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Mini Review

Chemical kinomics: a powerful strategy for target deconvolution

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Kinomics is an emerging and promising approach for deciphering kinomes. Chemical kinomics is a discipline of chemical genomics that is also referred to as "chemogenomics", which is derived from chemistry and biology. Chemical kinomics has become a powerful approach to decipher complicated phosphorylation-based cellular signaling networks with the aid of small molecules that modulate kinase functions. Moreover, chemical kinomics has played a pivotal role in the field of kinase drug discovery as it enables identification of new molecular targets of small molecule kinase modulators and/or exploitation of novel functions of known kinases and has also provided novel chemical entities as hit/lead compounds. In this short review, contemporary chemical kinomics technologies such as activity-based protein profiling, T7 kinase-tagged phages, kinobeads, three-hybrid systems, fluorescent-tagged kinase binding assays, and chemical genomic profiling are discussed along with a novel allosteric Bcr-Abl kinase inhibitor (GNF-2/GNF-5) as a successful application of chemical kinomics approaches. [BMB reports 2010; 43(11): 711-719]

INTRODUCTION

Kinomics is an emerging approach for deciphering kinomes. Chemical kinomics is a discipline of chemical genomics that is also referred to as "chemogenomics", which is derived from the merger of chemistry and biology. Chemical kinomics has become a relevant and promising approach to decipher phosphorylation-based complicated cellular signaling networks with the aid of small molecules that modulate kinase functions. The protein kinase family of the human genome (kinome) is critically involved in diverse regulation of cellular signal transductions. Aberrant activation and dysregulation of certain protein kinases caused by mutation and over-expression have been implicated in a broad spectrum of human diseases. Protein kinases are therefore one of the most important molecular targets for therapeutic intervention in cancer, inflammatory disease and autoimmune disorders (1-4). The human genome contains about 518 protein kinase genes, and kinases constitute about 1.7% of all human genes (5). Among these, approximately 30 kinases have been targeted for drug discovery and development, and over 80 protein kinase inhibitors are known to be undergoing human clinical evaluations (6). In approaching the conquest of cancer, one of the most effective strategies has been found to be identification of selective and potent small molecule inhibitors against oncogenic protein kinases (6, 7). Chemical kinomics using small molecules has played a pivotal role in the field of kinase drug discovery as chemical kinomics enables identification of new molecular targets of given kinase inhibitors (8, 9) and/or exploitation of novel functions of known kinases and has also provided novel chemical entities as hit/lead compounds. For example, the chemical kinomics approach enables identification and validation of the functional targets of dasatinib, which is a multi-targeted kinase inhibitor that becomes a frontline therapy along with imatinib and nilotinib for chronic myelogenous leukemia (CML) treatment, especially for imatinib resistant and intolerant patients. Rix *et al*. reported that dasatinib-interacting 38 proteins in human leukemic (K562) cells were pulled-down using drug-affinity matrices created by covalent attachment of dasatinib to resin and then identified by affinity purification and LC-ESI-MS/MS methods (10). More recently, Li *et al*. identified 40 different kinase targets of dasatinib in human lung cancer (H292, H441 and HCC827) cells by conducting immunoaffinity purification of tyrosine phosphorylated peptides and LC/MS/MS analysis (11). A variety of smart approaches for identifying molecular targets of small molecule kinase inhibitors of interest have been continuously developed. In this short review, recently devised chemical kinomics technologies will be discussed along with the introduction of an allosteric Bcr-Abl kinase inhibitor (GNF-2) as a successful application of chemical kinomics approaches.

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Protein kinases and forward/reverse chemical genomics

Protein phosphorylation, which is triggered in response to extracellular signals, plays important roles in regulating cellular signal transduction cascades including metabolic pathways, cell growth, apoptosis, membrane transport, and gene transcription (12, 13). Protein kinases modify protein functions by transferring a gamma-phosphate group from ATP or GTP to free hydroxyl groups of serine, threonine and tyrosine (1).

Among these, protein tyrosine kinases are a major mechanism for cellular signaling pathways that lead to cell proliferation, differentiation, survival, migration and fertilization (14). Dysregulated kinases such as over-activated kinases in cellular signal transduction cascades have been implicated in pathogenesis in human cancers (15). Protein kinases contain a structurally conserved kinase catalytic domain that comprises the N- and C-terminal lobes. The N-lobe contains several strands to form antiparallel beta-sheets and a single alpha-helix (alpha-C-helix), which accomplishes critical catalytic and regulatory functions in many protein kinases, whereas the C-terminal lobe is composed of several alpha-helixes. These two lobes are connected through a single polypeptide strand (the linker/hinge region) that acts as a hinge that enables the two domains to rotate with respect to one another upon binding of ATP and/or substrate. All protein kinases possess an aspartate residue located on the catalytic loop, which has been implicated in the catalytic mechanism (16, 17). Analysis of 3-dimensional structures of kinases has become a critical starting point in the rational design of small molecule protein kinase inhibitor drugs (18).

Forward chemical genomics, which becomes a central strategy for identifying specific molecular targets through biochemical and cell-based assays (9, 19), relies on the cellular phenotype changes caused by certain chemicals (19). Protein targets have traditionally been pulled down by using the affinity chromatography with their particular small molecule ligands conjugated to a solid matrix such as Sepharose. Proteins purified by a drug-affinity column can be identified by tandem mass spectrometry (MS/MS) enabling protein information such as peptide masses or amino acid sequences (20). However, contamination of nonspecific proteins might lead to misinterpretation in the course of target profiling procedures using affinity chromatography. Therefore, it is necessary to demonstrate that activity altered by the small molecule is significantly correlated with the cellular functions of the target proteins (21). Reverse chemical genomics has become a major approach for exploring novel cellular functions of certain molecular targets based on phenotypic changes created by small molecules. Thus, certain small molecules that have been known to interact with target proteins have been applied to phenotypic screening to identify the physiological roles of the target proteins.

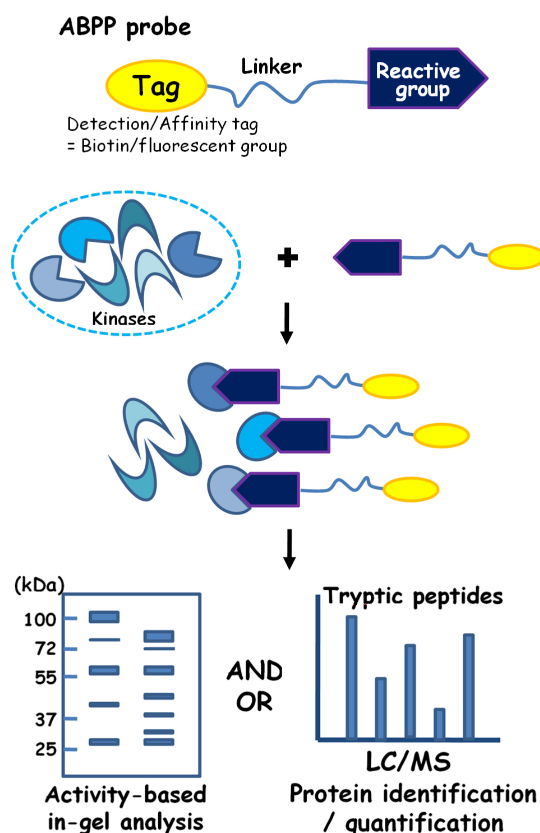

Fig. 1. (Modified from Fig. 1-3 in the reference 22). Activity-based protein profiling. Activity-based protein profiling (ABPP) utilizes active chemical probes that consist of a reactive group, linker, and affinity tag. The reactive group targets specific enzymes and the affinity tag usually employs a fluorophore such as a rhodamine or biotin for detection of the probe-labeled proteins. Basically, specific kinases are reacted with the ABPP probe and probe- labeled proteins are visualized by in-gel fluorescence scanning. In addition, probe-labeled kinases are digested with trypsin and the labeled peptides are then identified by liquid chromatography-mass spectrometry (LC-MS).

Activity-based protein profiling (ABPP)

ABPP is a chemoproteomic technology that utilizes active site-directed chemical probes to identify new molecular targets of given small molecules and to investigate the functional roles of certain enzymes (22). As shown Fig. 1, ABPP probes are composed of three compartments, a reactive group for binding to the active sites of their enzyme targets, a linker region, and a reporter tag portion such as biotin for detecting labeled enzymes. These probes typically possess reactive electrophilic or photoreactive functional groups that enable covalent binding to given kinase targets, which are trapped by active site nucleophilic amino acid residues such as lysine or cysteine (23). ABPP is connected with affinity gel- and mass spectrometry (MS)-based methods for identifying the reactive target kinase proteins (22) (Fig. 1). Patricelli *et al*. reported that 39 targets of staurosporine, a non-selective natural kinase inhibitor, have been identified in cancer cell lines (K562, MOLT4, MDA-MB-435, SK-MEL-5) using the kinase-directed activity-based probes, BHAcATP and BHAcADP, which consist of a biotin tag and an acyl phosphate reactive functional group containing ATP or ADP (24). These chemical probes contain nucleotides (ATP or ADP) that can irreversibly react with conserved lysine residues

of the ATP binding pocket in protein kinases. The kinases labeled with the biotinyl group of BHAcATP and BHAcADP undergo proteolytic digestion with trypsin. The generated biotinylated peptides are captured by streptavidin-agarose beads and the labeled target kinases as well as the specific labeling sites can be identified by LC-MS/MS based on their Xsite platform technology (25). This MS-based Xsite technology can be adopted to assess the kinase selectivity of certain ATP competitive kinase inhibitors in living cells or cell/tissue lysates, which could offer advantages over conventional kinase profiling approaches using recombinant kinases. Fluorophore tags have also been employed instead of biotin tags in ABPP chemical probes. Wortmannin, a PI3K inhibitor, was found to target mammalian Polo-like kinases (PLK1 and PLK3) with the aid of wortmannin conjugated tetramethylrhodamine (TAMRA) chemical probes, referred to as AX-7503 (26, 27). The soluble fractions derived from five breast cancer cell lines were treated with AX-7503, followed by SDS-PAGE separation and visualization of the in-gel using a flatbed laser-induced fluorescence scanner to reveal that AX-7503 labeled PLK1 along with PI3Ks and DNAPKcs from each of these cancer cell lines (27). It is well known that wortmannin irreversibly inhibits PI3K by forming a covalent bond to Lys802 of the PI3K catalytic subunit (28). Additionally, the sequence similarity between PLKs and PI3Ks is low (11%), and it might be difficult to predict that wortmannin could inhibit PLKs based on its potency against PI3Ks. It should be noted that chemical probes such as AX-7503 are of a great value, as indicated by the fact that AX-7503 revealed that different kinases with very low sequence similarity could have related catalytic sites to accept the same small molecules. Analysis of LC-MS/MS revealed that a conserved lysine residue (Lys82 for PLK1 and Lys91 for PLK3) in the ATP-binding site was labeled with TAMRA-wortmannin conjugate (AX-7503) (26).

Peptide-binding array (Peptide microarray)

Protein function microarrays can be divided into three categories, microarrays of protein interaction domains, peptide microarrays and whole proteome microarrays (29). Peptide microarrays that enable deconvolution of protein-protein interactions in high-throughput have become powerful tools for identifying substrates of kinases of interest and for assessing cellular activities of given kinase inhibitors (30-32). Hilhorst *et al*. characterized the substrate specificity of protein kinase A (PKA) utilizing a commercially available peptide microarray (33), while Houseman *et al*. demonstrated that c-Src kinase inhibitors such as quercetin, tyrphostin A47 and ATP noncompetitive inhibitor-PP1 inhibit c-Src kinase activity by assessing on-chip changes in the phosphorylation of the c-Src kinase substrate peptide (34). The peptide chip used in this paper was prepared by the Diels-Alder reaction-mediated immobilization of the c-Src kinase substrate peptide (AcIYGEFKKKC-NH_2) on self-assembled mono-layers (SAMs) of alkanethiolates on a gold surface. Phosphorylation of the immobilized peptides was assayed by measurement of phosphorimaging radioactivity, fluorescence and surface plasmon resonance (SPR) (34). Measurement of phosphorimaing was assessed by detecting the intensity of radioactivity from isotope-labeled ATP on solid surfaces. Fluorescence detection was conducted using the anti-phosphotyrosine antibody and fluorescent dye such as an AlexaFluor 532-labeled goat anti-mouse conjugate. In addition, evaluation through SPR was incorporated using the phosphotyrosine antibody on the chip (34). A notable peptide binding array in a microarray format was developed to detect the inhibitory activity of kinase by resonance light scattering (RLS) or surface-enhanced Raman spectroscopy (SERS) (35). Microarray-based SERS spectroscopic assay has been shown to be a high throughput kinase assay system by using PKA and its known substrates (36). This chip was designed using γ-biotin-ATP as a substrate followed by binding to avidin-modified gold nanoparticles. The authors have confirmed this assay system by demonstrating the efficiency of the known kinase inhibitor (H89, KN62, and SB203508) for the two kinases (PKA and CAMKII) in accordance with the known activity of these inhibitors (37). Due to the unique optical properties of gold nanoparticles, the kinase activity can simply be measured based on the color change.

T7-phage display

The phage display technique describes the display of a myriad of peptides or proteins on the surface of filamentous bacteriophage particles to elucidate molecular interactions of interest including protein-protein, protein-peptide, and protein-DNA (38). Fabian *et al*. adopted a phage display approach using T7 bacteriophage to identify the target protein kinases of certain kinase inhibitors that are currently on the market or in clinical investigation (39). Bacteriophage T7 is a double-stranded DNA phage that is easily growing and replicates rapidly that is also stable and robust (40, 41). An ATP binding site-dependant competition binding assay system employing the T7-phage display technique devised by Fabian *et al*. is composed of three components, T7 bacteriophages expressing almost all of the human protein kinases (T7 kinase-tagged phage), immobilized probe ligands (tethered 'bait' kinase inhibitors such as staurosporine) possessing a high binding affinity to various protein kinases and unmodified free test compounds including small molecule kinase inhibitors of interest. Probe ligands including staurosporine are biotinylated to be immobilized on streptavidin-coated magnetic beads. Test kinase inhibitors compete with immobilized probe ligands for certain kinases tagged on T7 phages (Fig. 2). If the test kinase inhibitors bind these T7 kinase- tagged phages, fewer T7 kinase-tagged phages are captured by the probe ligands immobilized on the beads. The reduced amount of T7 kinase-tagged phage can then be quantified by either phage plaque assays or quantitative PCR to generate the binding constant for the interaction between the test kinase inhibitor and the kinase. Allosteric kinase inhibitors and ATP-competitive kinase inhibitors can be applied to this meth-

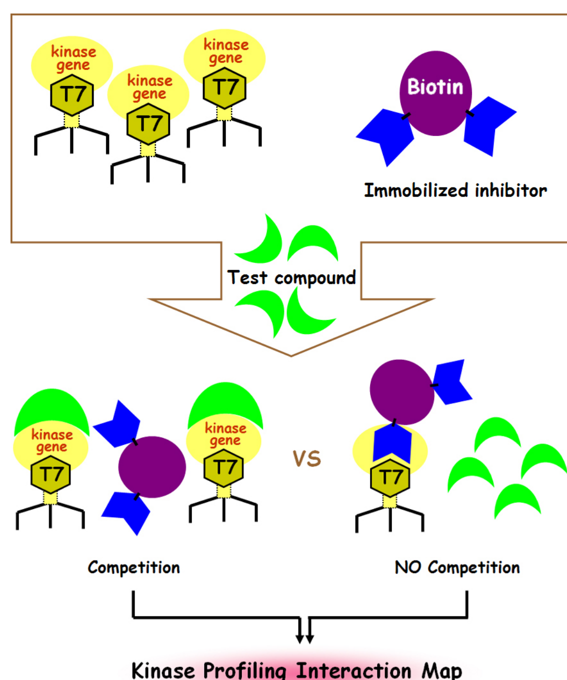

Fig. 2. (Modified from Fig. 1 in the reference 38 and 42). T7 bacteriophage-display based assay. *Escherichia coli*. T7 bacteriophage expressing kinase domains and known immobilized kinase inhibitors (bait compound) are mixed with the test compound, after which the binding ability of the test compound to compete with the bait compound for the kinase domain is measured. Binding profiles are used to develop an interaction map.

odology using T7 kinase-tagged phage (39). A more detailed discussion on allosteric kinase inhibitors will be taken up at a later date. Using this approach revealed that BIRB-796, an allosteric p38 kinase inhibitor, binds to T315I-Abl mutant, which is the most serious imatinib-resistant variant present in the Abl kinase domain. Moreover, LCK (lymphocyte-specific protein tyrosine kinase), is a Src kinase family that was found to be a molecular target (K_d = 62 nM) of imatinib using the T7 kinase-tagged phage approach. It is worth noting that Src and other Src kinase family members such as FYN and FRK have little or no binding affinity to imatinib. By the adoption of this methodology, it was also revealed that clinical EGFR kinase mutations do not affect the intrinsic interactions between the EGFR ATP-binding site and gefitinib or erlotinib. It is important to note that the entire panel of T7 kinase-tagged phages can be run in parallel. Griffin also emphasized the usefulness of an in vitro competitive binding assay using T7 bacteriophage as it enables rapid profiling of the specificity and selectivity of kinase inhibitors of interest (42).

Kinobeads (Mixed kinase-inhibitor matrix)

By using immobilized nonselective kinase inhibitor matrix (kinobeads), Drewes *et al*. developed an improved chemical proteomics approach that enables quantitative assessment of protein-affinity profiles of given kinase inhibitors in any type of cells/primary tissues and investigation of changes in phosphorylation induced by kinase modulators on the captured subproteome. Drewes *et al*. selected a few kinase inhibitors possessing a low kinase selectivity based on their target protein profiling data generated by incubating a collection of immobilized ATP-competitive kinase inhibitors with HeLa or K562 cell lysates. Kinobeads (mixed kinase-inhibitor matrix) were created by immobilizing a combination of the selected seven kinase inhibitors having a broad kinase selectivity profile. A total of 307 non-redundant kinases were identified from pull-down experiments using kinobeads and 14 different cell lines and tissues, indicating that kinobeads could cover a variety of protein kinases located on different branches of the kinome tree. Kinobeads capture a large set of protein kinases and their interacting proteins as well as ATP- and purine-binding proteins such as chaperones, ATPase and helicases. The proteins bound on the kinobeads are subsequently analyzed by tandem mass spectrometry (MS/MS) using the iTRAQ (isobaric tagging for relative and absolute quantification) methodology to generate quantitative affinity profiles of target proteins (43). It is important to note that this cutting-edge proteomics technology that employs kinobeads differs from conventional affinity chromatography methodology in that immobilized small molecules on kinobeads are not test compounds. Unmodified free test compounds including kinase inhibitors of interest compete with immobilized nonselective kinase inhibitors on kinobeads for ATP- and purine-binding proteins when incubated together in cell/tissue lysate. Kinobeads that compete with test compounds capture target proteins of test compounds in a reduced amount when compared with kinobeads treated with vehicle (DMSO). The differential quantity of target proteins captured by test compound treated- and vehicle treated kinobeads is assessed by measuring the signal intensity of the iTRAQ reporter ions. iTRAQ (44), which is a non-gel based proteomics approach, utilizes isotope coded covalent affinity tags, which enables identification and relative/absolute quantification of proteins derived from different sources in a single experiment. iTRAQ reagents composed of four isobaric isoforms form covalent bonds with amino groups of the N-terminus and lysine residue of peptides derived from digested proteins. With the aid of kinobeads, Drewes *et al*. identified receptor tyrosine kinase DDR1 (discoidin domain receptor 1) and NQO2 (quinone acceptor oxidoreductase-2) as unexpected targets of imatinib, dasatinib, and bosutinib in K562 (human immortalized myelogenous leukaemia cell line) (43). Peters and Gray (45) showed that this technology enables the simultaneous quantification of the on- and off-targets of certain kinase inhibitors in any cell type or tissue.

Three-hybrid system approach

The yeast three-hybrid assay system is a useful tool for identify-

ing novel targets including protein kinases of given small molecules in intact cells rather than *in vitro*. This screening system was devised by incorporating a small molecule hybrid ligand into an existing yeast two-hybrid system that has been utilized to deconvolute the protein-protein interactions in cells. As shown in Fig. 3, this assay system consists of three key components, a hybrid ligand, a DNA binding hybrid protein and a hybrid protein containing a transcriptional activation domain. Becker *et al*. reported a three-hybrid assay system employing an MTX (methotrexate)-based hybrid ligand (46). In this study, a hybrid ligand was prepared by conjugating MTX to a small molecule test compound including a kinase inhibitor via a polyethylene glycol repeat spacer. LexA-DBD (DNA binding domain) was fused to dihydrofolate reductase (DHFR) to create a DNA binding hybrid protein. The MTX of this hybrid ligand binds to DHFR (47). GAL4-AD (a transcriptional activation domain) was coupled to a polypeptide that could bind to the test compound to yield another hybrid protein. Small molecule hybrid ligands, which are also known as CIDs (chemical inducers of dimerization), include FK506 (tacrolimus) and MTX (48). FK506 is a potent immunosuppressive drug displaying affinity to FKBP12, which is the 12-kDa FK506-binding protein (49). Caligiuri *et al*. demonstrated a successful example of MASPIT (mammalian small molecule protein interaction trap) based on the cytokine receptor-associated JAK (Janus-activated kinase)/STAT (signal transducer and activator of transcription) signal transduction pathway (50). DHFR fused to the chimeric receptor including Epo receptor (EpoR) interacts with the MTX-fusion small molecule compound. Interaction of the small molecule with the gp130-prey fusion protein leads to recruitment of a docking site for STAT3 and STAT3 can subsequently be phosphorylated by JAK2. The small molecule-protein interaction was determined by measuring the activity of the STAT3-responsive reporter gene (50). This methodology enables identification of target proteins of unmodified small molecules in intact cells, which is an advantage of MASPIT. By adopting this approach, several ephrin receptor tyrosin kinases were found to be molecular targets of PD173955, an ABL kinase inhibitor. Most recently, Jester *et al*. reported a coiled-coil enabled split-protein three hybrid system to rapidly assess the relative binding affinities of kinase inhibitors that are either ATP-competitive or non-ATP-competitive (51). Targeted protein kinase and the coiled-coil Fos are attached to the C- and N-terminal fragment of luciferase, respectively, to yield Cfluc-kinase and Fos-Nfluc fused proteins. These two split-protein reporter fragments (Cfluc-kinase and Fos-Nfluc) and the coiled-coil Jun peptide conjugated to the staurosporine build up the ternary protein complex. This enables restoration of the activity of the split-luciferase. Certain CID-competitive small molecules interfere with the interaction between the Jun-staurosporine and Cfluc-kinase. The disassembly of these two fragments causes loss of the restored split-luciferase activity. The authors also emphasized that this split-protein based three hybrid system can identify the non-ATP binding site targeted allosteric kinase

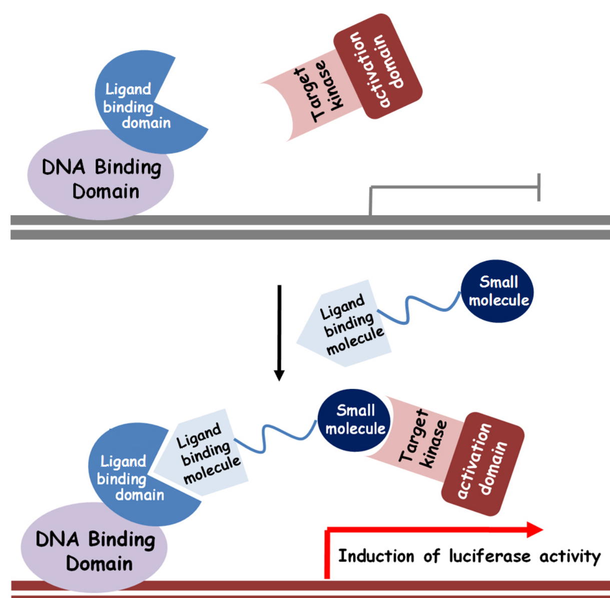

Fig. 3. (Modified from Fig. 1 in the reference 46). Three-hybrid system. The three-hybrid system consists of a DNA-binding domain, ligand-binding molecule linked to a small molecule, and target-kinase protein fused transcriptional activation domain. The DNA-binding domain was fused to a ligand-binding domain such as dihydrofolate reductase (DHFR). Binding of small molecules with target proteins results in the formation of three hybrid components, which then induces the activity of the reporter gene.

inhibitors by inclusion of the full-length kinases, which contain known regulatory domains (51). They found that Akt1/2, which is an allosteric AKT1 and AKT2 inhibitor, inhibits (>70%) the full-length AKT containing the PH domain (Pleckstrin homology domain) while not inhibiting the AKT kinase domain alone. Further, the authors utilized the three hybrid system to demonstrate that Akt1 allosteric inhibitors induce the inactive conformation through binding at the active site of a PH domain. Based on this cell-free split-protein based three hybrid system, further studies might result in cell-based versions of this methodology.

Fluorescent-tagged kinase binding assay

Type II small molecular kinase inhibitors (52) such as imatinib and sorafenib occupy both the ATP binding site and an immediately adjacent allosteric binding pocket that is created when the activation loop of the kinase domain containing the conserved DFG (Asp-Phe-Gly) motif is in an out conformation and becomes closed. A fluorescent-tagged kinase assay devised by Simard *et al*. enables identification of the Type II kinase inhibitors, which stabilize the inactive (DFG-out conformation) kinase conformation (53). They selected the serine/threonine kinase p38α to develop and validate this assay system. Fluorophores incorporated in fluorescent-conjugated proteins are

small and sensitive to conformational changes and/or polarity (54). The most critical point in developing this assay system is identification of the most suitable site in the activation loop to which fluorophores are covalently attached. The amino acid position to which fluorophores form covalent bonds should be significantly moved upon kinase inhibitor binding to facilitate detection of the conformational change and should be solvent-exposed to make fluorophores accessible during the formation of covalent bonds. Based on the results of a previous study (55) that found the movement of amino acids (DFG+1 and DFG+2) immediately following the DFG motif to be significant, Simard *et al*. selected the DFG+2 position (Ala172) of p38α for the fluorophores attachment site and mutated this alanine into a Cys enabling conjugation reaction with thiol-reactive fluorophores. Using fluorophores-tagged A172C- p38α kinase, they detected conformational changes in p38α and assessed dissociation (k_{off})/association (k_{on}) as well as binding (K_d) of kinase inhibitors to the allosteric pocket of p38α kinase (53). Furthermore, they revealed that sorafenib, a B-raf/KDR inhibitor, binds to p38α with a high affinity and demonstrated a unique Type II inhibitor binding mode of sorafenib on p38α Simard *et al*. also applied this methodology to c-Src kinase (56) to identify allosteric type II inhibitors. The results of this fluorescent-tagged binding assay were validated and confirmed by protein X-ray crystallography (56).

Chemical genomic profiling

The chemical genomic profiling approach adopted a direct comparison between microarray transcriptional signatures created by a certain kinase inhibitors with unknown cellular specificity and those created by specific inhibition of candidate analog-sensitive kinases (57). Cellular effects exerted by a kinase inhibitor of unknown specificity were compared with those caused by specific inhibitions of candidate protein kinases bearing a functionally silent mutation in the ATP active site (analog-sensitive allele). It should be noted that engineered analog-sensitive kinases, which are also referred to as "-as" alleles, accept ATP analogues such as 1-NA-PP1 that cannot suppress the kinase activity of wild-type kinases. With this chemical genomic profiling approach, Kung *et al*. identified a cyclin-dependent kinase Cdk1 and Pho85 as *in vivo* target protein kinases of GW400426 in *Saccharomyces cerevisiae* (57) by using analog-sensitive kinases (Cdk1-as1, Pho85-as1 and dual Cdk1-as1/Pho85-as1) and 1-NA-PP1, which is a naphthalene analogue of PP1. This budding yeast, *Saccharomyces cerevisiae*, has frequently been used as a unicellular organism model in genetic studies (58). Chemical genomic profiling using analog-sensitive alleles could be a powerful and general approach for identifying and validating *in vivo* molecular targets of certain kinase inhibitors of unknown specificity. In addition, previous reports have indicated that it is possible to identify the small molecule inhibitors in the analog-sensitive allele of protein kinases (59).

Allosteric kinase inhibitors, GNF-2/GNF-5

Most protein kinase inhibitors that have been developed as therapeutic agents to date are ATP-competitive small molecules that target the ATP-binding site of the kinase domain to compete with the high intracellular concentration of ATP. It is challenging to enhance the kinase selectivity of ATP-competitive small molecule kinase inhibitors as protein kinases share a high degree of homology in their kinase catalytic site. This kinase specificity issue has become a major hurdle in pre-clinical and clinical development of ATP-competitive small molecule kinase inhibitors because low kinase selectivity could potentially lead to drug toxicity. Much greater kinase selectivity could be achieved using allosteric kinase inhibitors that do not compete with ATP by targeting the non-ATP binding site. It should be emphasized that allosteric kinase inhibitors have also provided useful clues to reveal novel functions of kinases of interest. Next, GNF-2/GNF-5, a novel allosteric Bcr-Abl kinase inhibitor, will be discussed as a representative example of allosteric small molecule kinase inhibitors.

The phenomenal clinical success of the Bcr-Abl inhibitor imatinib (STI-571, Gleevec, Novartis) for the treatment of Chronic Myelogenous Leukemia (CML) has opened a new era in discovery of molecularly targeted therapeutic agents. Imatinib binds to both the ATP-binding site and its adjacent allosteric site in the Abl tyrosine kinase domain. Imatinib recognizes a distinctive inactive conformation of Abl kinase domain and freezes its inactive conformation to prevent conversion into the active conformation of the Abl kinase domain, thereby inhibiting Abl kinase activity (60, 61). One major problem facing imatinib treatment is the emergence of acquired drug resistance, which is mainly caused by several point mutations in the Abl kinase domain. This imatinib resistance decreases the clinical efficacy of imatinib (62-65). To overcome the imatinib resistance, Adrian *et al*. identified the GNF-2 compound using an unbiased differential cytotoxicity high-throughput screen evaluation of a combinatorial kinase directed heterocycle library (66, 67). GNF-2, which is a 4,6-disubstituted pyrimidine analogue, is an allosteric non ATP competitive Abl kinase inhibitor. GNF-2 is capable of strongly and specifically inhibiting Bcr-Abl-dependent cell proliferation. GNF-2 also has an exceptionally high degree of enzymatic specificity, as indicated by its not exhibiting any kinase inhibitory activity against a panel of 63 kinases during biochemical assay, even at high (10 μM) concentrations. GNF-2 strongly inhibits E255V-Bcr-Abl, a P-loop point mutant that causes imatinib resistance but does not have significant activity against T315I-Bcr-Abl 'gatekeeper' mutation, which is the most recalcitrant Abl point mutant. HSQC NMR analysis and X-ray crystallography revealed that GNF-2 binds to the myristate binding pocket located at the bottom of the C-lobe of the c-Abl kinase domain, and it has been proposed that GNF-2 might inhibit Bcr-Abl kinase activity by stabilizing the inactive conformation of Bcr-Abl upon binding to the myristoyl pocket of the Abl kinase domain. It was also reported that GNF-5, a GNF-2 analog pos-

sessing improved pharmacokinetic properties, showed good *in vivo* efficacy against wild-type Bcr-Abl in a bioluminescent xenograft mouse model using a transformed wt-Bcr-Abl-Ba/F3 cell line that has stable luciferase expression (67). Furthermore, combination of GNF-2 or GNF-5 with an ATP-competitive Abl kinase inhibitor such as imatinib and nilotinib resulted in remarkable synergistic activity against both wild-type and T315I-Bcr-Abl mutant by binding to its ATP and myristate-binding sites (67). It should be noted that individual treatment with GNF-2, GNF-5, or nilotinib did not induce appreciable potency against the T315I 'gatekeeper' Abl mutation. In the T315I-Bcr-Abl bone-marrow transduction/transplantation model, which resembles human CML disease, the combination of GNF-5 (75 mg/kg) and nilotinib (50 mg/kg) resulted in normalization of white-blood-cell counts and spleen weights and extended survival relative to those with treated with either GNF-5 alone and/or nilotinib alone. Moreover, hydrogen- exchange mass spectrometry revealed that binding of GNF-5 to the Abl myristate-binding site alters the conformation of the ATP-binding site of Abl. This method can measure protein dynamics such as conformational change through alteration of the enzyme activity during the binding of small molecules. Mass spectrometry measures the exchange of labile backbone hydrogens in a protein with deuterium atoms in a D_2O bulk solvent state (68). Indeed, binding of GNF-5 to the myristate-binding pocket causes significant hydrogen exchanges in several peptides near the myristate-binding cleft as well as the ATP-binding site (residue 306-316 and 317-324). This provides a reasonable mechanism by which synergistic effects caused by the combination of GNF-5 and nilotinib (a binder of Abl ATP-binding site) occur.

CONCLUSION

Phosphorylation is the cornerstone of cell signaling and is involved in all signal transduction cascades. Deciphering phosphorylation-based complicated cellular signaling networks has become a critical requirement in the field of kinase drug discovery. Dysregulation of the protein kinases is deeply implicated with a variety of diseases. Druggable protein kinases have therefore become relevant molecular targets for therapeutic drug discovery/development. Chemical kinomics using small molecules that modulate kinase functions have become a powerful approach to decipher phosphorylation-based cell signaling, which enables identification of new therapeutic molecular targets, as well as novel functions of known kinases and unique modes of action for given kinase inhibitors.

In this short review, contemporary technologies for chemical kinomics research, including activity-based protein profiling, T7 kinase-tagged phage, kinobeads, three-hybrid system, fluorescent-tagged kinase binding assay, and chemical genomic profiling, were discussed along with a representative allosteric kinase inhibitor, GNF-2/GNF-5.

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