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Sparking Signals

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Kinases as Molecular Signaltransducers and Pharmacological Drug Targets in Inflammation

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Preface

Almost all aspects of cell life and action are controlled by the reversible phosphorylation of proteins. The human genome projects have revealed that about one-third of mammalian proteins contain covalently bound phosphate, and there are likely to be around 570 protein kinases encoded by the human genome. Protein kinases, classified as either protein tyrosine kinases or serine/threonine kinases, have crucial roles in immune cell signaling, including T cell and Toll-like receptor signaling, that represent central pathways for adoptive and innate immunity. In these immunoreceptor-related pathways, kinases interact sequentially with substrate proteins, which by phosphorylation become activated to allow effective signal transduction to further downstream targets that are directly or indirectly involved in controlling proliferation, homing, and survival of immune cells. Protein kinases therefore play a pivotal role in the initiation, propagation, and regulation of immunological responses. The impressive progress in the past few years in the field of kinases has led to an improved understanding of the role and function of protein kinases as powerful signal transducers for the regulation of immune cell effector functions; kinases are now understood as key mediators for the induction of pathogenesis in a variety of immunological diseases.

The rapid advances in our understanding of the molecular structures of kinases, as well as the advances to unveil the biochemical tuning of kinases, are also the basis for the development of innovative therapeutic agents that target defined protein kinases involved in inflammatory diseases and cancer. The design of kinase inhibitors focuses mainly on the ATP-binding pocket and less conserved surrounding pockets and exploits differences in kinase structure to achieve selectivity. Almost all currently known small inhibitor molecules of protein kinases bind to the active ATP-binding site and act as competitive inhibitors. The high degree of structural similarity in the ATP-binding pocket of protein kinases, however, presents a major challenge to the development of selective inhibitors. Nevertheless, targeting specific kinases that are overexpressed or overactive in disease should allow for selective treatment as shown, e.g., with the success of the specific Abelson murine leukemia viral oncogene kinase inhibitor Gleevec in the treatment of chronic myelogenous leukemia.

The aim of this workshop was to present cutting-edge science in the fast developing kinase field, which is of special interest to basic researchers and the pharmaceutical industry. Within the rather broad field of kinase research, the focus was laid on Toll-like receptor and T cell receptor-mediated signaling. Luckily we were able to bring together internationally renowned experts from both academia and industry, who openly presented and discussed their most recent findings. Most of the presenting scientists agreed to publish their contributions in this book. We are convinced that the scientific forum made available during this kinase workshop allows us a better understanding of signaling events in relevant immune cell subsets, backed up as they are by innovative molecular, structural, and technical, e.g., high-throughput screening, aspects discussed during the meeting. Workshops such as this kinase symposium in Potsdam, therefore, should ultimately have a substantial impact on drug discovery, in particular for developing novel classes of selective inhibitors for the treatment of inflammatory diseases and cancer.

Finally, we would like to thank the Ernst Schering Foundation for the professional support in preparing the meeting, as well as during the workshop, which allowed the open and positive atmosphere for discussion that was present throughout the whole conference in a beautiful, perfectly fitting location close to Potsdam.

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Proteomics-Based Strategies in Kinase Drug Discovery

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Abstract. Studies of drug action classically assess biochemical activity in settings which typically contain the isolated target only. Recent technical advances in mass spectrometry-based analysis of proteins have enabled the quantitative analysis of sub-proteomes and entire proteomes, thus initiating a departure from the traditional single gene—single protein—single target paradigm. Here, we review chemical proteomics-based experimental strategies in kinase drug discovery to analyse quantitatively the interaction of small molecule compounds or drugs with a defined sub-proteome containing hundreds of protein kinases and related proteins. One novel approach is based on 'Kinobeads'—an affinity resin comprised of a cocktail of immobilized broad spectrum kinase inhibitors—to monitor quantitatively the differential binding of kinases and related nucleotide-binding proteins in the presence and absence of varying concentrations of a lead compound or drug of interest. Differential binding is detected by high throughput and sensitive mass spectroscopy techniques utiliz-

ing isobaric tagging reagents (iTRAQ), yielding quantitative and detailed target binding profiles. The method can be applied to the screening of compound libraries and to selectivity profiling of lead compounds directly against their endogenously expressed targets in a range of cell types and tissue lysates. In addition, the method can be used to map drug-induced changes in the phosphorylation state of the captured sub-proteome, enabling the analysis of signalling pathways downstream of target kinases.

1 Introduction

Traditionally, biochemical assays of drug action assess activity on the isolated, purified target. Typically, recombinant enzymes or protein fragments are used instead of the full-length endogenous protein. To correlate the activity of a compound determined in such assays with pharmacodynamic efficacy is often not straightforward, as an isolated recombinant protein fragment may not accurately reflect the native conformation and activity of the target in its physiological context (Hall 2006). Because of the lack of interacting regulatory proteins, expression of alternative splice forms, or incorrect folding or post-translational modifications, results from *in vitro* experiments are not always predictive for the effects of a compound or drug in cell-based or animal model systems. Moreover, although lead compounds are traditionally optimized against a single protein, many drugs act on multiple targets (Morphy et al. 2004). Such 'off-targets' may produce toxic side effects, but might also increase the therapeutic potential of a drug. Multiple targets and off-targets are likely to be the rule—rather than the exception—in large target classes with a high degree of structural conservation around the 'druggable' binding site, such as kinases, enzymes which catalyse the transfer of phosphate groups from ATP to the hydroxyl groups of proteins, lipids, or sugars. Protein kinases represent a class of drug targets of increasing importance particularly in oncology and inflammation (Cohen 2002). Small molecule inhibitors directed at the ATP-binding site of kinases are not likely to be selective for a single kinase, because there are around 500 protein kinases and more than 2000

other ATP- and purine-binding proteins encoded in the human genome, many of which share similarly shaped ATP binding pockets (Manning et al. 2002; Haystead 2006). Conventional strategies by and large rely on assay panels of 20–100 recombinant enzymes, and in addition on cell-based model systems, to address compound potency, selectivity and potential off-target liabilities, rather than to determine the *bona fide* targets of a drug in a direct, more unbiased manner (Fabian et al. 2005; Fliri et al. 2005).

Recent progress in affinity-based proteomic strategies has enabled the direct determination of protein-binding profiles of small molecule drugs under more physiological conditions (Szardenings et al. 2004). To date, most methods rely on the attachment of labels to the compound (immobilization, fluorescent or affinity tags) or to the proteins (Fabian et al. 2005; Godl et al. 2003; Knockaert et al. 2000), which may cause artefacts because of the altered properties of the modified compound or the labelled protein. We here describe how technical and methodical advances enabled a strategy involving the capturing of a defined sub-proteome, consisting of a large fraction of the expressed kinome and related nucleotide-binding proteins, on a mixed inhibitor matrix (*kino*me *beads* or Kinobeads), and subsequent quantitative analysis of this defined sub-proteome by mass spectrometry (Bantscheff et al. 2007). This methodology allows the parallel determination of protein affinity profiles of small molecule inhibitors in any cell type or primary tissue as well as the differential mapping of drug-induced changes of phosphorylation events on the captured sub-proteome.

2 Quantitative Mass Spectrometry-Based Proteomics

Proteomics is "the analysis of complete complements of proteins. Proteomics not only includes the identification and quantification of proteins, but also the determination of their localization, modifications, interactions, activities, and, ultimately, their function" (Fields 2001). Originally, proteomics approaches aimed at the identification of a list of typically several hundred proteins expressed in a given tissue or body fluid at a given time under a defined set of conditions. However, the analysis of complex proteomes remains daunting (Huber 2003), as body

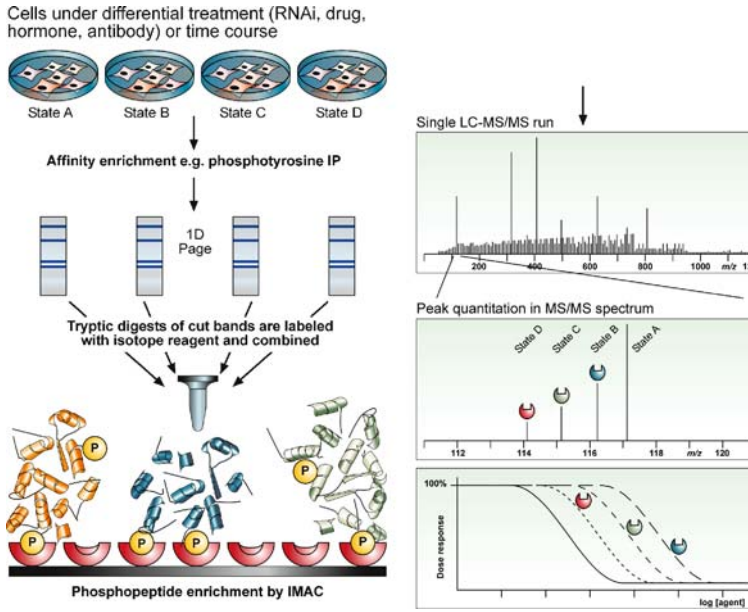

Fig. 1. Multiplexed differential analysis of sub-proteomes by quantitative mass-spectrometry. Up to four different treatments (e.g. hormones, siRNA probes, different compounds, several concentrations of one compound) are applied to a cell line in culture. After enrichment of proteins of interest (e.g. phosphotyrosine immunoprecipitation) proteins are separated by standard one-dimensional SDS polyacrylamide gel electrophoresis and the gel is stained, for example, by colloidal Coomassie dye. Depending on the type of experiment, individual protein bands are cut from the gel, or alternatively, the entire gel is cut into slices across the full separation range. Gel slices are then digested with trypsin and subsequently, the resulting peptide mixtures are labelled using stable isotope-containing tagging reagents (e.g. iTRAQ). After labelling, samples from the differentially treated conditions are mixed and subjected to enrichment of phosphopeptides. These peptides are then analysed using nano-LC separation and tandem mass spectrometry. Tandem mass spectra are used for identification of phosphorylated peptides and quantification is inferred from relative intensities of the low mass signature ions present in the MS/MS spectra

fluids or tissue lysates may contain in excess of 100,000 different proteins, with their relative abundances covering more than seven orders of magnitude, and the general use of 'complete proteome' approaches in drug discovery assays remains challenging (Rappsilber et al. 2002). Established methods tend to be biased towards proteins expressed at high levels, which are mostly structural and house-keeping proteins, and to discriminate against the typical 'signalling' proteins which are often expressed at low levels, with an additional bias against membrane proteins (Rappsilber et al. 2002). In contrast, the analysis of sub-proteomes, biochemically enriched samples of reduced complexity (tens to hundreds of proteins) that share functional context, has made significant progress recently and is being pursued more and more in quantitative fashion (Ong and Mann 2005). This chapter focuses on affinity proteomics methods that utilize affinity chromatography-based strategies for the analysis of sub-proteomes. The combination of novel affinity-based techniques with liquid chromatography (LC)-coupled mass spectrometry has become increasingly successful in analysing protein–protein interactions and protein complexes, in analysing post-translational modifications (e.g. 'phosphoproteomics'), and in elucidating the interaction of therapeutic compounds with their targets ('chemical proteomics'). Recent advances in the ability of mass spectrometry to quantify precisely proteins from complex samples now enables quantitative studies on the regulation of proteins and protein complexes under differential conditions such as hormone or drug treatment (Ong and Mann 2005; Zieske 2006). At present, stable isotope labelling techniques represent the gold standard in quantitative proteomics. There are several different methods that share the underlying principle that a sample of interest is labelled with heavy isotope containing tags or amino acids, and mixed with one or more reference samples that remain either unlabeled or tagged with a lighter isotope (Bantscheff et al. 2004; Gerber et al. 2003; Gygi et al. 1999; Ong et al. 2002; Ross et al. 2004; Schmidt et al. 2005; Zieske 2006). After tryptic digestion, mass spectrometric analysis is performed and proteins are quantified by comparing the ion intensities of heavy versus light forms of isotopically labelled peptides. The iTRAQ method uses a set of four amine-reactive isobaric tagging reagents, and quantification is based on low mass signature ions in tandem mass spectra. The multiplexing capability of the iTRAQ reagents is

particularly useful for a variety of applications including temporal analysis or the elucidation of concentration dependency of drug-induced-protein expression, modifications, and interactions, and for the discovery and elucidation of disease markers or markers for drug efficacy. A representative experimental strategy is illustrated in Fig. 1.

3 Chemical Proteomics: Systematic Analysis of Protein–Compound Interactions

Here, we use the term chemical proteomics for methodologies that measure the interaction of small molecule compounds with the proteome, or with defined sub-proteomes (Ding et al. 2003; Szardenings et al. 2004). These approaches, often based on classical affinity labelling or affinity chromatography procedures, are typically applied in drug discovery to elucidate the efficacy targets of compounds with an unknown mechanism of action. For some interesting examples, see the classical work by Schreiber and colleagues on the mechanism of immunosuppressants (Brown et al. 1994; Harding et al. 1989) or the more recent chemogenomic approach to the Wnt pathway (Emami et al. 2004). At early stages in the drug discovery process, the target of hit compounds originating from phenotypic screens in cell-based assays or whole animal studies in most cases is unknown, which makes it much more difficult for the medicinal chemist to optimize such hit compounds (Burdine and Kodadek 2004). However, even lead compounds and approved drugs sometimes lack an established mode of action. Also, it is important to note that small molecule drugs are rarely specific for a single target; they may bind to and modulate other proteins with similarly shaped binding pockets, and they will usually bind to members of the body's complement of xenobiotics—modifying enzymes. Knowledge about these 'off-targets' may alert the researcher to the risk of certain side effects, and may even be used for the reprofiling of the drug for a different therapeutic indication. The general principle of the direct determination of the profile of proteins binding to a given compound of interest is theoretically straightforward (Darvas et al. 2004; Graves et al. 2002). Compounds are tethered to a solid support, covalently or via affinity labels containing biotin, using a suitable chemical linker, and

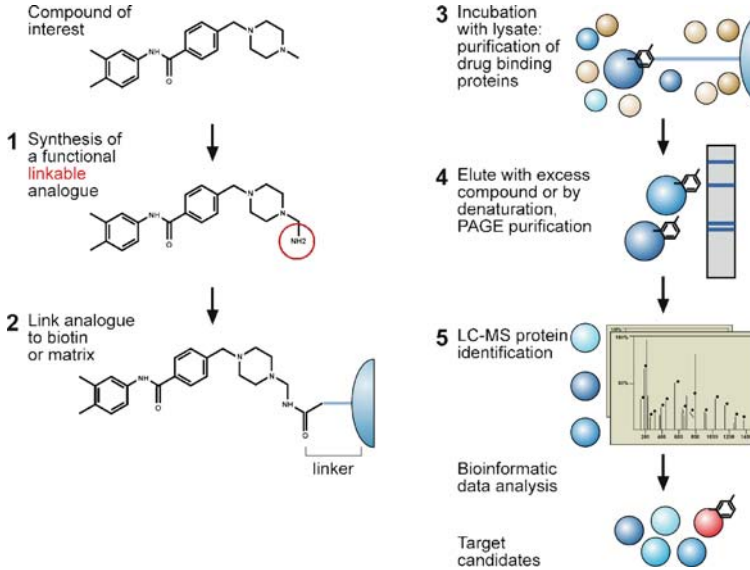

Fig. 2. Drug-affinity purifications. The compounds of interest are tethered to a solid support, covalently or via biotin-avidin methods, using a suitable chemical linker. It is often necessary to synthesize one or more analogues of the original compound, which contain the necessary functional group for coupling, and to test these analogues to ensure that their activity is preserved. The resulting drug-affinity matrix is incubated with cell or tissue lysate to capture specific drug-interacting proteins. After washing, bound proteins are eluted either by a large excess of the free compound or simply by denaturation, and are subsequently identified by LC-coupled tandem mass spectrometry. Usually, several experimental parameters such as the length and nature of the linker, the ligand density of the resulting affinity matrix, and the amount of tissue lysate, will require some empirical optimization in order to achieve an acceptable background level of non-specific proteins. Note that non-specific proteins interacting with the affinity matrix can be determined in a parallel experiment where an excess of the free compound of interest is present during the incubation with the lysate

incubated with cell or tissue lysate to capture specific interacting protein targets. After washing, bound proteins are eluted either by an excess of the free compound or simply by denaturation, and are subsequently

identified by mass spectrometry (Fig. 2). Non-specific proteins interacting with the affinity matrix can be determined in a parallel experiment where an excess of the free compound of interest is present during incubation with the lysate. However, a few potential complications must be taken into account. The main types of functional groups that permit the attachment of the linker—amines, carboxylic acids, thiols, and hydroxyls—are often not present in the compound of interest, or cannot be modified without the complete or partial loss of binding to the target. Hence, it is often required to synthesize one or more analogues containing the necessary functional group and to test these analogues to make sure that their activity is preserved (Daub 2005). Usually, several other experimental parameters such as the length and nature of the linker, the ligand density of the resulting affinity matrix, and the amount of tissue lysate, will all require optimization in order to achieve an acceptable background level of non-specific proteins. Target candidates identified by affinity labelling and affinity chromatography can fall into one of many categories, including efficacy targets, mediators of adverse events, proteins involved in absorption, distribution, metabolism, excretion of the compound, or proteins that do not directly interact with the compound but with any of the aforementioned proteins. Complexity of the experimental outcome makes detailed bioinformatics analysis and functional confirmation (using, for instance, RNA interference in a cell-based assay) often mandatory. In reality, affinity purification strategies do not work equally well for proteins from different target classes. The approach appears to be particularly suited to the profiling of protein and lipid kinases, ATP-binding proteins (e.g. chaperones, ATPases, helicases, or transporters), flavoenzymes, phosphodiesterases, proteases, and peptidomimetics and other agents targeting protein–protein interactions. In contrast, the approach appears to be less suited for ion channels, nuclear receptors, or G-protein coupled receptors. Here, we will focus on the application to kinases and related ATP-binding proteins.

In our laboratory, we have to date profiled a collection of more than 100 ATP-competitive kinase inhibitors including chemical scaffolds, research tool compounds, drug candidates in development, as well as approved drugs, by tethering them to a solid support using a suitable chemical linker, and incubation with cell or tissue lysates to capture and identify their interacting proteins (Bantscheff et al. 2007). Some exam-