embryonic stem cells [35].

The introduction of high performance mass spectrometers such as Fourier transform ion cyclotron resonance (FT-ICR) [36] and orbital ion trap [37,38] instruments that have high resolution and high mass measurement accuracy have significantly advanced phosphoproteomics [39]. Methods that employ higher mass accuracy and higher resolution instruments have been demonstrated to identify over twice as many unique phosphopeptides [40]. These instruments have also enabled relative quantification using extracted ion chromatograms (EIC); the high resolution and accurate mass measurement enable peak matching across samples, particularly when MS/MS data are available for verification. The process for EIC analysis is shown in Fig. 3. The LC–MS/MS elution profile for each identified peptide can be extracted using its

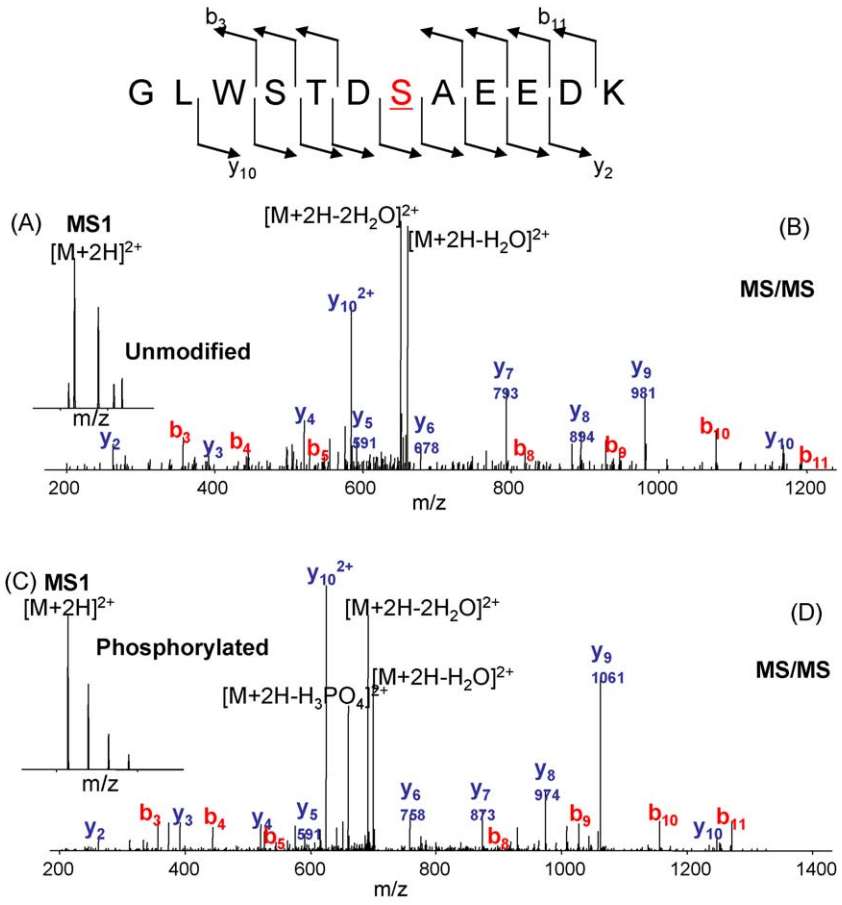


**Fig. 2.** Comparison of LC–MS/MS data for unmodified and phosphorylated peptides. When a phosphate group is added to a serine, threonine or tyrosine residue the result is an 80 Da mass shift. LC–MS/MS is a powerful tool to sequencing phosphopeptides because not only can it measure the mass difference between unmodified and phosphorylated peptides, it can also provide peptide sequencing information that will lead to assignment of the specific phosphorylation site on that peptide. The mass measurement of doubly charged unmodified peptide precursor is 669.2988, measured with 3.1 ppm error (A) can be paired with the doubly charged phosphorylated peptide precursor is 709.2833, measured with 4.7 ppm error (C), because of the 80 Da mass shift. The tandem mass spectrum of unmodified GLWSTDSAEEDK (B) can be compared with the MS/MS spectrum of phosphorylated GLWSTDSAEEDK (D) to localize the phosphorylation to one of the three candidate sites. The difference in mass-to-charge ratio between $y_5$ and $y_6$ indicates the site of the phosphorylation on a serine residue.

precursor mass and retention time. The integrated peak area is used to quantify the relative amount of the peptide in each sample. The intensities can be normalized against internal standards spiked prior to mass spectrometry analysis; modified peptides can also be normalized by other unmodified peptides from the same protein to normalize for differences in sample loading.

The identification of phosphopeptides relies heavily on commercial and academic software packages for matching tandem mass spectra with theoretical peptide fragment patterns generated *in silico* from protein databases. Although some progress is being made in this field, manual verification of MS/MS spectra remains necessary in order to reduce false positive results and ensure high quality data is reported.

## 4. Chemical enrichment strategies for phosphopeptides

If target protein has a low stoichiometry of phosphorylation, a phosphopeptide enrichment step can be carried on before mass spectrometry analysis. Immobilized metal affinity chromatography (IMAC) captures phosphopeptides based on the phosphate group's affinity for metal ions. The stationary phase of IMAC contains iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA), which chelates triply charged iron ($Fe^{3+}$) [41] or gallium ($Ga^{3+}$) ions [42]. In 1994, Watts et al. demonstrated the utility of this technique by detecting *in vitro* sites of tyrosine phosphorylation induced on the T cell-specific protein tyrosine kinase ZAP-70 [43]. In phosphoproteomics, nonspecific binding is commonly observed, particularly for acidic peptides. Chemical derivatization methods have been introduced to reduce the nonspecific binding, *e.g.* conversion of carboxylic acids to methyl esters [44]. The selectivity of IMAC has also been shown by effective enrichment of low abundance phosphopeptides (2%) from a background of unmodified protein [42]. Large scale *in vivo* phosphoprotein profiling experiments are also feasible using IMAC phosphopeptides isolated from *Arabidopsis thaliana* plasma membranes with greater than 75% purity, as determined by LC–MS/MS [45].

Metal oxide affinity chromatography (MOAC) provides a similar mechanism for phosphopeptide extraction that has slightly different selectivity [46,47]. Titanium dioxide particles can extract femtomole amounts of phosphopeptides from proteolytic digests [48]. Zirconium dioxide was used to successfully enrich phosphopeptides from β-casein and α (S1)-casein [49]. Aluminum hydroxide enrichment has recovered phosphopeptides from α-casein and protein extracted prepared from *A. thaliana* leaves. For MOAC, selectivity can be improved by adding glutamic acid and aspartic acid in the loading buffer [42,50]. Loading peptides with the presence of 2,5-dihydroxybenzoic acid (DHB) has been shown to be a very efficient way to reduce nonspecific peptide binding to $TiO_2$ while retaining high affinity for phosphorylated peptides [46].

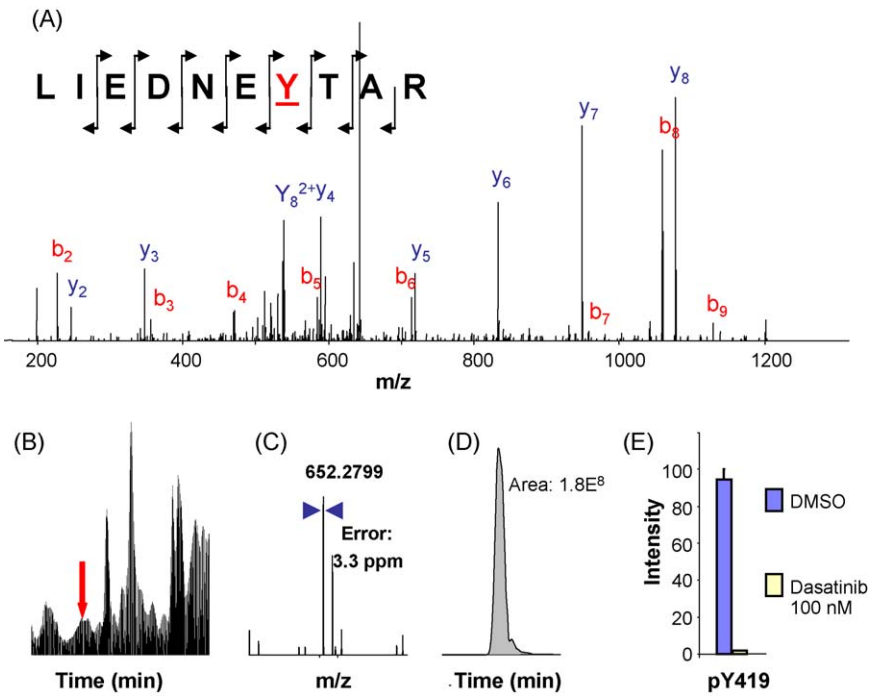


**Fig. 3.** Phosphopeptide quantification using extracted ion chromatograms (EIC). Using accurate mass measurements and relying on reproducible LC gradients, the ion chromatograms of phosphopeptides can be extracted across the sample set. Integrated peak areas can be used to quantify the relative phosphorylation intensities between samples. First, confident phosphotyrosine peptide identification was achieved (A). The tandem mass spectrum is located in the data (B); here, the peptide was identified at 23.7 min. The assignment can be verified using the mass measurement error, 3.3 ppm, and isotope ratio matching (C). Integrated peak areas for peptide quantification are calculated from extracted ion chromatograms (EIC) using QuanBrowser from Xcalibur 2.0 (D). These values were restricted by $m/z$ (±0.02) and retention time (±60 s). By applying EIC, the phosphorylation on SRC pY419 was observed to decrease dramatically after 100 nM Dasatinib treatment (E).

## 5. Chemical derivatization mechanisms for phosphopeptide isolation

Different synthetic schemes for replacement or coupling of phosphorylation sites have also been successfully demonstrated for enrichment. The phosphoprotein isotope-coded affinity tag (PhIAT) method used β-elimination and Michael addition to replace the phosphate with ethanedithiol. The remaining sulfhydryl is biotinylated for purification with avidin–biotin affinity [51]. Phosphoramidite chemistry formed the basis for another recovery mechanism using polyamidoamine dendrimers. A six-step derivatization and purification protocol was employed to react the carboxylate and phosphate groups on peptides to form amide and phosphoramidite bonds, followed by phosphate regeneration [52]. Although enrichment is successful, the number of steps of chemical derivatizations and purifications would increase the time and effort required for each experiment and could decrease overall phosphopeptide yield.

## 6. Phosphotyrosine profiling

Tyrosine kinases are encoded by approximately 100 genes in humans [53]. One family, cell surface receptor protein tyrosine kinases (RTKs), acts to transduce extracellular stimulus by tyrosine phosphorylation cascades (*e.g.* EGFR) [54]. Once the receptors are phosphorylated, a variety of nonreceptor kinases are recruited and activated to induce downstream cellular signaling pathways by phosphorylating cytoplasmic kinases, such as Janus kinase (Jak) or Src [55]. In every step of the signaling cascade, tyrosine phosphorylation is involved in the recruitment of multiprotein complexes and activation of downstream targets. To date, more than half of protein tyrosine kinases have been linked to human cancers caused by constitutively active mutants [56]. Despite the promising drugs that specifically target these signaling molecules, clinical efficacy remains low because the mechanisms of signaling pathways are not fully understood. During clinical application, drug resistance and patient relapse are commonly observed [57]. Therefore, phosphotyrosine profiling can still play a critical role in elucidation of tumor biology and prediction clinical outcomes, specifically the response to targeted therapy.

The characterization of the phosphotyrosine proteome is challenging, because tyrosine phosphorylation represents only 0.05% of total protein phosphorylation [58]. Therefore, enrichment of phosphotyrosine-containing proteins or peptides must occur by a more specific mechanism than the IMAC or MOAC affinity enrichment for total phosphopeptides. Anti-phosphotyrosine antibody development has created the required affinity substrate for phosphotyrosine peptide recovery. Substrates of EGFR that are tyrosine-phosphorylated upon EGF stimulus in HeLa cells have been identified using affinity selection with immobilized anti-phosphotyrosine antibodies, followed by SDS-PAGE separation, in-gel digestion, and LC–MS/MS analysis; among many known substrates, a novel signaling molecule, STAM2, was also shown to be associated with the EGFR signaling pathway [59]. The combination of immunoaffinity purification of phosphotyrosine-containing proteins, two-dimensional gel separation, and mass spectrometry, tumor-specific phosphoproteome profiles in primary human breast cancers were shown to include Vimentin, HSP70, and actin [60].

Recently, a peptide-based phosphotyrosine profiling strategy has been developed using immunoaffinity purification of phosphotyrosine-containing peptides prior to LC–MS/MS analysis [61]. In this strategy, the cells are lysed in denaturing buffer and the proteins are reduced and alkylated, followed by trypsin digestion. After reverse phase peptide extraction, the digest mixture is resuspended in immunoaffinity buffer for purification of phosphotyrosine-containing peptides by immunoprecipitation. The eluted peptides are analyzed with LC–MS/MS, leading to the identification of 628

phosphotyrosine sites from three different cell lines. Similar efforts have been used to profile signaling pathways in human mammary epithelial cells, integrating phosphotyrosine immunoprecipitation with subsequent IMAC phosphopeptide enrichment to identify 222 phosphotyrosine sites [62].

Because this peptide-based strategy generates the highest yield in terms of identified peptides and modification sites with increased specificity, the method has been applied to examine the phosphotyrosine-dependent signaling pathways in cultured cancer cells and patient tumors. Using phosphotyrosine profiling of 41 lung cancer cell lines and 150 tumor tissue samples, 4551 phosphotyrosine sites were identified; nearly all of 266 phosphotyrosine sites on 56 different tyrosine kinases were previously associated with kinase activities [11]. This study provides valuable information about oncogenic signaling pathways in lung cancer by detection of tyrosine kinases and their substrates. In addition, classification schemes can be determined for grouping different cell lines and tumor types by activated tyrosine kinases.

## 7. Phosphoserine/phosphothreonine profiling

Unlike phosphotyrosine profiling, IMAC/MOAC enrichment of the entire proteome recovers more peptides than can be detected and sequenced in a single LC–MS/MS analysis. Therefore, pre-fractionation prior to phosphopeptide enrichment is required to increase the number of peptides and modification sites detected. Strong cation exchange (SCX) chromatography at low pH is commonly used to separate tryptic digests prior to phosphopeptide enrichment. When compared with the unmodified sequence, phosphopeptide retention on SCX columns decreases and phosphopeptides will elute earlier [32]. The combination of SCX with either IMAC or $TiO_2$ has been proven to be the most comprehensive technology to profile the phosphoproteome. By applying SCX and IMAC, Gygi's group identified 5635 nonredundant phosphorylation sites in mouse liver [63] and 13,720 phosphorylation sites in proteins extracted from *Drosophila* embryos [64]. The advantage of this combination is that SCX groups peptides based on charges; so in each fraction, competition between peptides is more uniform and there should be little or no discrimination between singly and multiply phosphorylated peptides[65]. SCX fractionation and MOAC enrichment were applied in the study of phosphorylation dynamics in signaling networks of HeLa breast cancer cells stimulated with epidermal growth factor (EGF), in which 6600 phosphorylation sites were identified [66]. The combination of SCX and MOAC offered a 4-fold improvement when compared to SCX alone [67]. Titanium dioxide was also used in a study that identified a total of 4002 unique phosphopeptides from SKBr3 breast cancer cells [42].

## 8. Sequential enrichment of phosphotyrosine and total remaining phosphopeptides

In our effort to profile and phosphoproteome of cancer cells before and after administration of targeted therapeutic agents, a two-step enrichment strategy is applied, as diagrammed in Fig. 4

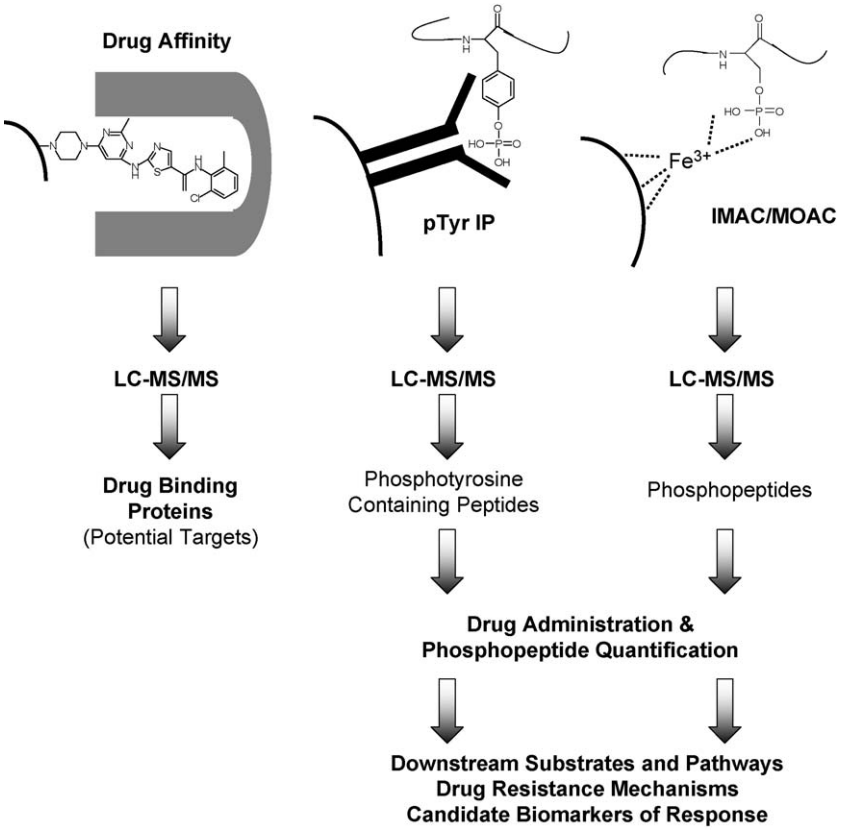


**Fig. 4.** Implementation of affinity chromatography and mass spectrometry in selection of kinase inhibitor therapy. In chemical proteomics (left), drug affinity matrices are prepared by immobilizing the drug molecule of interest or a derivative structure. After incubation with cell lysates, the proteins that bind to the drug are identified with LC–MS/MS and database searching. Downstream substrates and pathways can be identified by phosphoproteomics (right), which couples affinity selection methods (*i.e.* anti-phosphotyrosine antibodies and immobilized metal affinity chromatography) with LC–MS/MS phosphopeptide sequencing. These techniques yield catalogs of modification sites, and extracted ion chromatograms can also be used to derive quantitative information. Because these phosphorylation sites correlate to the activity of the proteins, these techniques can be used to quantify the drug response, enabling dose response or timescale studies. The final outcomes of these experiments are candidate biomarkers of response and initial data to examine drug resistance. Additional experiments can be implemented to gain additional information, including SH2 Domain Profiling (binding partners for each phosphorylation site), activity-based protein profiling (active kinases). The data from these experiments can be funneled into targeted quantification workflows using LC–MRM.

and described in the supplementary material. First, immobilized anti-phosphotyrosine antibodies are used to pull down phosphotyrosine-containing peptides. The flowthrough from the phosphotyrosine immunoprecipitation buffer exchanged and separated with SCX chromatography into 11 fractions. Phosphoserine and phosphothreonine peptides in each fraction are enriched using IMAC. Both enrichment steps are followed by LC–MS/MS analysis on an LTQ-Orbitrap and database searching. We are able to routinely identify 2500–3500 phosphopeptides; quantitative information can also be obtained using extracted ion chromatograms.

## 9. Quantitative phosphoproteomics

Although phosphoproteomics experiments are capable of generating catalogs of modified peptide containing more than ten thousand of phosphorylation sites, the value of the data is increased significantly when quantitative results are also available. When performed with quantitative methods, the results from such large phosphoproteome profiling experiments suggest that only a small fraction of total phosphorylation sites that respond to stimulus [68].

For examination of downstream effects of kinase inhibitor therapy, the changes of the phosphorylation status in response to external stimulus (*e.g.* activation by ligand) or drug treatment can be monitored on targeted phosphorylation sites using LC–MS/MS [69]. Temporal phosphorylation can also be quantitatively monitored. Dynamic profiles of 81 signaling proteins, including nearly all known epidermal growth factor receptor substrates, have been created over five time points subsequent to ligand stimulation [70].

Stable isotope labeling by amino acids in cell culture (SILAC) [71] is a widely used technique, which enables relative quantification. Control cells are cultured in "light" medium containing amino acids with elements in their natural abundances; the experimental group is metabolically labeled with "heavy" amino acids containing stable isotopes (*e.g.* $^{13}C$ and $^{15}N$ labeled lysine and arginine). The proteins from both cell cultures are combined and analyzed with LC–MS/MS. The chemically identical peptide pairs can be differentiated by mass spectrometry because of their mass difference. Comparison of the peak areas in the mass spectra for each peptide pairs is used to calculate the relative abundance of the two proteins in the two samples, which is expressed as a heavy to light ratio. SILAC has been applied to quantify the modification of known phosphorylation sites [72] as well as unknown phosphorylation events [60]. Guided by peptide sequencing results, SILAC data could be manually interpreted in an extremely arduous process. The open source software, MaxQuant [73], was recently introduced; it automatically identifies and quantifies SILAC peptides from LC–MS/MS data.

Chemical labeling approaches have also been successful for quantitative phosphoproteomics. Isobaric tagging for relative and absolute quantification (iTRAQ) [74] labels peptides from different samples with different amine-reactive isobaric tags. The fragmentation of the tags generates reporter ions in the low mass region that can be used for relative quantification of each peptide from 4 or 8 samples. In the Kinobeads study described above, iTRAQ was applied to quantify on- and off-target effects of kinase inhibitors [20]. The binding of specific proteins to the beads was monitored as competing kinase inhibitor concentration increased. The affinity binding constants for hundreds of kinases from multiple cell lines and tissues were determined. In an effort to study the temporal phosphorylation changes in EGFR signaling cascade upon stimulation, iTRAQ reagents were used to label peptides from four different time points. The iTRAQ reporter ion ratios provided temporal phosphorylation profiles for 78 phosphotyrosine sites in EGFR and downstream substrates [75].

## 10. Complementary emerging techniques

Additional quantification and functional biochemistry experiments are required to validate the responses associated with particular phosphorylation events on selected proteins [76]. Several recent developments can also contribute complementary information about kinase inhibitor therapy, including SH2 domain binding profiling, activity-based protein profiling (ABPP) and liquid chromatography coupled to multiple reaction monitoring mass spectrometry (LC–MRM).

Tyrosine-phosphorylated signaling proteins are specifically recognized by Src Homology 2 (SH2) protein binding domains. More than 100 SH2 domains are encoded in the human genome; each SH2 domain binds to a different group of tyrosine-phosphorylated ligands and provides information on the signaling state of those substrates. A competitive binding assay based on the far-Western blotting has been developed, in which Src homology 2 (SH2) domain probes are used to detect patterns of tyrosine-phosphorylated sites in cell lysates [77]. This tool may have value in molecular diagnostics to classify different cell lines or tumors. SH2 domain binding profiles were also shown to be able to distinguish highly related multiple myeloma (MM) cell lines that have different resistance to anticancer agents [78]. This study implies that tyrosine-phosphorylation plays important role in the mechanism of drug resistance and those proteins could potentially serve as biomarkers.

Activity-based protein profiling (ABPP) is a functional proteomic technology that uses specially designed chemical probes that bind to active sites of enzymes and then react forming a covalent linkage [79,80]. Tagged probes can be imaged using fluorescence or recovered by avidin–biotin complex formation. Because the labeling is based on enzyme's catalytic properties and it is independent of protein expression level, ABPP technology distinguishes active enzymes from their inactive counterparts and can provide access to low abundance proteins because of the specific selection mechanism. When biotinylated ABPP probes are combined with tandem mass spectrometry, the identification of hundreds of active enzymes from a single sample can be achieved. Probes are available for profiling cysteine proteases [81] and serine hydrolases [77]. By applying such technology to molecular classification of tumors, three enzymes (protease FAP/Seprase, lipase PAF-AH2 and hydrolase KIAA1363) were found to be highly elevated in ER(−)/PR(−) human breast cancer specimens, when compared with other breast tumors [82]. The probes that target serine hydrolases have been used to profile enzyme activities in different human breast and melanoma cancer cell lines, leading to a classification scheme based on tissue of origin and the state of invasiveness [83]. Although no probes have been published for kinases to date, two probes for protein tyrosine phosphatases (PTPs) have been synthesized for imaging using a dansyl fluorophore and protein recovery using biotin [84]. Another highly specific PTP probe that consists of α-bromobenzylphosphonate as the PTP-specific binding site, a linker and a biotin tag [85]. The use of these probes with in-gel imaging reveals patterns of active enzymes in each biological sample and could be used for rapid screening of proteins for later identification with LC–MS/MS after using a similar biotinylated probe for protein recovery.

Most LC–MS/MS analyses collect data using data-dependent acquisition (DDA) mode. In this mode, the mass spectrometer acquires a full $MS^1$ spectrum, followed by several tandem spectra by selecting and fragmenting the most abundant precursor ions in $MS^1$ spectrum. The whole cycle repeats continuously throughout the analysis. In complex samples, multiple peptides eluted every

retention times, so the instrument will not select and fragment the same set of precursors every time, especially if the abundances of the peptides vary in different samples. The overall result is poor reproducibility in both peptide identification and quantification using DDA LC–MS/MS. Variations in peptide identifications between technical replicates are typically in the range of 20–30% [63,64]. An alternative strategy is to apply LC–MRM, in which the instrument constantly monitors transitions from selected peptide precursors to specific fragment ions producing extremely sensitive measurements of peptide quantity. The presence of chromatographic coelution of multiple transitions provides high confidence for assignment of selected peptides. Integrated workflows with both DDA LC–MS/MS and LC–MRM are capable of identifying more than 200 phosphotyrosine sites in the EGFR signaling network, and quantifying these phosphorylation events across 7 stimulation time points with high reproducibility [62]. When compared with DDA analyses, in which only 34% of the phosphorylation events can be reproducibly quantified, significant improvement is shown in this study as 88% of the phosphorylation events can be reproducibly quantified using LC–MRM.

## 11. Summary

Over the last several decades, techniques and technology have developed to support the combination of affinity methods and LC–MS/MS, enabling our current capabilities in chemical proteomics and phosphorylation profiling. Powerful enrichment technologies and extensive separation strategies enable researchers to probe deeper into the phosphoproteome. Biological (SILAC) and chemical (iTRAQ) labeling strategies allow relative quantification of the differences in protein amount and modification between different samples. Optimized workflows are now available to tackle a wide variety of questions from detecting the spectrum of drug targets to quantification of the changes in phosphorylation in response to biological signaling events and kinase inhibitor treatment. The current state of the art combines affinity purification schemes with LC–MS/MS to enable unbiased, systems level views of signaling pathways.

Additional technical improvements can still be made; new methods continue to be developed. Despite these advances in phosphoproteome profiling, no single technology is currently comprehensive in profiling the entire phosphoproteome, as illustrated by the comparison of three phosphoproteome profiles obtained for *Drosophila* [64,67,86]. Each study generated a large dataset containing around ten thousand phosphorylation sites, but the overlap in phosphoprotein identification between these studies is around 70% and in phosphorylation sites is around 50% [64]. The combination of LC–MS/MS-based techniques with emerging technologies, like SH2 domain profiling and activity-based protein profiling, can be used to develop complementary strategies for the assessment of tumor biology and drug response.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.03.027.

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