Point of VIEW

ACS chemical biology

# Chemical Proteomics Identifies Unanticipated Targets of Clinical Kinase Inhibitors

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**ABSTRACT** Kinases represent one of the most important target classes of current drug discovery efforts. However, because the vast majority of potential small-molecule therapeutics is directed toward the highly conserved ATP-binding cleft, kinase inhibitors often exhibit significant unintended off-target effects. A recent report describes a chemical proteomics methodology that enables the simultaneous *in vivo* quantification of the on- and off-binding targets of kinase inhibitors across hundreds of nucleotide-dependent enzymes.

The discovery of novel protein kinase inhibitors continues at a relentless pace, fueled both by their promise as therapeutic agents and by their potential to elucidate the biological function of new kinases. Currently, seven kinase inhibitors have received clinical approval, while hundreds of compounds are in clinical or preclinical testing. However, determining the full complement of intracellular targets for these kinase inhibitors is a daunting challenge, because the vast majority of these small molecules are directed toward the highly conserved kinase ATP-binding cleft. In addition, kinases represent only one class of nucleotide-dependent enzymes, which also include polymerases, methyltransferases, chaperones, reductases, motor proteins, chaperones, and ATPases, among others. Taken together, this diversity represents a vast array of potential unintended intracellular targets for an unsuspecting ATP-mimetic compound. Drewes and coworkers (*1*) have now reported a significant methodological advance that combines the displacement of potential targets from a kinome-enriched pool with quantitative mass spectrometry (MS). This technique enables the simultaneous quantification of the on- and off-targets of kinase inhibitors across hundreds of nucleotide-dependent enzymes in any cell type or tissue.

Traditionally, the specificity profiling of kinase inhibitors has involved the use of recombinant enzymes in parallel *in vitro* phosphorylation assays. However, these measurements are performed in an environment that is clearly quite different from conditions *in vivo*. By contrast, affinity-chromatography-based techniques have enabled the evaluation of the kinase selectivities of immobilized small molecules under more physiological conditions (*2*). Although this approach remains the most direct and time-honored mode for target identification, it does suffer from some serious deficiencies. For example, it is laborious and sometimes impossible to modify a target compound with a linker molecule to enable its immobilization onto an affinity matrix without significant deleterious consequences to the biological activity of the target compound. In addition, many of the proteins identified are simply highly abundant rather than functionally relevant targets (*3*).

In recognition of these issues, several groups have reported strategies that first affinity-purify a subproteome of interest. They then subsequently determine specificity profiles for a given small molecule by identifying those proteins whose isolation efficiency is reduced when the compound of interest is present in solution during the affinity enrichment. For example, Haystead *et al.* (*4*) isolated the purine-binding proteome by using γ-phosphate-linked ATP-Sepharose, and they employed displacement affinity chromatography to discover novel targets of quinoline drugs. Given the large number of nucleotide-dependent enzymes encoded in the human proteome, later studies sought to further tailor the

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**Published online October 19, 2007**
10.1021/cb700203j CCC: $37.00

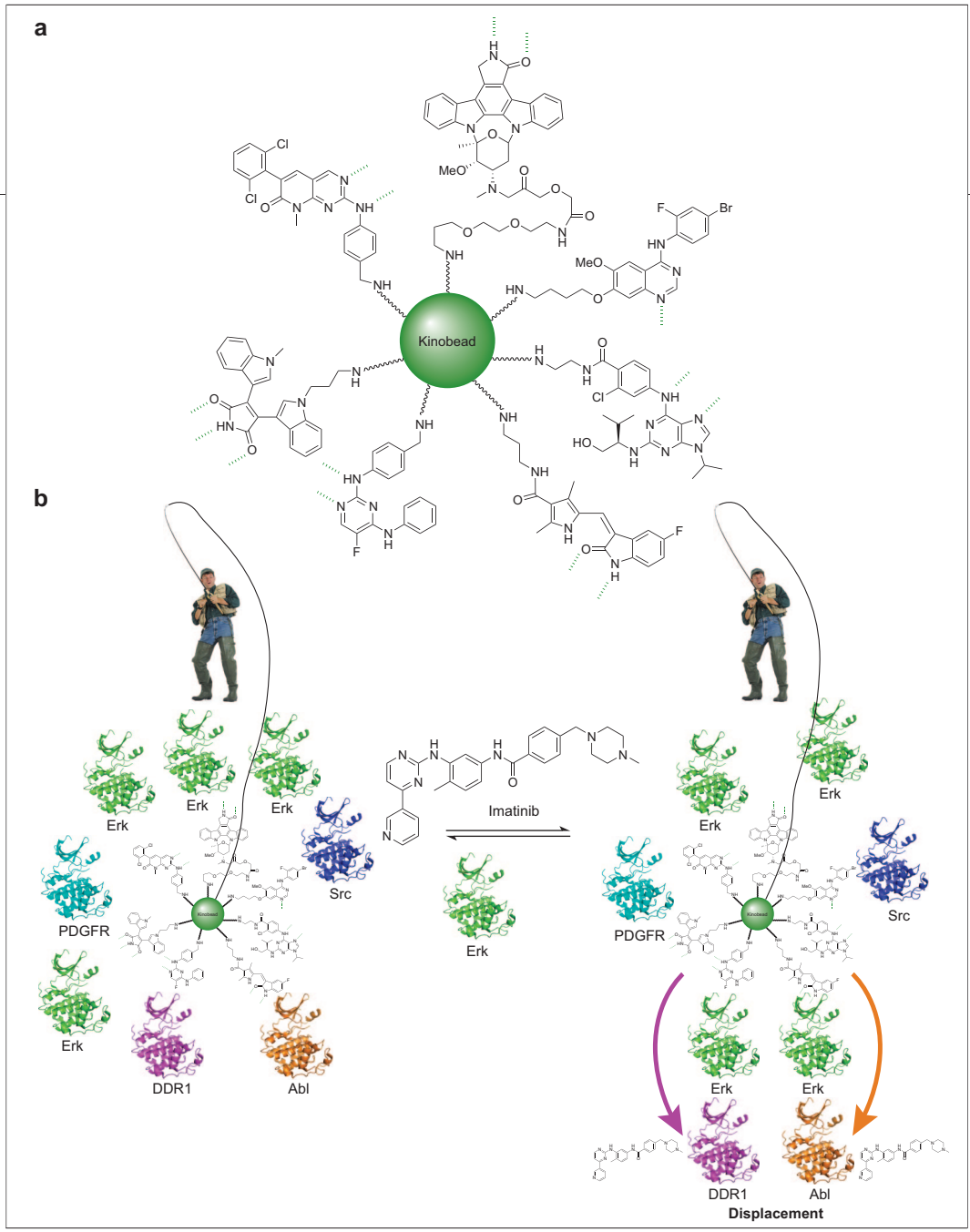


**Figure 1. a) Chemical structures of the seven ATP-competitive kinase inhibitors used to construct the "kinobead". Hashed green lines show groups that hydrogen bond to the kinase hinge resion based on crystallography. b) Kinobeads can be used to capture many diverse kinases and ATP-dependent enzymes (left). In the presence of a competitor such as imatinib, specific targets (DDR1 and Abl) are selectively eluted from the beads (right).**

affinity enrichment step toward the isolation of kinases. Thus, Daub and coworkers (*5*) employed multiple affinity resins containing four structurally different protein kinase inhibitors connected in series. With this approach, >100 kinases and >200 sites of phosphorylation could be identified from a single cell line. Patricelli *et al.* employed a variation of the activity-based proteomic profiling strategy (*6*) to selectivity enrich kinases by using acyl-phosphate-containing nucleotide probes (*7*). These probes were designed such that the acyl phosphate reactive group was positioned to selectively react with conserved lysine residues in the ATP-binding pocket, thus covalently labeling kinases and enabling their subsequent facile enrichment. Using this technique, the authors identified >400 different kinases in various tissues and cell lines The selectivity profiles of several inhibitors were measured by using quantification of the MS signals of species of interest after the generation of normalization factors based on the signals of neighboring peaks.

Building on the results of such chemical proteomics reports as well as their own experience in this area, Drewes and coworkers describe and meticulously evaluate the performance of their methodology that combines a broadly applicable kinome enrichment scheme with the latest advances in quantitative MS. The affinity enrichment scheme utilizes "kinobeads" consisting of seven distinct ATP-binding-site ligands immobilized onto a single solid matrix (Figure 1). The ligands used were selected because of their promiscuous interactions with kinases from across the various branches of the kinase phylogenetic tree. As an example, the kinobeads were capable of affinity-purifying 174 and 183 protein kinases from HeLa and K562 cell lysates, respectively, along with hundreds of other ATP- and purine-binding proteins. When this survey was extended to 14 other mouse and human cell lines and tissues, 307 kinases were identified. The ability of this methodology to quantify the binding partners of various small-molecule

inhibitors across hundreds of kinases and other nucleotide-dependent enzymes was demonstrated through the use of two commercial (imatinib and dasatinib) as well as one clinical candidate (bosutinib) inhibitor of the tyrosine kinase Abl. Specifically, the compound to be profiled was added at three different concentrations to separate cell lysates of K562 cells, and these spiked lysates as well as a control sample with no added compound were subjected to separate kinobead precipitation. The kinobead-bound materials from each of the four conditions were individually digested and subsequently labeled with different forms of the iTRAQ reagent (*8*). The four digested and labeled lysates were then recombined and analyzed by LC/MS/MS, such that the relative ratios of signals arising from a particular protein present in the samples could be determined by measuring the relative intensities of the MS/MS reporter ions. If a spiked compound did indeed interact with a particular protein, then less of this protein would bind to the kinobeads, resulting in a decreased signal for this species in the mass spectrometer compared with the control (Figure 2). By repeating this process with differing compound concentrations always referenced to the same control sample, the authors constructed $IC_{50}$ curves for >500 different proteins for each of the three compounds tested based on nine different concentration measurements ranging over 5 orders of magnitude (100 pM to 10 μM). Of importance is that this same process could immediately be applied to other small-molecule candidates of interest, because the inhibitor being evaluated does not need to be immobilized to an affinity resin.

The power of this technique is clearly observed in the specificity profiles that were obtained for the three compounds tested. For example, the presence of free imatinib affected only two of the 141 kinases quantified in the K562 lysate, a reflection of the highly selective profile of this compound, whereas dasatanib and bosutinib exhibited much broader target profiles, with 39 and 53 proteins, respectively, showing >50% inhibition at 1 μM. Of more importance, this approach enabled the discovery of both a new kinase target as well as a non-kinase target of imatinib. The new kinase target is the discoidin domain receptor 1 (DDR1), which was subsequently verified as a true target *via* various enzymatic and cellular assays. DDR1 is believed to be involved in tumor progression, metastasis, atherosclerosis, fibrosis, and lung inflammation. Because imatinib has been shown to counteract some of these phenomena, it will be quite exciting to determine whether these observations are indeed a direct consequence of DDR1 inhibition. The new non-kinase target identified is NQO2, an abundant NADPH-dependent oxidoreductase that protects against oxidative stress. Interestingly, this exact species was also found to be an off-target of quinoline drugs as earlier reported by Haystead (*4*) using γ-phosphate-linked ATP-Sepharose to enrich the purine-binding proteome. NQO1, a close homologue of NQO2, was also identified by affinity chromatography by using an ATP-mimetic trisubstituted purine that altered the structure of the mitotic spindle in *Xenopus* egg extracts (*9*). The authors also demonstrate the ability of these binding assays to differentiate between distinct conformations of a target in the same cell. This was shown by the lack of competition observed by imatinib for kinobead-bound wild-type c-KIT but the detection of submicromolar competition for activated c-KIT, in this case measured by using a phosphospecific antibody against Tyr703 rather than MS. Finally, the authors also demonstrate the ability to measure downstream effects of inhibi-

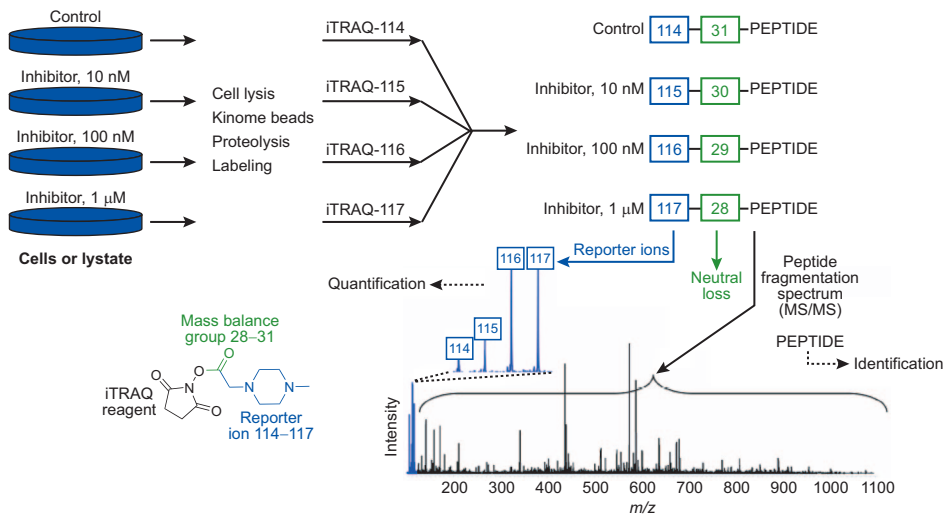


**Figure 2. Process for discovering new cellular targets using kinobeads. Cells are incubated with an inhibitor at a range of concentrations. Cellular extracts are prepared in the presence of the inhibitor and incubated with kinobeads. The beads are washed, and the proteins retained on the beads are subject to tryptic digestion and subsequent labeling with the iTRAQ reagent. The samples are combined and analyzed by LC/MS/MS to determine the identity and relative abundance of the retained proteins. Proteins that are lost in a dose-responsive manner are potential specific targets of the inhibitor of interest. The iTRAQ tags allow the samples to be tracked and quantified during the tandem MS portion of the procedure.**

tion on signaling pathways, as measured by the down-regulation of several sites of phosphorylation among hundreds of such sites detected after phosphopeptide enrichment of iTRAQ-labeled peptide mixtures.

For laboratories without access to personnel highly experienced in MS, a word of caution should be noted. Namely, the pulsed-Q disassociation technique used to enable the detection of the iTRAQ reporter ions in the linear ion trap mass spectrometer in this study has historically been plagued by poor sensitivity because of inefficient fragmentation. The success of the technique is anecdotally related to both the skill of the operator and the instrument itself. Although several reports cite improvements either in this technique or upon implementation of alternative fragmentation mechanisms (*10*), this issue should be kept in mind when determining what instrument to use.

Nevertheless, the technique as described represents the state of the art for real-world chemical proteomics studies. Further improvements in targeted affinity enrichments and/or the sensitivity of the MS methods used should lead to the detection and quantification of targets of even lower abundance, because of overall copy number, distribution among several conformational states, and so forth. In addition, the development of ligands with broad selectivity for other protein classes of interest, such as ubiquitin ligases, phosphatases, and proteases, could greatly extend the overall utility of this approach.

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