

Strategies for kinome profiling in cancer and potential clinical applications: chemical proteomics and array-based methods

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Received: 19 February 2010 / Revised: 6 April 2010 / Accepted: 24 April 2010 / Published online: 8 June 2010
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Abstract Kinases are key enzymes involved in deregulated signal transduction associated with cancer development and progression. The advent of personalized medicine drives the development of new diagnostic tools for patient stratification and therapy selection Ginsburg and Willard (Transl Res 154:277-287, 2009). Since deregulation of kinase-mediated signal transduction is implied in tumorigenesis, the analysis of all kinases (the kinome) active in a particular tumor may yield tumor-specific information on aberrant cell signalling pathways. Tumor tissue kinase activity profiles may correlate with response to therapy and therefore may be used for future therapy selection. In this Trend paper we describe peptide array and mass spectrometry-based technologies and new developments for kinome profiling, and we present an outlook towards future implementation of therapy selection based on kinome profiling in clinical practice.

Keywords Kinase · Kinome profiling · Chemical proteomics · Peptide substrate array · Kinase inhibitor

Introduction

Kinases are key enzymes involved in deregulated signal transduction associated with cancer development and progression. The advent of personalized medicine drives the development of new diagnostic tools for patient stratification and therapy selection [1]. Since deregulation of kinase-mediated signal transduction is implied in tumorigenesis, the analysis of all kinases (the kinome) active in a particular tumor may yield tumor-specific information on aberrant cell signalling pathways. Tumor tissue kinase activity profiles may correlate with response to therapy and therefore may be used for future therapy selection. In this paper we describe peptide array and mass spectrometry-based technologies and new developments for kinome profiling, and we present an outlook towards future implementation of therapy selection based on kinome profiling in clinical practice.

Protein kinases

The human kinome

Phosphorylation and dephosphorylation are the central mechanism for intra- and intercellular signal transduction. Kinases are key enzymes catalyzing protein phosphorylation by transferring a phosphate group from adenosine triphosphate (ATP) to specific substrates. In the human genome 518 protein kinases have been identified, of which 90 are tyrosine kinases [2]. Deregulation of kinase activity by gene amplification or mutations has been implicated in cancer and immune system diseases as well as in metabolic and infectious diseases. Virtually all cellular signalling processes involve

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transfer of phosphate groups, implying that inhibition of aberrant kinase activity can repair deregulated cellular signalling. Therefore, kinases and especially tyrosine kinases have become one of the most intensively targeted enzyme classes for therapy, illustrated by the development of numerous small-molecule inhibitors for the treatment of cancer by the pharmaceutical industry.

Protein kinases

Protein kinases catalyze the transfer of the γ -phosphate of ATP to a serine (Ser), threonine (Thr), or tyrosine (Tyr) residue of the target protein. Kinases have an active site where ATP is bound by hydrogen bonds to the adenine moiety. An Mg^{2+} ion is required at the active site for phosphotransfer to occur. ATP binding takes place in the so-called hinge region of the kinase connecting the N-terminal and C-terminal domains [3]. In the hinge region kinases have a conserved activation loop whose degree of phosphorylation is highly correlated with catalytic activity [4]. Most kinase inhibitors are ATP mimics that bind competitively in the ATP binding cleft, mimicking the adenine hydrogen-bonding network [3]. Several kinase inhibitor classes can be distinguished: type 1 inhibitors bind the kinase with the activation loop in the active conformation, whereas type 2 inhibitors bind the kinase with the activation loop in the inactive conformation. A third class of inhibitors binds outside the ATP binding site to modulate kinase activity (allosteric inhibitors). The fourth class of inhibitors is formed by irreversible or covalent inhibitors targeting an active site cysteine residue present in a subgroup of kinases [3].

Kinases and cancer

Many kinases have been associated with tumor cell proliferation, migration, and survival [5]. Some kinases have gained transforming capability by gene amplification, mutation, or translocation and are considered to be oncogenic [6]. Their constitutive activity is essential for cancer cell survival or proliferation. Inhibition of these oncogene-addicted cells by small-molecule inhibitors has proven very effective for the treatment of selected tumor types. Examples of oncogenic kinases are BCR-ABL1 fusion protein in chronic myelogenous leukemia (CML) [7], PI3KCA in cervical cancer [8], and the V600E mutation in the activation loop of BRAF involved in development of melanoma and ovary, thyroid, colon, and pancreatic carcinomas [9]. Other non-oncogenic or mutated kinases are required for cell growth, cell division, and/or proliferation, and are often found downstream of transforming oncogenes. Examples of these kinases are MEK1, MEK2, mTOR, RSK, FGF, and CDKs. Other protein

kinases are involved in auxiliary processes for tumor maintenance and survival, e.g., for new blood vessel formation mediated by vascular endothelial growth factor receptor (VEGFR).

Kinases and anticancer treatment

In the past decade, multiple agents that target specific signalling proteins important for tumor growth have been developed and reached clinical approval, thereby importantly extending the therapeutic arsenal for patients with advanced (inoperable or metastasized) cancer. In tumors where the oncogenic kinase driver genes are known, selective small-molecule inhibitors of the deregulated kinase have significant impact. The most well-known example is the BCR-ABL1 fusion protein, the causative transforming event in chronic myelogenous leukemia. Upon treatment with imatinib up to 80% response rates in CML patients have been reported [10].

Additionally, inhibitors of angiogenic receptor tyrosine kinases have been developed as a systemic treatment strategy for cancer. These agents target the formation of new blood vessels required for tumor growth and the formation of metastases, a process called angiogenesis. The small-molecule tyrosine kinase inhibitors (TKIs) sunitinib and sorafenib target multiple tyrosine kinase receptors, including VEGFR and platelet-derived growth factor receptor (PDGFR), and have demonstrated clinical benefit in patients with advanced renal cell carcinoma [11, 12]. Compared with previous standard therapy, these agents doubled progression-free survival and increased response rates from 5–10 to 30%, with durable disease regression in individual patients. Moreover, sunitinib has been approved for the treatment of gastrointestinal stromal tumor (GIST) after disease progression or intolerance to imatinib [13]. In addition to tyrosine kinase inhibition, sorafenib was originally developed as a Raf serine/threonine kinase inhibitor and has been approved for the treatment of hepatocellular cancer [14].

Other clinically approved TKIs include erlotinib, which targets epidermal growth factor receptor (EGFR), for the treatment of advanced non-small cell lung cancer, and lapatinib, a dual EGFR (ErbB1) and HER2/Neu (ErbB2) inhibitor, for the treatment of metastatic breast cancer. Numerous clinical trials are ongoing with these and other TKIs in various cancer types, both as single agents and in combination with other targeted agents or chemotherapy.

Although small-molecule TKIs have dramatically improved therapy in a selection of advanced cancer patients, only a subgroup of patients will actually benefit from these agents. A significant proportion of patients will not respond to targeted treatment, demonstrating initial resistance. In

addition, tumor regression and clinical improvement are temporary phenomena as well, as most patients will eventually acquire resistance [15] and subsequently experience disease progression.

In this paper we discuss strategies for kinase activity profiling to acquire new insights into molecular mechanisms of drug resistance and response to therapy and with potential for accomplishing treatment selection in patients.

Kinome profiling

Global kinome analysis

Kinome profiling or ‘kinomics’ [16] is the global analysis of all kinases in cells or tissue with respect to abundance, activity, substrate specificity, phosphorylation pattern, and mutational status. Several strategies can be followed including the chemical proteomics approach for kinase abundance and phosphorylation analysis, large-scale (>300 kinases) analysis of recombinant kinases [17, 18] for substrate specificity analysis, peptide substrate arrays for upstream kinase activity profiling, and qPCR for mutational analysis. In this paper, we will focus on the emerging strategies for protein-level kinome profiling using chemical proteomics, reverse protein arrays with immunoassay readout, and peptide substrate arrays (Fig. 1, Table 1).

Chemical proteomics for TKI target identification and kinome profiling

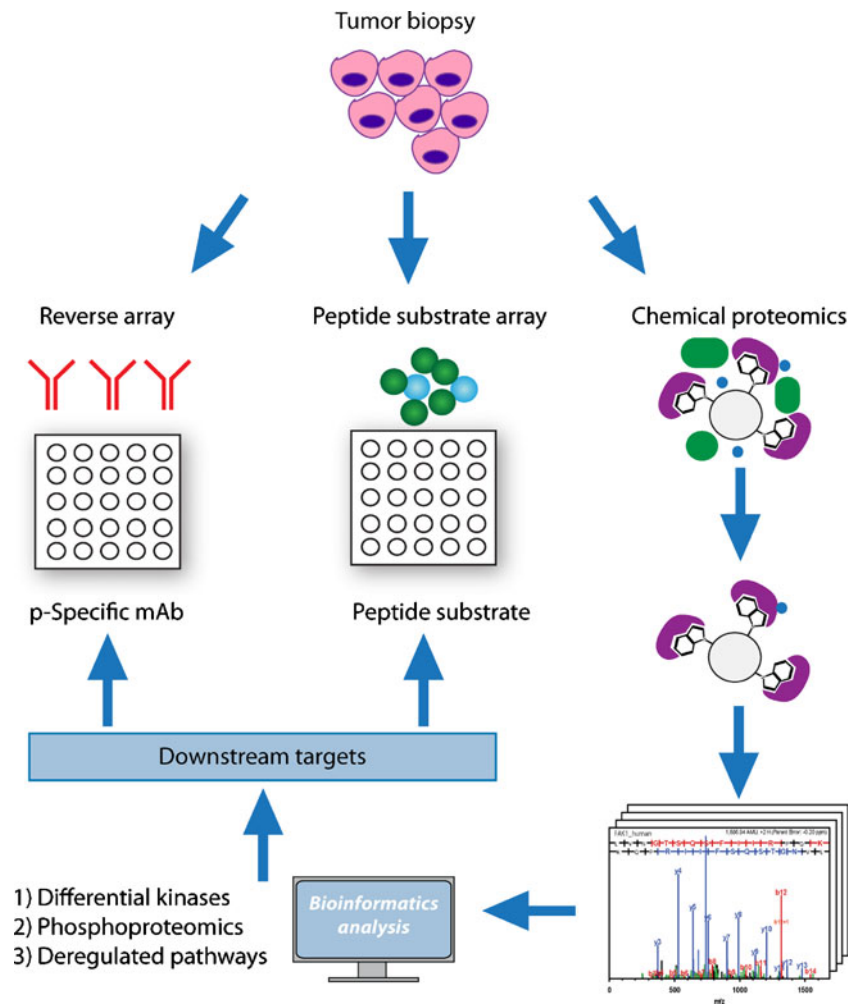
Conventionally, small-molecule kinase inhibitors are tested against a panel of recombinant kinases to identify drug targets and to assess inhibitor specificity and selectivity [16]. However, these *in vitro* assays include only a subset of all protein kinases and do not take into account physiological kinase binding partners, post-translational modifications, mutated forms, cellular concentrations, or compartmentalization.

Chemical proteomics is emerging as a novel comprehensive kinome-wide strategy for drug target identification in disease-relevant cells and tissues. Chemical proteomics is an enrichment strategy that combines an immobilized drug affinity pull-down approach with mass spectrometry-based proteomics for protein identification, quantification, and phosphorylation analysis (for recent reviews, see [19, 20]). A suitable broad specificity ATP-competitive protein kinase inhibitor such as purvalanol B, bisindolyl maleimide, and staurosporine or more specific inhibitors such as imatinib or dasatinib

are covalently immobilized on a biocompatible matrix such as sepharose or agarose through a free hydroxyl, carboxyl, sulfhydryl, or primary amine group present on the inhibitor. If the inhibitor does not have a linkable group, an analogue must be synthesized containing a linkable moiety to confirm the inhibitory activity of the analogue. The group used for immobilization should not interfere with binding of the inhibitor to the kinase. Cell lysates, consisting of proteins in their native state, are incubated with the affinity matrix and washed extensively prior to elution to reduce protein background. Bound proteins are eluted and identified by using nanoLC–MS/MS-based proteomics (Fig. 2). When focusing on phosphopeptide identification, a phosphopeptide enrichment step can be applied by using immobilized metal ion affinity chromatography (IMAC) or titanium dioxide beads prior to LC–MS/MS [21]. The main advantages of chemical proteomics over conventional methods are: (1) it provides an unbiased method for finding drug-interacting proteins; (2) the method is applicable to protein kinases in their native cellular environment, (3) at natural abundances, and (4) in the presence of *in vivo* interaction partners; and (5) post-translationally modified forms, splice variants, and mutated forms are also captured. Successful examples of chemical proteomics pull-downs employing immobilized TKIs dasatinib, imatinib, and nilotinib show that dasatinib inhibits the Tec kinases Btk and Tec at nanomolar concentrations but not Ltk, in addition to its target ABL and SRC kinases [22]. For the highly specific TKI nilotinib DDR1 was identified as additional major target and NQO2 as off-target [23], showing the power of chemical proteomics for TKI target and off-target identification.

A drawback of chemical proteomic approaches has been the high amount of required input material, tens of milligrams of total protein. However, recently, scaling down to the 2–8 mg protein level has been demonstrated [24], opening up the chemical proteomics approach for a much wider range of applications. Further streamlining the chemical proteomics workflow allowed Fernbach et al. to identify 40 bosutinib-interacting proteins from as little as 2×10^6 K562 cells, which is equivalent to ~0.5 mg protein [25]. Most likely the near future will see further miniaturization of methods, e.g., with magnetic bead-assisted pull-downs. Another complicating factor is the presence of abundant, sticky proteins that may obscure detection of low-abundance kinases. Bead control experiments can be performed but most of the time interference can be attributed to (usually) high-abundant proteins that bind aspecifically to the hydrophobic inhibitors with medium affinity. Optimization of wash stringency is important for optimal detection of kinases. However, non-kinase ATP-binding proteins will always be ob-

Fig. 1 Overview of kinome profiling approaches. Tumor biopsy material can be profiled by chemical proteomics yielding kinase abundances, along with associated signalling complexes and phosphorylation sites. Kinases that are differential for responders vs. non-responders to therapy can be used to design custom substrate peptide arrays or reverse protein arrays. These custom arrays can be used, when validated, for therapy selection in the clinic



served in the immobilized kinase pull-downs, and the same holds for kinase-binding proteins (secondary and ternary binders). For comprehensive reviews discussing chemical proteomics for (off-)target identification, see [19, 20].

Chemical proteomics for kinome profiling

To profile the kinome comprehensively, a mixture of immobilized inhibitors must be formulated such that all kinases expressed by a cell are quantitatively captured.

Table 1 Comparison of methods for kinase (activity) profiling

Method	Characteristic(s)	Information content	Kinase activity	Detection	Throughput
Array-based					
Peptide array (PepScan)	In vitro	Medium (1,000 peptides)	Yes (indirect)	Radiolabeling	High
Peptide array (PamGene)	In vitro	Low (144 peptides)	Yes (indirect)	Ab-based	High
Protein array (Zeptosens)	Targeted, in vivo	Low (~100 p-proteins)	Yes (direct)	Ab-based	Medium
MS-based					
Kinobead-based profiling	Unbiased, in vivo	High (1,000s peptides)	Yes (+phosphoproteomics)	MS/MS	Low
KAYAK approach	In vitro	Low (~100 peptides)	Yes (indirect)	MS/MS	Medium
MRM-based kinome profiling ^a	Targeted, in vivo	Medium (~500 peptides)	Yes (direct)	MS/MS	Medium

KAYAK kinase activity assay for kinome profiling, MRM multiple reaction monitoring, Ab antibody

^a Anticipated method for the human kinome; a kinome-wide MRM-based analysis has been reported for yeast [43]

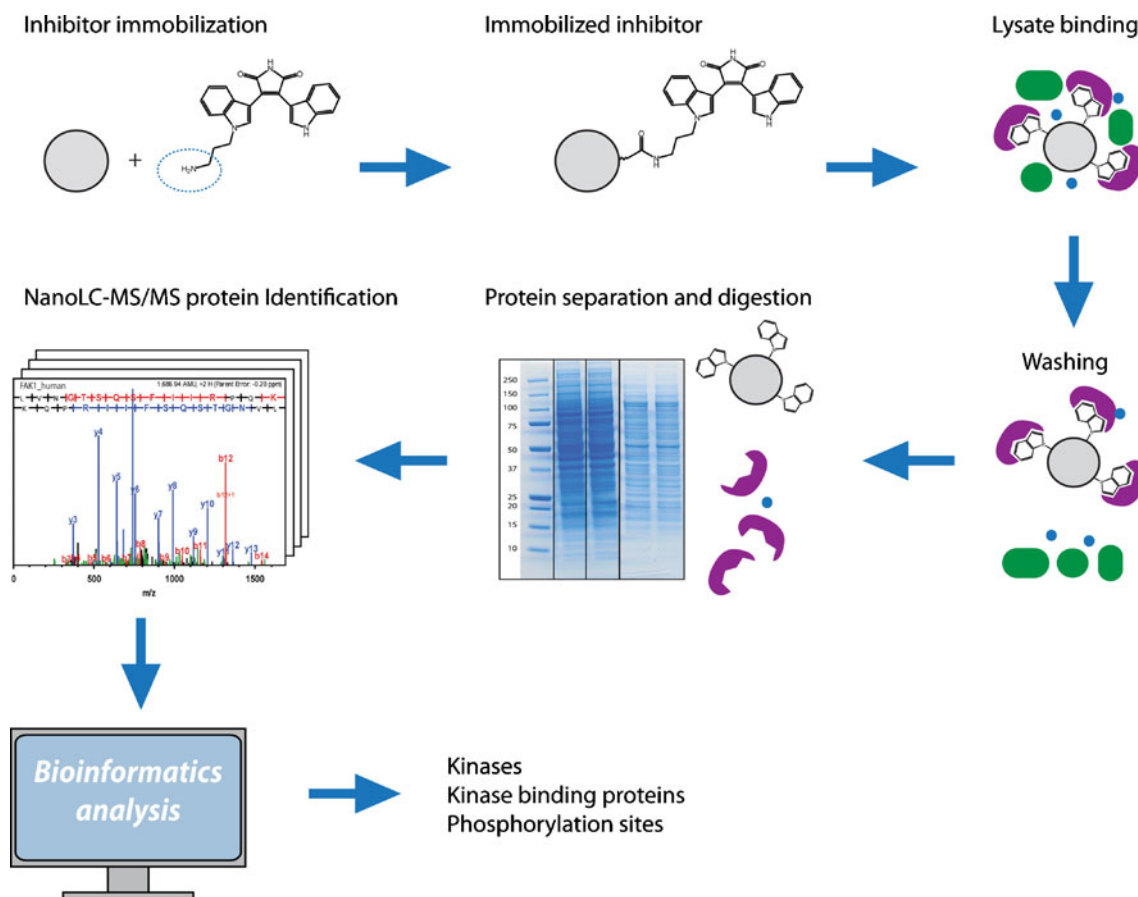


Fig. 2 Chemical proteomics workflow. A small-molecule kinase inhibitor is covalently linked to a resin. Lysate is incubated with the immobilized inhibitor, washed and bound proteins are eluted. Eluted

proteins are identified and quantified by LC-MS/MS yielding kinase abundances, phosphorylation sites, and interacting proteins

Broad-spectrum inhibitors should be combined with more specific inhibitors in order to capture the kinases present in cell lysate. In this respect kinome profiling by chemical proteomics differs from drug target identification, where a single immobilized target inhibitor is employed. For quantitative analysis of kinase abundance and phosphorylation levels several quantitative chemical proteomics approaches have been reported recently. Bantscheff et al. demonstrated in a seminal paper the application of kinobeads, a mixture of seven immobilized protein kinase inhibitors, combined with isobaric tags for relative and absolute quantitation (iTRAQ) for quantitative proteomic analysis of protein kinases [26]. In a competition assay with clinically relevant free kinase inhibitors the known cellular targets of imatinib were identified, i.e., BCR-ABL1, but also unexpected binders DDR1 and NQO2. The method yielded kinome-wide affinity constants for imatinib and identified known targets as well as novel off-targets [26]. Additionally, the paper reported a total of 307 distinct protein kinases (296 human and 196 rodent) from 14 human and rodent cell lines and tissues [26]. In addition to kinome profiles, downstream pathway information was also

obtained including specific phosphorylation site information of kinases and kinase targets. Both kinases as well as kinase-binding proteins are captured, which is a plus for pathway discovery. A recent state-of-the-art chemical proteomics study applied a quantitative analysis of the interactome of immobilized staurosporine analogue K252A. By preincubating cell lysate with soluble inhibitors, true binders were distinguished from aspecific binders [24]. A specific variation of chemical proteomics is activity-based profiling. The kinase-binding ligand (without solid matrix) is linked to a (photo)reactive cross-linker group to form a covalent bond with the interacting protein upon binding. A biotin group is also included in the structure to enable selective capture of the ligand-kinase complex by streptavidin [27, 28].

Kinome profiling combined with phosphoproteomics

In addition to the presence (identification and quantitation) of kinases, their regulation and catalytic activity are relevant for their *in vivo* functions. Recently, combined chemical and phosphoproteomic methods have been used to characterize the activity of dasatinib in lung cancer [29].

Regulatory phosphorylation sites are of importance for in vivo function of kinases and many kinase phosphorylation sites have been reported [30–32]. Even more relevant is the activation loop phosphorylation level which can be used as a surrogate readout for in vivo kinase activity. Oppermann et al. recently performed large-scale quantitative proteomics analysis of three different cancer cell lines by using three immobilized kinase inhibitors yielding quantitative kinase measurements of 170 different kinases. Additionally, the ATP-competitive V116832 ligand was used in a pull-down for phosphopeptide analysis yielding 1,200 distinct phosphorylation sites on 200 kinases, including more than 50 activation loop phosphopeptides [21].

Finally, the above studies show that kinase-selective isolation combined with various proteomics strategies opens a detection window for low-abundance kinases. We envision that correlating kinase abundance, associated protein complexes, and phosphorylation patterns in patient material to therapeutic efficacy and prognosis will be key for development of novel diagnostics tests for patient stratification. These novel tests may be MRM mass spectrometry-based or, alternatively, may make use of phosphorylation-specific antibodies against key phosphorylation sites or key peptide substrates to be implemented in measurements employing reverse protein arrays of peptide arrays (see below).

Reverse protein arrays

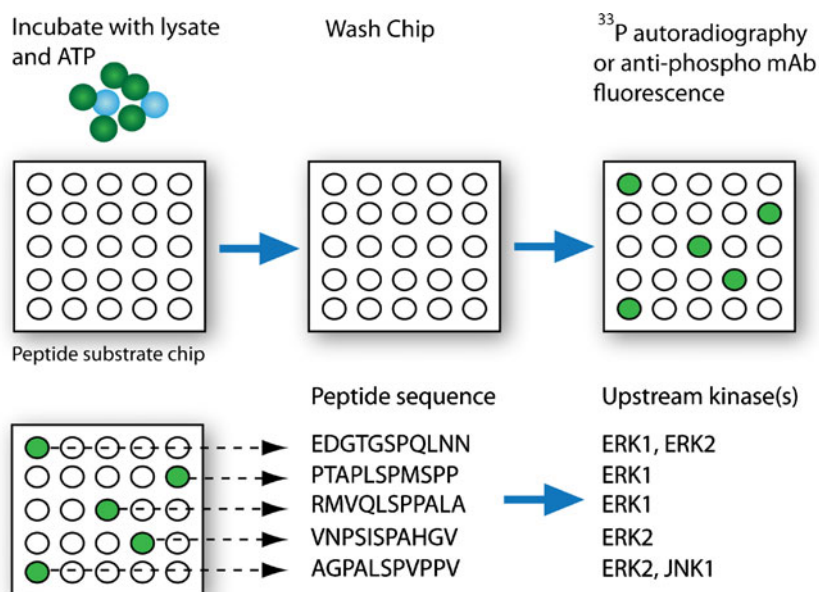
Phosphorylation site-specific antibodies can be used to detect (activated) kinases or kinase substrates in active signalling pathways. These phosphorylation site-specific antibodies combined with western blotting are the classical tools for pathway analysis. A parallel high-sensitivity reverse protein array format using phosphorylation site-specific antibodies has been developed by the company Zeptosens [33]. Tissue or cell lysates (1×10^5 cells) are spotted in an array format on a high-adsorbance chip surface, blocked, and incubated with antibodies. The fluorescence readout makes use of planar waveguide technology to selectively measure target-bound antibodies with very low background signal. In a proof of principle paper Weissenstein et al. reported sensitivities (LOD) of 33 pg/ml for plasminogen activator inhibitor-1, 1 pg/ml for VEGF, and 1 pg/ml urokinase-type plasminogen activator in breast tumor extracts [34]. Recently, reverse arrays have been applied to analyze the effect of succinate signalling via receptor GPR91 on dendritic cells. For pathway analysis the reversed array was probed by phospho-specific antibodies against Erk1/2, Mek1 and Mek2, STAT1, STAT2, STAT3, STAT5, STAT6, and P38 [35]. Sensitivity of the Zeptosens platform is compatible with tumor core biopsies [34] and can yield cancer-relevant pathway coverage depending on availability of high-quality

phospho-specific antibodies. Currently, antibodies against 109 different target proteins are available with a total of 98 different phosphorylation sites covered.

Kinase peptide substrate arrays

Microarrays of kinase substrate peptides have been developed to study (recombinant) kinase specificity and potentially cellular signal transduction by detecting up- and down-regulation of peptide phosphorylation in an array-based format. By generating information on kinase-induced peptide phosphorylation, these arrays may provide new insights into specific downstream signalling pathways, e.g., involved in proliferation and migration of tumor cells, and identify potential druggable targets. Ideally, kinase peptide substrate arrays provide kinase activity values across the kinome. Originally, these arrays were used to determine the substrate specificity pattern of individual kinases, and later to determine kinase activity in complex mixtures such as cell lysates and tissues [36]. Kinase peptide substrate arrays consist of immobilized peptides containing Ser, Thr, and Tyr residues with additional sequence context for phosphorylation by their upstream kinases and can therefore reveal kinase activity in vitro (Fig. 3). Commercially available peptide arrays (e.g., PepChip, PamChip) contain typically between 144 and $\sim 1,200$ peptides with Ser, Thr, or Tyr residues available for phosphorylation and additional amino acid sequence context. Peptide sequences are derived from literature or computational predictions and are correlated with one or multiple upstream kinases. After incubating the array with cell lysate, phosphorylation is determined by phosphoimaging (radioactive [^{33}P]ATP) or fluorescence microscopy (anti-phospho antibodies). Advantages of peptide arrays include parallel readout of up to 3,000 spots, requirement of small (clinical) quantities of cell lysate and tissue lysate (~ 10 – $250 \mu\text{g}$ protein) [37, 38] as well as assay speed (incubation time 2 h, phosphoimaging time 24 h). Spot intensities can be correlated with kinase activity; however, inference of in vivo upstream kinase activity from peptide arrays is not straightforward. (1) Kinases are promiscuous, multiple kinases phosphorylate the same peptide. Each spot intensity on the array corresponds to the sum of the accumulated kinase activities acting on the peptide immobilized on the spot. Only few peptides are unique for a single kinase and allow for direct correlation of spot intensity and kinase activity. (2) Only a limited subset of the 518 human protein kinases is accounted for in currently available peptide arrays. For example, the PamChip shows an overrepresentation of EGFR signalling-related substrate peptides. (3) There are peptides on the array for which the upstream kinases are currently unknown (but which are phosphorylated by cell lysate). (4) Cellular compartmentalization is lost. (5) Array-based methods are unable to determine the actual site of

Fig. 3 Peptide substrate array workflow. Peptide substrate arrays are incubated with lysate, washed, and probed by autoradiography or fluorescence microscopy yielding peptide phosphorylation patterns



phosphorylation, if more than a single Tyr, Ser, or Thr residue is present in the peptide sequence. Although the relation between kinase activity and peptide substrate spot intensity is not 1:1, patterns of spot intensities could be useful for pattern-based diagnostics of kinase activity. Sikkema et al. recently demonstrated tyrosine kinase profiles for 29 pediatric brain tumors, yielding previously reported enhanced tyrosine kinase activity of EGFR, VEGFR, and c-Met. In addition, peptide substrates for Src family kinases demonstrated high phosphorylation levels. Subsequent application of Src family kinase inhibitors PP1 and dasatinib induced a decrease of survival in pediatric brain tumor cell lines but not in control cell lines, suggesting therapeutic potential for Src activity inhibition [39].

In another recent report peptide substrate chip profiling of chondrosarcoma cell lines and primary cell cultures indicated enhanced activity of the Akt/GSK3B pathway, PDGFR, and Src family kinases [37]. It was shown that the small-molecule kinase inhibitor dasatinib but not imatinib decreased chondrosarcoma cell viability at nanomolar concentrations, leading to the proposal of using dasatinib as a therapeutic option in inoperable patients. These recent studies illustrate the potential of peptide substrate arrays for the identification of druggable targets and therapy selection. Versele et al. reported an application of the PamChip for detection of cytoplasmic and receptor tyrosine kinase activities in cell lysates in the presence and absence of a kinase inhibitor. In a panel of 27 cell lines and 8 xenografts, the authors were able to identify signature peptides (16 and 8, respectively) that resulted in clusters accurately predicting response to a multitargeted kinase inhibitor [40].

By correlating array phosphorylation patterns with clinical outcome, kinase peptide substrate arrays have the potential to become a diagnostic tool for disease management. The key factor in the design of kinase peptide substrate arrays is target peptide selection. The choice of peptides determines the relevance of the array. Target peptides must be selected such that the major signalling pathways are sufficiently covered. All nodes of the pathway should be represented by one or more peptides. Pathway target peptides should be phosphorylated exclusively by the designated upstream kinase. In addition to peptide choice, high reproducibility and robustness are requirements for application of kinase peptide substrate arrays as a diagnostic platform in clinical practice. Recently, a promising variation on the peptide substrate array has been reported in which high resolution MS was used as the quantitative readout. The method builds on the seminal paper of Cutillas et al. [41], which reported on ultrasensitive and absolute quantification of phosphoinositide 3-kinase/AKT signal transduction by MS. In the approach termed kinase activity assay for kinome profiling (KAYAK) 90 selected synthetic kinase substrate peptides are pooled and incubated in a single reaction with nanogram to microgram amounts of cancer cell or tissue lysate [42]. Following *in vitro* phosphorylation of peptides by endogenous kinases present in the lysate, the phosphorylated peptide pool is spiked with corresponding heavy-isotope labelled peptides, enriched by IMAC, and identified and quantified by nanoLC-MS/MS. The MS/MS spectra, combined with isotopically labelled peptides, enable determination of phosphorylation site-specific phosphorylation rates; no other method including peptide substrate arrays is able to deliver this. The authors foresee application of the KAYAK approach for therapy

selection and assessment of aberrant signalling in patient tumors [42].

Outlook

Although small-molecule tyrosine kinase inhibitors have changed therapeutic options in selected tumor types considerably, these agents induce clinical benefit in a subgroup of patients. It is of crucial importance to develop new clinical tests to determine which patients will respond to specific targeted agents. So far, no adequate and reliable test to predict response is available.

As each patient has a unique genomic and proteomic tumor profile, it is assumed that responses to targeted agents depend on specific receptor and protein signalling activities in tumor tissues. We therefore envision that kinase activity profiling may be a potential clinical diagnostic tool to predict tumor response to targeted therapy with kinase inhibitors.

Kinase profiles of individual patients may give insight into the deregulated signalling processes and may have diagnostic value and aid patient therapy selection. This application is not restricted to cancer therapy but may be extended to other diseases with deregulated kinases. With ten kinase inhibitors registered for treatment and an additional few dozen under development, there is demand for technology platforms that can faithfully map kinase activities in patient material and the effect of TKIs *ex vivo*. Chemical proteomics techniques can yield individualized kinase passports. The kinobeads reported by Bantscheff [26] are the first achievements in the development of a ligand mixture capable of kinome-wide enrichment. Future application of chemical (phospho-)proteomics may identify differential kinases along with associated signalling complexes and phosphorylation patterns for responders vs. non-responders. The downstream targets of these differential kinases can be translated into dedicated peptide substrate array tests that, when validated, may be implemented in clinical practice for therapy selection. Custom-made arrays with clinically relevant peptides may provide an accurate readout of tumor deregulated signalling pathways and aid in TKI selection to provide the optimal therapy based on patient-specific pathway activity. The currently available therapeutic options drive development of platforms that will enable the oncologist to select the best targeted therapy for each individual patient.

Acknowledgments This work was supported by the VUmc-Cancer Center Amsterdam (CRJ). The Aegon Foundation is acknowledged for financial support (ML).

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