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Reactivity-Based Chemical-Genetic Study of Protein Kinases

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Abstract

The human protein kinase superfamily comprises over 500 members that operate in nearly every signal transduction pathway and regulate essential cellular processes. Deciphering the functional roles of protein kinases with small-molecule inhibitors is essential to enhance our understanding of cell signaling and to facilitate the development of new therapies. However, it is rather challenging to identify selective kinase inhibitors because of the conserved nature of the ATP binding site. A number of chemical-genetic approaches have been developed during the past two decades to enable selective chemical perturbation of the activity of individual kinases. Herein, we review the development and application of chemical-genetic strategies that feature the use of covalent inhibitors targeting cysteine residues to dissect the cellular functions of protein kinases.

Introduction

Protein kinases participate in central signaling pathways by controlling reversible phosphorylation of substrate proteins in eukaryotic cells. Due to the large number of kinases and their ubiquitous presence in physiological processes, these enzymes represent a major class of targets for drug discovery and development.[1] While significant progress has been achieved in understanding the regulation and function of numerous kinases, a large portion of the "kinome" remains understudied, with less than 10% of all protein kinases being targeted by FDA-approved small-molecule drugs.[1,2] Identification of potent and selective kinase inhibitors is a challenge because of the highly conserved nature of the kinase active site where the majority of kinase inhibitors bind. Having selective kinase inhibitors available, however, is critical to expand our understanding of kinase functions and mechanisms in biological processes, which in turn will accelerate the development of therapeutics for this protein family. About twenty years ago, chemical genetics emerged as an innovative means for deciphering the roles of kinases in biology and disease and, therefore, aid in the discovery and development of selective kinase inhibitors. Chemical genetics is a valuable and powerful tool that marries genetic selectivity to rapid pharmacological perturbation. As an emerging discipline, it incorporates diverse areas of research, including genetics, protein engineering, medicinal chemistry, and cell biology, that together allow the generation of potent and specific small-molecule inhibitors capable of selectively and rapidly perturbing the functions of one or few protein targets at a time.[3,4]

A number of chemical-genetic approaches have been successfully developed and applied to the study of protein kinases over the past 25 years. They involve engineering a kinase for specific recognition and inhibition by a rationally designed inhibitor. Ideally, these inhibitors are not

recognized by wild-type (WT) kinases to diminish off-target effects and confidently attribute the observed phenotypes to inhibition of the mutant proteins. The selective inhibition of the engineered kinase is based on either shape or reactivity complementarities between the mutant kinase and the inhibitor afforded by these chemical genetic approaches. The first approach, termed "bump-hole", was developed earlier, and found wide applications in the functional characterization of protein kinases. The latter approach has found complementary and unique applications compared to bump-hole using electrophilic compounds to afford covalent inhibition of the engineered kinases. In this review, we highlight structural and functional features of protein kinases that are important for inhibitor development and then describe various chemical-genetic strategies that have been used to study protein kinases in cells, with a focus on those featuring covalent inhibitors targeting cysteines.

Protein kinases

The human kinome contains 538 distinct protein kinases, accounting for approximately 3% of all human genes.[5,6] Protein kinases are enzymes that catalyze the transfer of the γ-phosphoryl group of ATP to serine, threonine, and tyrosine residues on a substrate protein. Reversible phosphorylation is an essential post-translational modification that plays key roles in cellular signal transduction and metabolism. Phosphorylation catalyzed by protein kinases is involved in nearly all eukaryotic cellular pathways, and is crucial to normal cell growth and development.[7,8] Dysregulation of kinase function, therefore, contributes to cancers and a variety of other human disorders, including neurological, metabolic, and immunological diseases. As a result, protein kinases represent an important class of drug targets.[9]

Structurally, serine/threonine and tyrosine kinases share a highly conserved active site that is located in a deep cleft between two lobes, the N-terminal and C-terminal domains (**Figure 1A**). These are connected by a short and flexible conserved loop known as the hinge region. The N-lobe is composed of β-sheets and one α-helix, termed helix αC, as well as a glycine-rich phosphate-binding loop (P-loop). The C-lobe is predominantly α-helical and contains the conserved DFG motif, the activation loop (A-loop), and the catalytic loop (C-loop).[10,11]

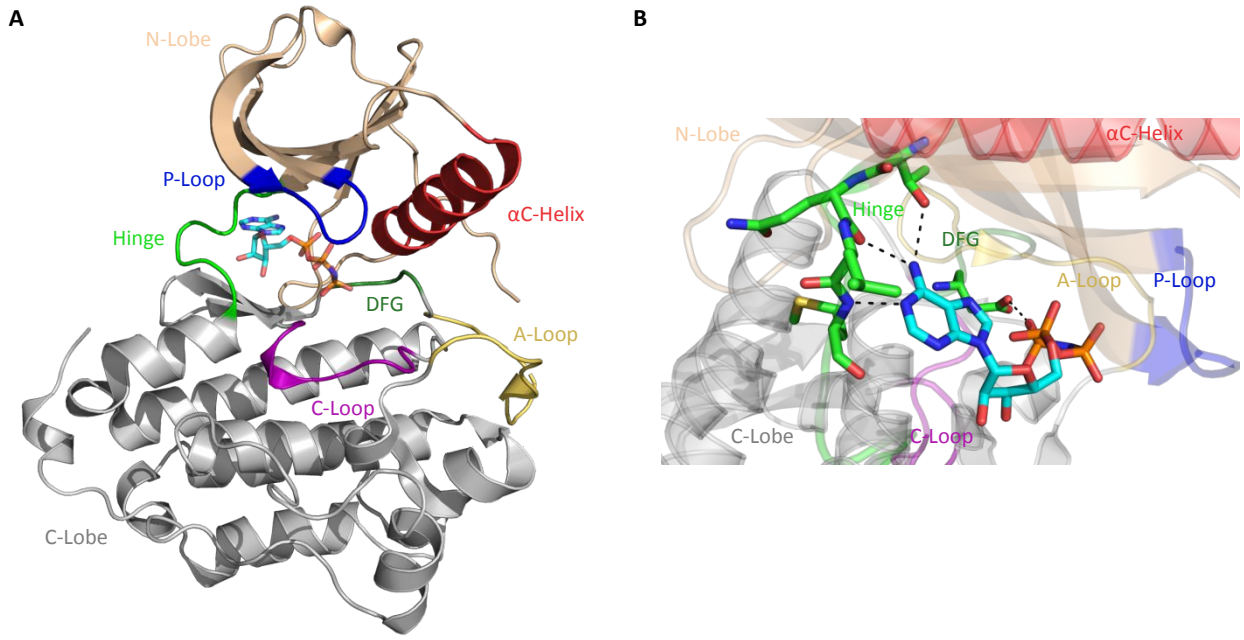

Figure 1. General structure of the catalytic domain of protein kinases. (**A**) Ribbon representation of the kinase domain of the epidermal growth factor receptor (EGFR) in its active state with key structural elements highlighted in different colors as follows: activation loop (A-loop), yellow; αC helix, red; phosphate-binding loop (P-loop), blue; catalytic loop (C-loop), pink; hinge region, green; DGF motif, dark green. Adenylyl-imidodiphosphate (AMP-PNP) is bound to the ATP-binding pocket (PDB ID: 2GS6). (**B**) Hydrogen bonds between the ATP-binding site of EGFR and AMP-PNP are indicated by black dashed lines.

Most protein kinases adopt similar active conformations upon activation, while inactive kinases adopt distinct conformations, reflecting the diversity in their regulation. Active kinases require a

specific alignment of key elements in the kinase domain for a successful phosphoryl transfer. ATP binding is stabilized by interactions with conserved amino acid residues located on the hinge region, αC helix, DFG motif, and P-loop (**Figure 1B**). The adenine ring of ATP hydrogen bonds to the peptide backbone of the hinge region, with the N^6 position binding to a large and conserved amino acid side chain, termed gatekeeper, located N-terminal to this region. The phosphate groups are stabilized by interactions with the glycine-rich P-loop and a conserved lysine residue in the N-lobe (not shown in **Figure 1B**). Peptide substrate binds close to the γ-phosphate of ATP near the A-loop. This loop is phosphorylated on either serine/threonine or tyrosine residues when the kinase is in its active state. The activation segment varies its conformations substantially depending on the state of the kinase. In the unphosphorylated state (inactive), it collapses into the active site and blocks the binding of both ATP and peptide substrate. When the kinase is activated by phosphorylation, it moves outwards from the active site, exposing the phosphorylated residues and allowing substrate binding and catalysis.[10–12]

The majority of kinase inhibitors are ATP-competitive small molecules that lack specificity due to the highly homologous nature of the ATP-binding pocket within the protein superfamily. They interact with the kinase active site often mimicking the hydrogen bonds that the adenine group of ATP forms with the protein main-chain atoms located in the hinge region (**Figure 1B**). These compounds can either bind to the active/DFG-in (type I) or the inactive/DFG-out (type II) conformations of the kinase. Other compounds bind to a nonconserved allosteric site that can be adjacent to (type III) or remote from (type IV) the kinase active site.[13,14] Small molecules that target the same conserved ATP-binding pocket usually exhibit cross-reactivity, resulting in promiscuous inhibitors. Allosteric inhibitors can circumvent this hurdle by binding to distinct

sites involved in regulatory mechanisms that are unique to an individual kinase, thus presenting higher selectivity.[9] Only a fraction of protein kinases have been shown to be amenable to allosteric inhibition, however.

Another way to achieve selectivity is with covalent inhibitors – compounds that form an irreversible bond with a nucleophile on the kinase, usually a cysteine residue.[15,16] The efficiency of a covalent inhibitor is determined by the initial reversible interactions with the protein and the rate of subsequent covalent bond formation with a nearby nucleophilic amino acid residue in the target kinase.[16,17] The former depends on its reversible binding kinetics, while the latter depends on the reactivity of the electrophile and its accurate positioning.[18,19] Using structure-guided design, one can predict the optimal position to attach an electrophile to a known non-covalent inhibitor. The initial step of reversible binding serves to orient the reactive warhead in proximity of a cysteine residue for covalent bond formation. This combination of noncovalent and covalent binding factors often results in a highly specific permanent interaction between the irreversible inhibitor and the target kinase.[20–22] Many kinases have cysteines in and near the ATP-binding site, which are collectively known as the cysteinome of protein kinases.[23] Different studies have mapped these residues revealing the positions of the cysteines, which are largely distributed among the hinge region, the glycine-rich P-loop, and near the DFG motif.[9,24–26] These cysteines are all non-catalytic residues that do not directly participate in phosphoryl transfer, hence being well-suited for covalent targeting with electrophilic inhibitors.[23]

Genetic techniques including knockout/knock-in and transgenic animals, gene mutations or deletions, and RNA interference (RNAi) technology have been instrumental in functional

characterization of kinases.[27] The advantages of genetic approaches are the high specificity and portability, given that a single gene can be modified in different organisms. Despite its usefulness in dissecting biological functions in lower organisms, traditional genetics presents difficulties when applied in mammals due to their large physical size, large diploid genome, prolonged gestation periods, and slow reproduction rate. Another limitation is the functional compensation effect produced when related genes mask a phenotype caused by the knockout of a non-essential gene. Gene deletions can also impair the study of protein functions that are modulated by post-translational modifications and, in the case of an essential gene, can be lethal, compromising the further study of the organism. Furthermore, most genetic mutations are not conditional, i.e., they cannot be turned on and off as needed. Although conditional mutations, like temperature-sensitive (*TS*) alleles, can be employed to achieve reversibility, they can induce undesirable side-effects, such as the heat shock response, making the resulting phenotypes difficult to interpret.[3,28,29]

Pharmacological approaches to study protein kinases present advantages when compared to genetic techniques such as rapid and conditional target inhibition. The poor selectivity of small-molecule kinase inhibitors, however, is a limitation of these methods because it is always possible that the observed pharmacological phenotypes reflect an off-target effect of the drug. Combining pharmacological and genetic approaches, chemical genetics surfaced as an alternative to overcome the difficulties found in classical genetics and pharmacological methods. This approach allows the study of genes and/or proteins by using small molecules to induce a particular phenotype.[30] The use of chemical genetics to dissect complex signaling pathways offers several advantages over traditional genetics. First, small molecules act rapidly and

reversibly, usually functioning in different cell types and organisms. Second, this approach is conditional; ligands can be added or removed at will, as well as used at different concentrations, allowing for real-time temporal control and direct kinetic analysis of the protein modulation by the chemical compound. Third, small molecules can act as either gain-of-function or loss-of-function mutations, depending on whether they act as agonists or inhibitors of the target protein. Finally, rather than shutting down entire pathways (as with classical genetics), specific binders make it possible to observe the effects of perturbing individual activities of multifunctional enzymes and receptors.[27,29,31]

Dissecting protein kinase functions with chemical-genetic approaches

The bump-hole approach

The use of small molecules to perturb biological functions is limited to compounds that are available and characterized. Although this was an issue when the field of chemical genetics first appeared, the number of small molecules that became accessible through commercial libraries and public repositories increased dramatically in the past two decades.[29,32] A more significant issue, however, is to find compounds that specifically interact with a protein of interest. This is especially challenging when the target protein shares a high degree of homology with others, as it happens with protein kinases. As a means to overcome this problem, a combination of medicinal chemistry with molecular biology to generate non-natural ligand/protein pairs can be used.[33] Using genetic techniques, it is possible to introduce point mutations or even entire domains in proteins to confer specificity to a high-affinity promiscuous inhibitor.[3] Shokat and colleagues have previously established a combined chemical-genetic approach to afford highly specific inhibition of kinases by modifying the protein to exclusively recognize rationally

designed inhibitors.[34,35] The approach is known as "bump-hole", with the engineered kinase referred to as analog-sensitive (*AS*) allele (**Figure 2**). In this context, a functionally silent but structurally significant mutation is introduced to the kinase active site, specifically, to the gatekeeper residue. The gatekeeper is a conserved, bulky, and mostly hydrophobic amino acid (methionine, leucine, threonine, phenylalanine, among others) located near the hinge region (**Figure 2A**). Replacement of this residue with glycine or alanine (smaller residues) creates an extended pocket (or "hole") that can be uniquely accessed by a bulky (or "bumped") inhibitor (**Figure 2B**). The inhibitor is designed by modifying non-specific kinase inhibitors with substituents that are complementary to the new pocket only, thus not inhibiting WT kinases due to steric clash.[36–39] To expand the kinome coverage of the *AS*-kinase technology, different heterocyclic scaffolds have been derivatized, such as quinazoline[40] and indazole[41]. In the attempt of producing more potent and selective *AS*-kinase inhibitors, however, pyrazolopyrimidine-based (PP) analogs (**Figure 2C**) were found to be the ones with not only the best potency and selectivity but also generality toward protein kinases.[42]

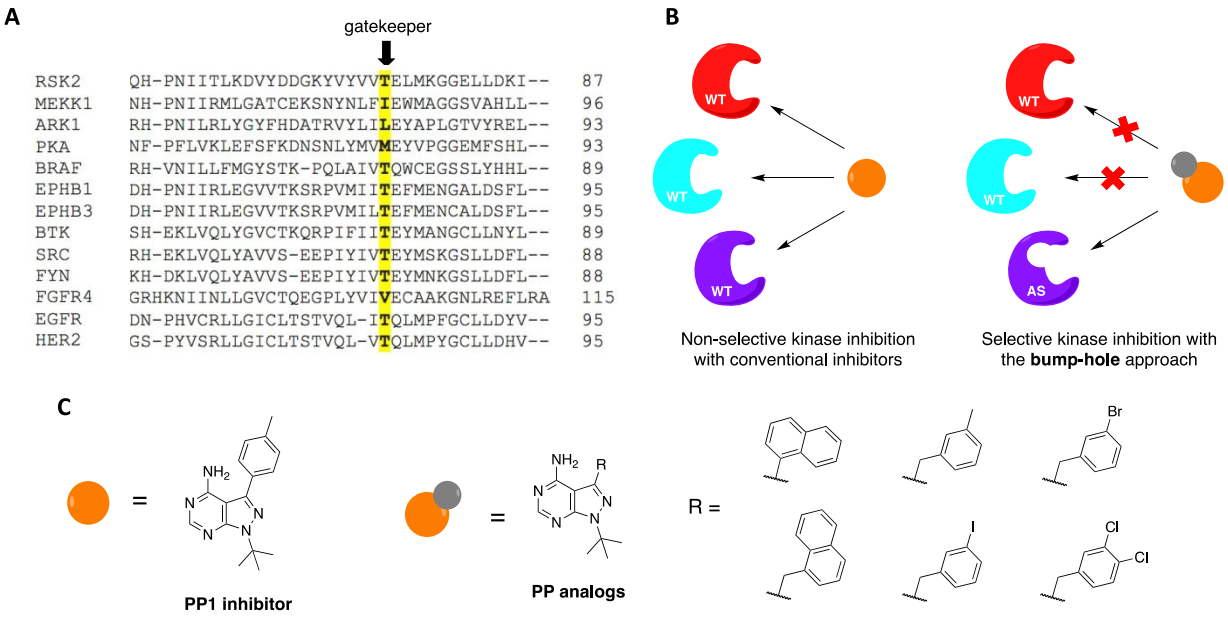

Figure 2. Bump-hole approach allows for selective inhibition of a single engineered protein kinase. (**A**) Partial sequence alignment of different kinase domains highlighting the conserved gatekeeper residue. (**B**) Mutation of this residue to glycine or alanine creates an extra pocket ("hole) in the active site of the analog-sensitive kinase (*AS*-allele) that can be specifically targeted by a bulky ("bumped") inhibitor. (**C**) Chemical structures of non-selective kinase inhibitor PP1 and rationally designed PP analogs.

The bump-hole method has been applied to the study of numerous kinases in diverse organisms including mammals and yeast.[43–51] Its use allowed the dissection of kinase functions, the elucidation of novel signaling mechanisms, as well as the discovery of highly selective and potent kinase inhibitors. For a kinase of interest to be amenable to the approach, however, some requirements must be met. First, the stability and activity of the mutant protein should be comparable to that of the WT. Second, it must be possible to introduce the mutant allele into the organism of interest. Third, a potent, bioavailable, and orthogonal inhibitor analog to the protein of interest should be identified.[3,42] Despite the successful application of this approach to dozens of kinases from diverse organisms, there are two limitations in its applications. First, a portion of enzymes do not tolerate the required gatekeeper mutation.[52] The gatekeeper residue is in direct

contact with the N^6 group of ATP. It governs inhibitor and substrate specificity in the kinase active site by controlling access to a deep hydrophobic pocket that is not in contact with ATP (thus the name "gatekeeper").[53] Upon mutation of this residue to glycine or alanine, certain kinases suffer a significant or severe loss of catalytic activity and cellular function. Second, although tolerant to the gatekeeper mutation, some kinases are not effectively inhibited by available bumped inhibitors.

Over the past two decades, researchers have developed complementary techniques to overcome these limitations. For instance, introduction of second-site suppressor mutations (or suppressors of glycine gatekeeper – *sogg*) was able to restore the catalytic activity or cellular function of weakened *AS*-kinase alleles.[52] This strategy has been successfully applied to kinases that play important roles in diverse signaling pathways, such as stress-activated MAPK signaling (MEKK1), mitotic regulation and cytokinesis (Cdc5), G protein-coupled receptor signaling (GRK2), and plant disease resistance (Pto). Although these strategies improved the tolerance of some kinases to bump-hole, this technique still does not work for all kinases. As a result, a significant portion of the kinases are not amenable to the bump-hole method, leaving researchers with the challenging quest to discover alternative chemical-genetic approaches that could fill in this gap.

Cysteine-targeting chemical-genetic approaches

For protein kinases that were not amenable to the aforementioned methods, the use of covalent inhibition has proven effective in achieving selectivity without compromising kinase activity or biological function. These covalent chemical-genetic approaches all involve the use of

electrophilic compounds to target a nucleophilic cysteine residue, natural or engineered, that is in or near the active site of the target kinase. They differ in the positions of the covalently targeted cysteine residue, however. These covalent chemical-genetic studies are grouped based on the position of the covalently targeted cysteine and reviewed in detail below.

Kinases containing an EGFR-like cysteine

The discovery of irreversible kinase inhibitors dates back to the 2000s when neratinib and pelitinib were reported as the first covalent inhibitors of EGFR.[54] These compounds are 4-anilino-3-cyano quinoline derivatives that contain a Michael-acceptor group at the 6-position to target Cys797 of EGFR.[55] Cys797 is located within the kinase hinge region, seven residues C-terminal to the gatekeeper residue. Neratinib was approved by the FDA for the treatment of early-stage HER2-positive breast cancer in 2017.[56] Covalent kinase inhibitors have advantages over their reversible counterparts in cancer clinics due to their superior efficacy and selectivity, relative safety, and the ability to overcome drug resistance.[57] Other examples that have been approved by the FDA over the past few years include afatinib[58] (2013, EGFR and human epidermal growth factor receptor 2-HER2), ibrutinib[59] (2013, Bruton's tyrosine kinase-Btk), osimertinib[60] (2015, EGFR), and, more recently, a combination of neratinib and capecitabine[61] (2020, advanced or metastatic HER2-positive breast cancer).

The successful application of these compounds in oncology triggered more research efforts not only into the generation of novel covalent kinase inhibitors to treat an increased number of tumor types but also into other areas of research involving protein kinases, such as chemical genetics. In covalent chemical genetics, a cysteine residue can be irreversibly targeted by an electrophilic

inhibitor. Covalent inhibition can be employed alone or in combination with bump-hole as a second filter to further improve selectivity (**Figure 3A**). While bump-hole works with shape complementarity, cysteine-targeting approaches are based on reactivity complementarity. Some advantages of this approach over bump-hole are the possibility of targeting cysteine residues located at different positions other than the gatekeeper (**Figure 3B**), achievement of a more complete inhibition due to the formation of an irreversible bond between cysteine and electrophile, higher selectivity due to targeting of rare or even unique cysteine residues on protein kinases, and the potential of developing orthogonal chemical-genetic systems to inhibit distinct kinases in the same cell (**Figure 3C**).

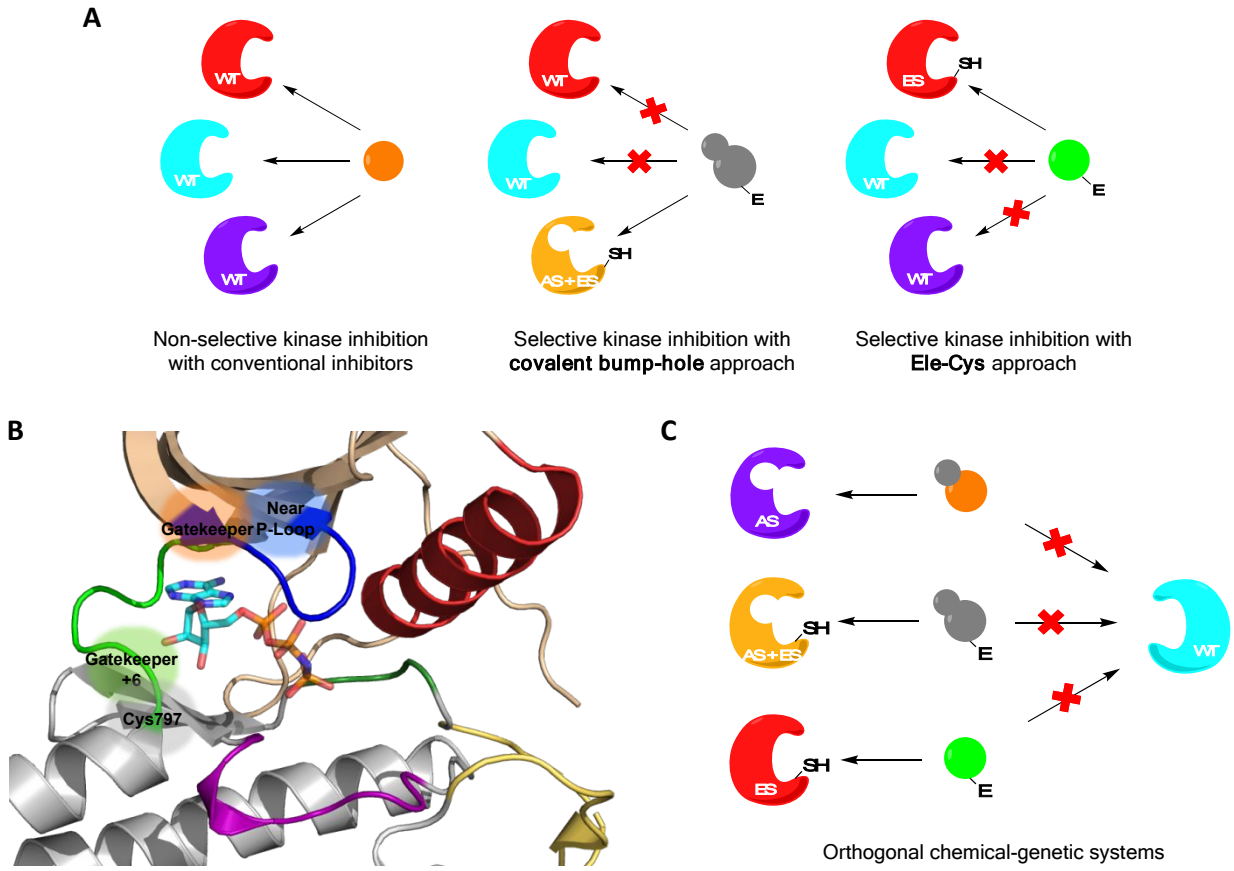

Figure 3. Cysteine-targeting covalent approaches to selectively inhibit protein kinases. (**A**) Covalent chemical-genetic approaches achieve selectivity by relying on the covalent complementarity between an engineered cysteine and an electrophilic inhibitor. (**B**) Cysteine residues at different positions can be used instead of relying on just the gatekeeper. A few previously reported positions are highlighted in and near the ATP-binding pocket in the kinase domain of EGFR (PDB ID: 2GS6). (**C**) Covalent complementarity makes it possible to develop orthogonal pairs of engineered kinases and electrophilic inhibitors that allow the study of signaling pathways involving multiple kinases in the same cell.

One covalent chemical-genetic strategy to identify specific inhibitors of protein kinases combines shape complementarity with covalent complementarity. Here, kinases are rationally designed to bear a space-creating mutation at the gatekeeper position and a cysteine at a position analogous to Cys797 of EGFR in the active site (native residue or engineered), which functions as an irreversible anchor point for a space-filling kinase inhibitor (**Figure 4A, B**). The first

admits binding of bulky small molecules that WT kinases do not tolerate due to steric clash, and the latter increases potency and target specificity. Some protein kinases may already have one or both selectivity filters, requiring either none or only one mutation to be introduced in its active site for this method to work. Using this strategy, c-Src was sensitized to 6-acrylamido-4-quinazoline analogs containing bulky aryl groups (**Figure 4C**) by engineering the tyrosine kinase with both a glycine gatekeeper and a cysteine residue at a position analogous to Cys797 of EGFR, located at the C-terminus of the hinge region (**Figure 4A)**.[62] These analogs were designed based on the structure of EGFR's reversible inhibitor Erlotinib. The rationally designed compounds showed higher potency against analog-sensitive c-Src alleles (both *AS*-kinase allele and double mutant) while being less effective against a c-Src-cys mutant that did not have the expanded binding pocket. After confirming the inhibitors' selectivity for kinases containing both specificity elements, an irreversible fluorescent affinity probe was also designed to specifically label an analog-sensitive of EGFR in cells (**Figure 4D**). With a covalent probe in hand, it was possible to measure target engagement after probe treatment and subsequent downstream signaling activation specifically caused by the kinase allele. In another study, fission yeast Aurora kinase (Ark1) was also genetically engineered for selective inhibition by covalent anilinoquinazolines.[63] Both alanine gatekeeper and a cysteine residue analogous to EGFR Cys797 were introduced in the protein's active site, but additional suppressor mutations were necessary to rescue kinase function.

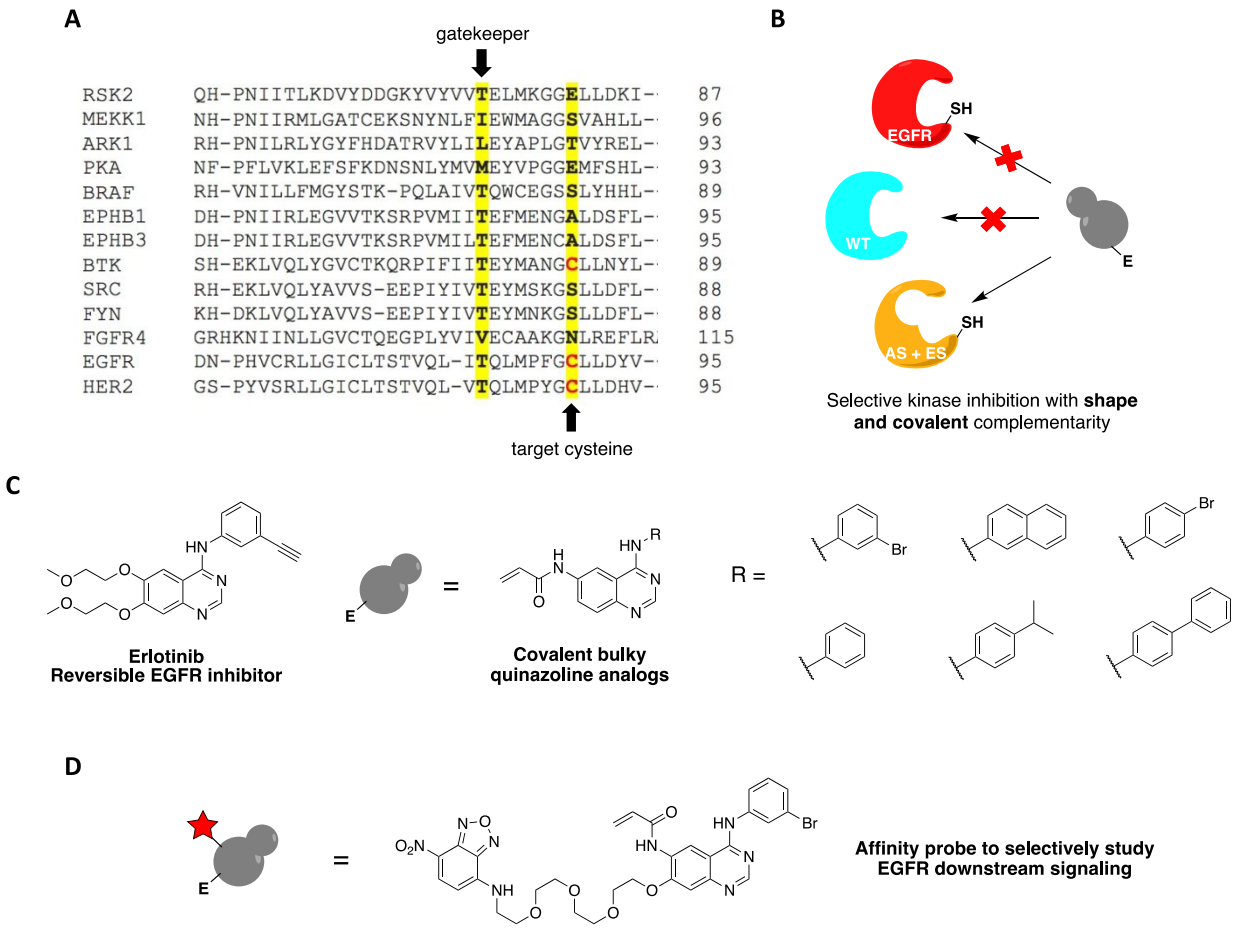

Figure 4. Covalent chemical-genetic approach to specifically target tyrosine kinases. (**A**) Partial sequence alignment of several protein kinases showing the chosen sites for the two-point mutations: the gatekeeper and a rare cysteine residue (Cys797 in EGFR). (**B**) By introducing two mutations into its active site, only the sensitized kinase (AS + ES) will bind to the rationally designed inhibitor since these features are not found together in any WT kinase. (**C**) Chemical structures of 6-acrylamido-4-anilinoquinazoline irreversible inhibitors used in the studies. The 4-anilinoquinazoline core is common in many EFGR inhibitors, such as Erlotinib. (**D**) Chemical structure of covalent affinity probe to measure target engagement and study the downstream signaling of EGFR allele in cells. A nitrobenzoxadiazole (NBD) fluorophore is linked to the quinazoline scaffold at C7 via a PEG chain.

A previous work by Poulikakos and coworkers utilized covalent complementarity with other chemical-genetic systems in a mechanistical study of RAF kinases. Here, a panel of engineered RAF kinases to investigate the mechanism behind an observed MEK/ERK signaling increase in cells expressing WT BRAF but not mutant BRAF(V600E) when treated with ATP-competitive

RAF inhibitors (**Figure 5A, B**).[64] This finding was observed with six ATP-competitive RAF inhibitors, including PLX4032 and PLX4720 (vemurafenib and its precursor) (**Figure 5D**). The authors proposed that the paradoxical activation of the enzyme by inhibitors was due to drug-mediated transactivation of RAF dimers. To test this hypothesis, RAF mutants with different kinase properties, such as catalytic inactivity, disrupted drug binding, electrophile-sensitive, and inability to dimerize, were created. The first series of experiments were performed with the drug PLX4720 to analyze the requirements for the paradoxical activation. Using CRAF mutants that could not bind to either RAS or the drug, the study showed that MEK/ERK activation by PLX4720 is RAS dependent and requires direct drug binding to the catalytic domain of RAF. Besides, knockout of *Braf* or *Craf* proved that only CRAF expression is necessary for significant signaling induction while BRAF is not required. The paradoxical activation was further confirmed by the observed increase on phosphorylation of both CRAF WT and a kinase-dead mutant upon binding of PLX4720, but not of the drug-resistant CRAF mutant. This induced phosphorylation led to activation of the enzyme and, subsequently, ERK signaling. After confirming the activation hypothesis, a covalent chemical-genetic approach was introduced to study the mechanism by which RAF inhibitors activate RAF dimers. For this, a cysteine residue was introduced into RAF at the position that is homogolous to EGFR Cys797 (**Figure 5C**) to achieve isoform selectivity through covalent inhibition by electrophilic quinazolines (JAB analogs) (**Figure 5E**), which would not be possible with the available reversible inhibitors of the kinase. Co-expression of JAB34-sensitive, kinase-dead CRAF and JAB34-insensitive, catalytic active CRAF proteins in 293H cells led to pronounced induction of ERK phosphorylation, showing that drug binding to the kinase-dead mutant transactivated the catalytically competent RAF. In contrast, when the drug bound to a catalytically active mutant that dimerized with

another that was kinase-dead but JAB34-insensitive, high concentration of the drug (10 μM) inhibited ERK signaling. Transactivation from CRAF to BRAF was also confirmed when co-expressing BRAF and the JAB34-sensitive, kinase-dead CRAF mutant in 293H cells. This model suggested a dependency on RAF dimerization for transactivation to occur. This was further validated with JAB34 failing to induce ERK signaling in cells co-expressing catalytically competent kinase and a JAB34-sensitive CRAF mutant with compromised ability to dimerize.

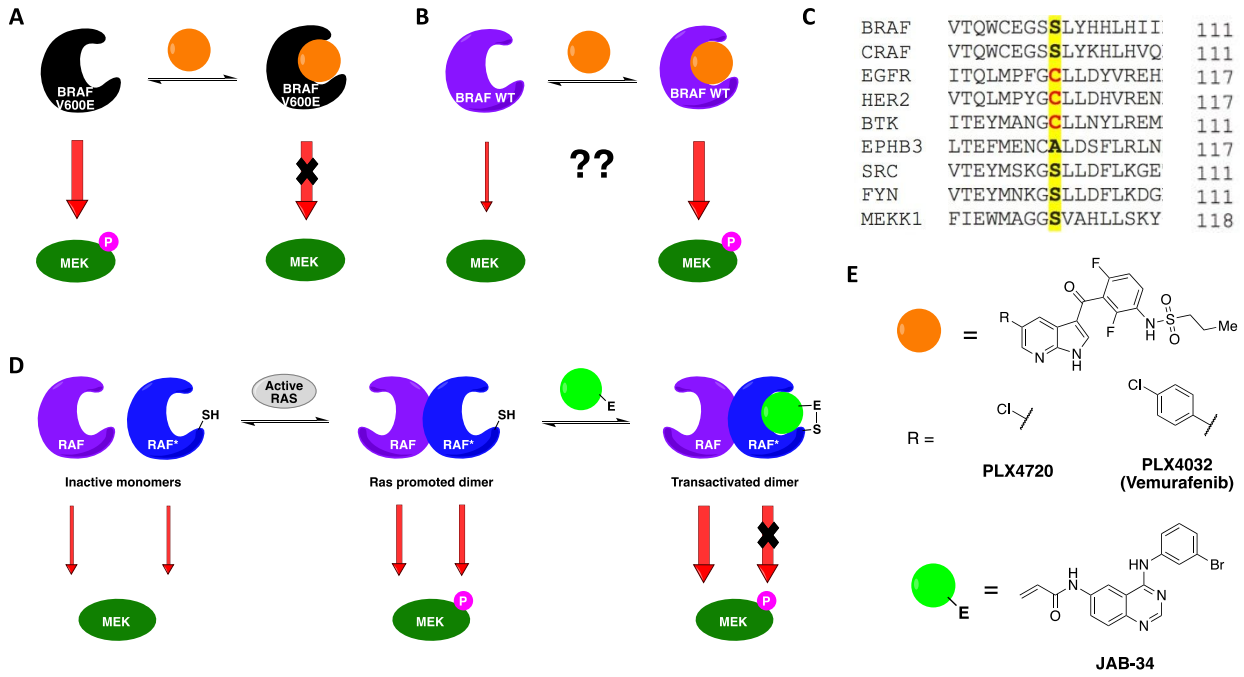

Figure 5. Constitutively active BRAF(V600E) is inhibited by ATP-competitive drugs (**A**) but not BRAF(WT) (**B**). (**C**) CRAF kinase was engineered with a cysteine residue analogous to EGFR Cys797 to achieve isoform selectivity through covalent inhibition by electrophilic quinazolines. (**D**) Covalent chemical genetics explained the paradoxical activation of BRAF(WT). Binding of the covalent inhibitor to the electrophile-sensitive protomer* within a RAF dimer produced both abolition of the catalytic activity of the inhibitor-bound RAF and transactivation of the drug-free RAF. (**E**) Reversible and irreversible ATP-competitive inhibitors that were employed in the chemical-genetic study.

Together, these chemical-genetic tools successfully showed that induction of RAF signaling in cells with WT BRAF is caused by the paradoxical transactivation of RAF dimers by ATP-competitive RAF inhibitors. Binding of the drug to the ATP-binding site of one promoter within a RAF dimer (either CRAF-CRAF or CRAF-BRAF) caused both abolition of the catalytic activity of the inhibitor-bound RAF and transactivation of the drug-free RAF (**Figure 5D**). The use of EGFR inhibitors in this specific study was not ideal because EGFR is situated upstream to MAPK kinases in this cellular signaling pathway, which might interfere with the RAF-MEK-ERK pathway outputs. Nonetheless, the specificity of the cysteine-targeting approach granted orthogonality to the combined systems and the rigorous use of WT RAF as a control in most experiments still allowed the authors to successfully test their dimer transactivation hypothesis. These findings contributed profoundly to the development of RAF inhibitors for use in the clinics. The transactivation model reported in this study predicted in which cases RAF inhibitors would be effective or not, and when they could cause toxicity or drug resistance. Some of these predictions were later confirmed in the clinical trial of Vemurafenib, the first RAF-selective inhibitor, and pointed directions for the development of second-generation BRAF inhibitors, such as pan-RAF inhibitors and dimer breakers (or paradox breakers).

Kinases containing an RSK-like cysteine

The employment of EGFR Cys797 residue in covalent chemical-genetic approaches has the advantage of being more tolerant to a broad range of protein kinases. However, many kinases share this cysteine residue in their active site, lowering the selectivity of this method. Another site that has been explored as an alternative is the cysteine immediately after the glycine-rich loop (P-loop) of RSKs (**Figure 3B**). In a 2005 study, Cohen and colleagues developed

irreversible inhibitors of p90 ribosomal protein S6 kinases (RSKs) using structural bioinformatics to identify unique features on the kinase that could be selectively targeted by a rationally designed inhibitor.[65] From a primary sequence alignment, they discovered the simultaneous presence of a threonine gatekeeper and a reactive cysteine immediately after the glycine-rich loop (P-loop) of RSKs (**Figure 6A**). By exploiting the two filters (small gatekeeper and nucleophilic cysteine), a pyrrolopyrimidine electrophilic analog, fluoromethylketone (fmk), potently and selectively inhibited RSK1 and RSK2 while sparing other kinases that naturally had only one selectivity filter (**Figure 6B, C**). An additional example involved sensitizing the budding yeast Polo-like kinase, Cdc5 (a homolog of human Plk1) to another irreversible pyrrolopyrimidine analog, chloromethylketone (cmk), by replacing a leucine gatekeeper with glycine.[66] The covalent complementarity was achieved by a native nonconserved cysteine present in the Cdc5 active site that is homologous to the aforementioned cysteine residue in RSKs. Specific irreversible targeting of Cdc5 by cmk both unveiled a new function of Cdc5 in mitosis and identified a new endogenous substrate of the kinase.

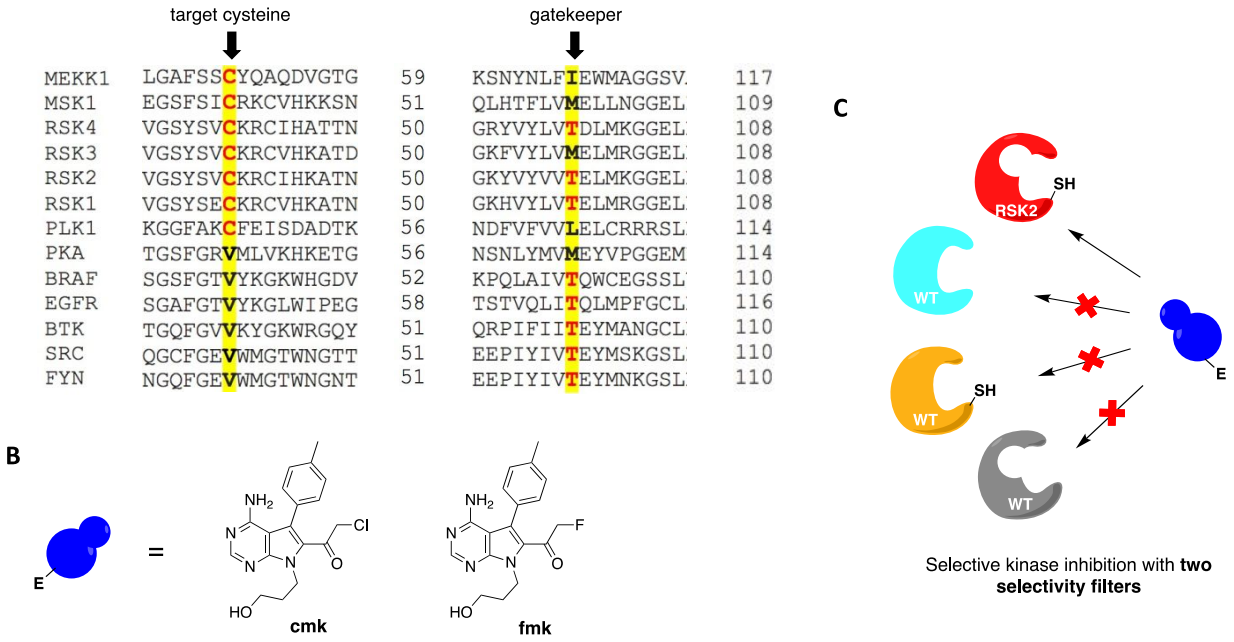

Figure 6. Covalent chemical-genetic approach exploiting two selectivity filters to target RSK kinases. (**A**) RSK2 is amongst the few protein kinases bearing a reactive cysteine at the P-loop C-terminal end and a small threonine gatekeeper residue. (**B**) Structures of the rationally designed halomethylketone pyrrolopyrimidines, cmk and fmk. (**C**) The two filters combined afforded selective targeting of RSK2 with the bulky electrophilic inhibitor.

A more recent work reported a chemical-genetic strategy named Cysteine Installation for Modulating Allostery and Targeted Inhibition of Kinases (CystIMATIK).[67] This method combines shape complementarity and covalent complementarity with comprehensive mutagenesis of Src's catalytic domain to probe the conformation of Src kinases. Using Deep Mutation Scanning, they identified amino acid residues that are important for regulating the protein's activity and studied the phenotypes corresponding to gain-of-function mutations. For example, the E381T mutant led to increased phosphotyrosine levels in multiple mammalian cell lines and promoted non-apoptotic membrane blebbing. The chemical-genetic method was then used to study the mechanism that caused a mutant's specific observed phenotype. CystIMATIK consisted of the installation of a cysteine mutation at the homologous position to RSK2 Cys436

into the kinase active site for selective targeting with a set of electrophilic probes. These compounds can stabilize different ATP-binding conformations, such as helix αC-out or DFG-out, that are responsible for modulating intramolecular regulatory interactions and global conformations of the kinase. They are also ATP-competitive inhibitors that will block phosphotransferase activity to allow the investigation of phosphotransferase-independent phenotypic effects. Using inhibitor **1** as a starting point, a panel of covalent pyrrolopyrimidine derivatives were designed to contain different bulky substituents at the C-5 position to specifically bind to a certain kinase conformation, and an α-cyano-substituted acrylamide as the warhead (**Figure 7A**). Probe **1** is known to bind to kinases containing both a cysteine on the P-loop and a threonine at the gatekeeper position, which are residues V284 and T341 in Src, respectively (**Figure 7B**). The rationally designed inhibitors exhibited potent and selective activity against the V284C mutant Src kinase. They were also shown to control the confirmation of Src's catalytic domain precisely and specifically, allowing for the study of the protein's global conformation separately from its phosphotransferase activity. As expected, **1** caused minimal perturbation to Src's global conformation. Helix αC-out-stabilizing inhibitor **2** promoted a closed global conformation of Src. Finally, DFG-out-stabilizing inhibitor **3** produced an open Src global conformation and consequently an increased SH3 domain accessibility (**Figure 7C**). The study showed that promoting Src's open global conformation is responsible for the previously observed membrane blebbing, which requires Src's membrane interaction but not its phosphotransferase activity. Moreover, it unveiled a direct regulatory interaction in Src caused by a previously unknown intramolecular interaction between Src's N-terminal SH4 domain and the αF pocket in the C-terminal lobe of the catalytic domain. This engagement seems to be

responsible for modulating the conformation, activity, localization, and effect of the kinase in cells.

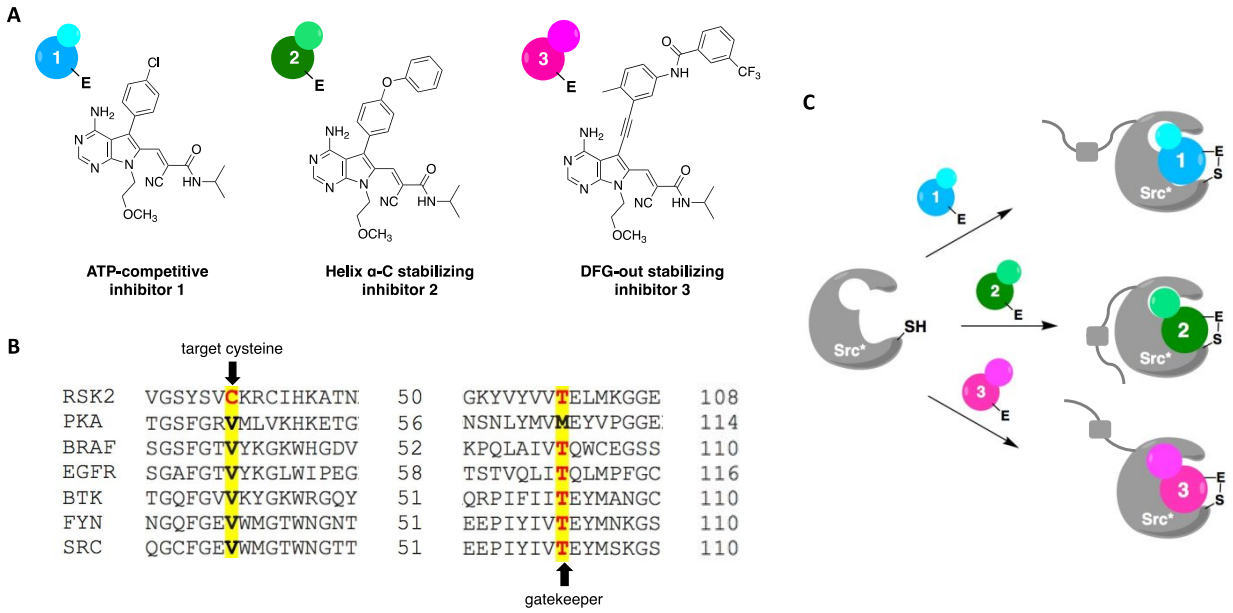

Figure 7. Cysteine Installation for Modulating Allostery and Targeted Inhibition of Kinases (CystIMATIK) method to probe kinase conformation. (**A**) Compound **1** was used as a starting point to generate conformation-selective probes **2** and **3**. By changing the C5 substituent on the pyrrolopyrimidine scaffold, CystIMATIK probes stabilize different ATP-binding site conformations. (**B**) Cysteine residue at the P-loop C-terminal end (V284 in Src) and a threonine gatekeeper residue (T341 in Src) were chosen as the two selectivity filters to generate CystIMATIK-sensitive kinases. (**C**) CystIMATIK was used to modulate Src's global conformation to investigate phosphotransferase-independent activity. Mutant Src* (V284C), that has a small gatekeeper residue, was selectively targeted by the inhibitors while sparing most wild-type protein kinases, allowing for the specific dissection of the engineered kinase's cellular activities.

Kinases containing a cysteine gatekeeper residue

Covalent inhibitors targeting a gatekeeper cysteine have also been alternatively employed in an approach termed electrophile-sensitive (*ES*)-kinase (**Figure 3B**).[68] Putatively, the introduction of a cysteine gatekeeper mutation instead of glycine or alanine would address the challenges posed by the bump-hole method (reduced kinase stability and activity) because the thiol-containing side

chain would not substantially affect the geometry of the ATP-binding site. To test this hypothesis, Garske and collaborators generated a cysteine gatekeeper mutant (c-Src-*ES* allele) of the tyrosine kinase c-Src and compared it with a glycine gatekeeper allele (c-Src-*AS1* allele). The kinetic properties of the *ES*-kinase were similar to those of the WT protein, whereas the *AS1*-kinase showed a 14-fold decrease in catalytic efficiency compared to c-Src-*ES*. Electrophilic analogs of 3-phenyl-substituted pyrazolopyrimidines were shown to irreversibly inhibit the gatekeeper cysteine of c-Src-*ES* both *in vitro* and in cells with few off-targets. Additional alleles bearing second-site mutations were also engineered and showed increased inhibitor potency. Another study employed the Cys-gatekeeper strategy for targeting with covalent type II kinase inhibitors.[69] In this study, analog-sensitive "DFG-out" kinase inhibitors (ASDO) were rationally designed to bind irreversibly to the inactive conformation of kinases mutated with a cysteine gatekeeper. This method was validated both *in vitro* and in cells with different kinases such as Aurora-A kinase, Greatwall (GWL) kinase, and cyclin-dependent kinase-1, and it displayed bio-orthogonality when the ASDO compounds inhibited the Cys-gatekeeper alleles but not *AS* alleles, and vice-versa.

Kinases containing an EphB3-like cysteine

The studies mentioned so far have designed electrophilic inhibitors to target cysteines located at either the gatekeeper, near the P-loop, or the C-terminus of the hinge region (**Figure 3B**). Although these molecules were successfully sensitized to their respective allele kinase partner, the methods did not prove general enough, and the inhibitors' potency, as well as selectivity, were moderate, likely because other kinases, including EGFR, HER2, and Btk, present homologous cysteine residues.

In 2016, Kung and collaborators identified a cysteine residue at a position six residues after the gatekeeper (gatekeeper +6), Cys717, that is unique to receptor tyrosine kinase EphB3 among all human kinases.[70] The authors tested a panel of electrophilic quinazolines against EphB3 and found that one quinazoline-chloroacetamide compound inhibited Cys717 with high potency and selectivity. As an alternative to overcome the aforementioned limitations, a recent study reported a novel covalent chemical-genetic approach, termed *Ele-Cys*, which yielded potent and specific interactions between an electrophilic small molecule and an engineered cysteine in the target protein.[71] A position homologous to Cys717 of EphB3 was chosen to introduce the cysteine mutation on EphB1 (**Figures 3B** and **8A**). Because only EphB3 contains a native cysteine at the same location, covalent targeting of a thiol group at this position should allow for potent and specific inhibition of an EphB1 Cys mutant (G703C) by compound **1** (**Figure 8B-C**), which caused the most potent inhibition of the kinase amongst a panel of electrophilic quinazolines. Furthermore, EphB3 expression is limited to a few tissues, maximizing inhibitor selectivity in most cell types. The authors also created an *AS* allele (T697G) of EphB1, to be targeted by the bumped inhibitor 3MB-PP1, as a comparison to the *Ele-Cys* approach in their experiments (**Figure 8B-C**). The cysteine mutation caused less perturbation of the enzyme's catalytic activity than the gatekeeper mutation, suggesting that the first is more well-tolerated than the latter. Both compounds inhibited their respective allele kinase target with great potency *in vitro* and in cells. While the two chemical-genetic systems showed cross-inhibition *in vitro*, this effect was significantly lower *in situ* likely due to an increased efficacy of covalent inhibition in a cellular environment. Other protein kinases, such as FGFR4 and RAF (unpublished data), were amenable to the *Ele-Cys* approach when introduced with a homologous cysteine mutation. Finally, the

study revealed that it is possible to control two separate kinases within the same cell by combining the two approaches (**Figure 8C**). Covalent inhibitor **1** and bumped inhibitor 3MB-PP1 targeted EphB3(WT) and EphB1(T697G), respectively, and specifically inhibited their autophosphorylation. With these results, the authors discovered not only that there is little trans-phosphorylation occurring between the two kinases, but also that they have a preference for homodimerization over heterodimerization (**Figure 8D**). Overall, this approach demonstrated generality when different kinases with homologous cysteine mutations were sensitive to the inhibitors, and orthogonality to the established bump-hole method when WT EphB3 and a Gly-gatekeeper mutant of EphB1 were expressed in the same cell and separately inhibited by the covalent small-molecule and a bulky PP1 analog, respectively.

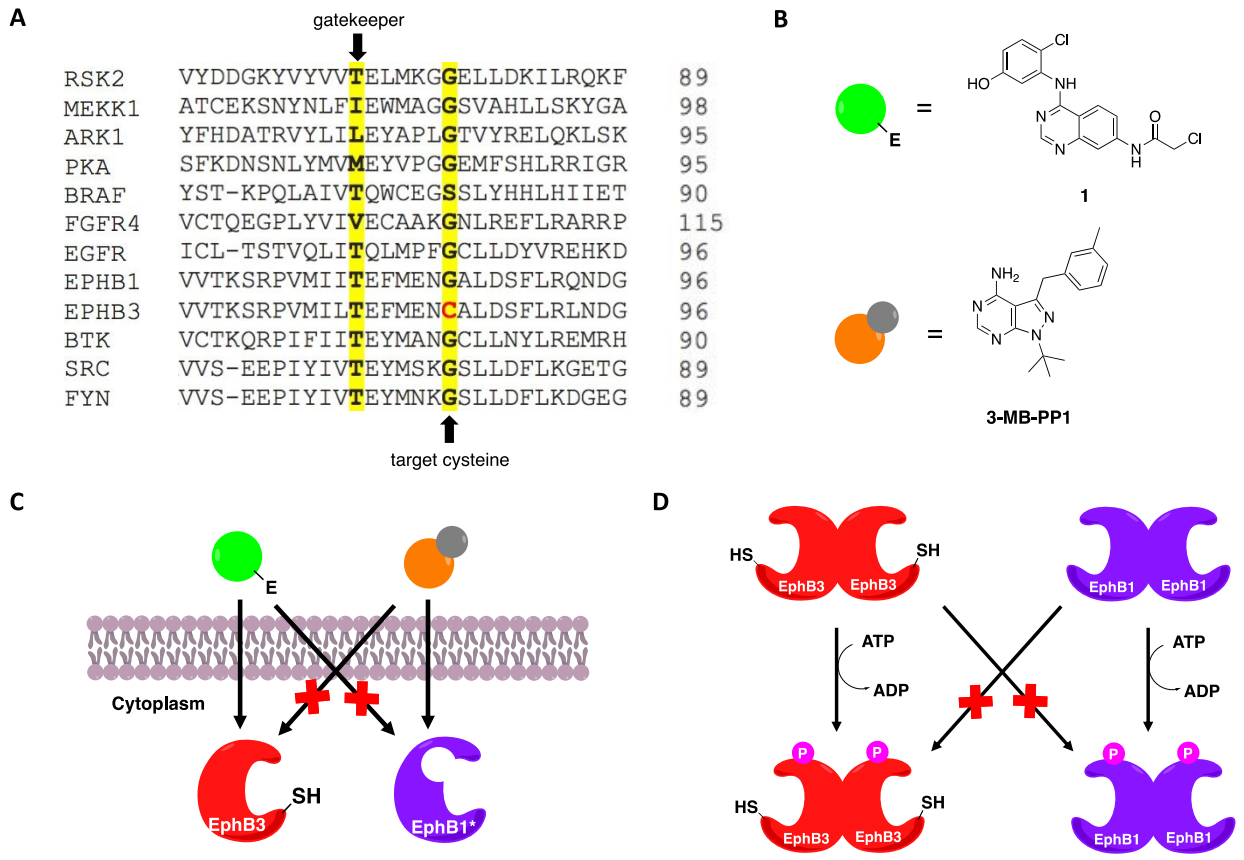

Figure 8. Covalent chemical-genetic approach named *Ele-Cys* to identify selective covalent inhibitors of protein kinases. (**A**) A position six residues C-terminal to the gatekeeper was chosen to install the cysteine, which is only found in a single human kinase, EphB3. (**B**) Chemical structures of the inhibitors utilized in the study. Electrophilic quinazoline **1** and bumped inhibitor 3-MB-PP1 to serve as a comparison. (**C**) Both *AS* and *Ele-Cys* chemical-genetic approaches afforded orthogonal inhibition of two kinases in the same cell. (**D**) The two strategies were employed together to examine the communications between two distinct Eph kinases: EphB3 and EphB1. Selective inhibition of EphB3-WT and EphB1-*AS* upon treatment with the two inhibitors shown in (**B**) caused selective abolishment of autophosphorylation of only the respective Eph kinase, indicating that there's little trans-phosphorylation between them.

Conclusions and Perspectives

Protein kinases are an important class of drug targets for the treatment of several diseases. Although protein kinases comprise one of the largest enzyme family being encoded by the human genome[72,73], only a small portion (<10%) of the kinome is targeted by current FDA-approved kinase inhibitors. As of January 2021, about two dozen distinct protein kinases were targets of 62 therapeutic agents, with the majority being prescribed as anticancer therapies, and only 7 being used in the treatment of non-malignant disorders such as rheumatoid arthritis and pulmonary fibrosis.[74,75] Protein kinases have been strongly linked to cancer since the 1970s when vSrc was identified as the first oncogene.[76] The complexity of kinase signaling and the successful history of kinase inhibitors in cancer therapy since then have perhaps biased kinase drug discovery towards anticancer drug development.[77] Nonetheless, the physiological functions of the remaining untargeted kinases and their direct substrates are still poorly understood. Consequently, the untargeted kinome represents a major opportunity to expand the range of kinase targets for the development of novel therapies for other disease areas.

A key step in the process of drug development is preclinical target validation.[78] This involves target engagement studies to monitor the interaction of an inhibitor with its target protein and correlate it to pharmacological and phenotypical effects in the cell. Quantifying protein-ligand binding is also important to determine the inhibitor's target occupancy, i.e., the extent of target engagement. This is crucial to differentiate the doses that produce efficacy while limiting side effects from those that generate toxicity, as well as to attribute the physiological effect to the perturbation of the protein of interest as opposed to other targets.[79,80] The controlled manipulation of proteins with chemical probes through chemical genetics is a powerful approach

to study target engagement and uncover protein functions in biological systems. Great progress has been made in the field of chemical genetics and its applications in the past two decades. Chemical genetics has proven a more advantageous tool to interrogate proteins and pathways than genetic and pharmacological models because they offer a combination of higher target specificity with rapid and reversible spatiotemporal control of kinase activity.[81] Besides, engineered protein kinases and their ligands have been successfully applied to a variety of scenarios to dissect the functions of these important biological regulators. For instance, unique findings from chemical-genetic studies include the identification of new functions and endogenous substrates of several protein kinases[45,62,63,66], the elucidation of dimerization and phosphorylation mechanisms between distinct isoforms of the Eph kinase subfamily[70], as well as of intramolecular interactions between different domains on the Src protein that regulate its global conformation and cellular activity[67]. A chemical-genetic study of RAF kinases had significant clinical implications on the development of RAF inhibitors predicting in which cases they would be effective, cause toxicity and even drug resistance.[64] Additionally, in a recent work, Remenyi and colleagues describe a chemical-genetic model in which a C905S mutant of JAK3 in mice was created to study the function of this kinase and its inhibitors in vivo.[82] A summary of the different cysteine residues that have been explored thus far in covalent chemical genetics with the respective protein kinases can be found in **Figure 9**.

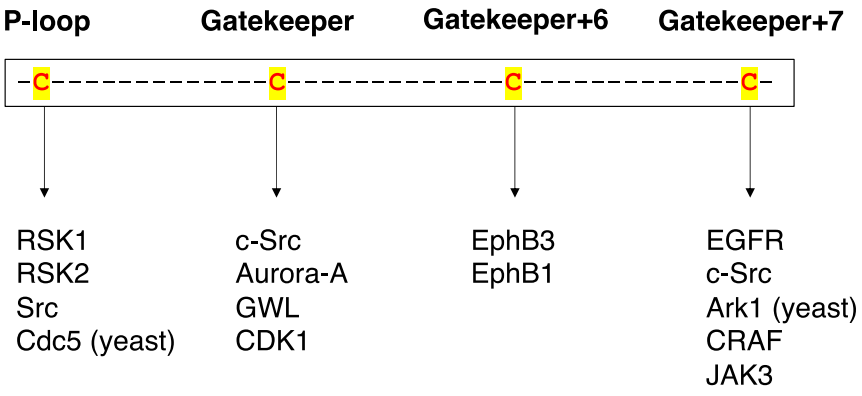

Figure 9. Summary of the protein kinases that have been studied by covalent chemical-genetic methods according to the targeted cysteine positions (either native or mutated).

These unique chemical-genetic findings were made possible with the use of chemical probes with a covalent, irreversible mode of action. The initial chemical-genetic studies employed the bump-hole approach to introduce shape or steric complementarity between the engineered kinase-ligand pair and achieve selectivity. Although this method proved effective in some cases, it later showed drawbacks such as impaired kinase activity and function, and non-specific interactions of the bumped inhibitor with WT proteins. These difficulties inspired scientists to search for different approaches and introduce the concept of covalent chemical genetics. Based on reactivity or covalent complementarity, inhibitors containing an electrophilic warhead were rationally designed to specifically and irreversibly bind to a non-catalytic cysteine residue, native or mutated, on the ATP-binding pocket of a target protein kinase. Different than bump-hole, which involves engineering the kinase in only one site (the gatekeeper) for the method to work, cysteine residues can be introduced at multiple positions in covalent chemical-genetics. Consequently, covalent complementarity, either alone or combined with steric components, has demonstrated superior generality and orthogonality across the kinome than bump-hole, thereby becoming the approach of choice in the majority of chemical-genetic studies since its discovery.

In most examples of the covalent approach, the unique nucleophilicity of the thiol group of cysteine has been explored. While this strategy has been successful so far, it restricts the number of proteins that can be targeted by electrophilic inhibitors. Cysteine residues, although highly reactive, are among the lowest naturally abundant amino acids found in proteins and, in several cases, are present within disulfide bonds, thus reducing their accessibility for covalent modification.[83] This shortcoming has encouraged researchers to develop novel methodologies that target other amino acid residues for covalent inhibition. Lysine, tyrosine and serine are amidst the residues with increasing interest in this context and hold the potential to be employed as chemical warheads in the development of novel covalent probes for chemical-genetic studies. For example, a sulfonyl fluoride probe (XO44) was employed in the first broad-spectrum chemoproteomic study of the human kinome in intact cells.[84] Probe XO44 was shown to covalently label lysine residues in the active sites of about 133 diverse endogenous kinases, and was used to monitor the intracellular target engagement by dasatinib, an FDA-approved BCR-ABL inhibitor for chronic myeloid leukemia. More recently, a sulfonyl-triazole analog of XO44 (KY-26) was designed to modify both lysine and tyrosine residues on protein kinases in another chemoproteomic analysis.[85] This shows that irreversible inhibitors are well-suited not only for chemical genetics but they also serve as ideal scaffolds for the development of chemical probes for use in other applications such as activity-based protein profiling (ABPP) or high-throughput screens (HTS).

Another current advancement in this field is the use of gene editing technologies such as CRISPR/Cas9. So far, reported chemical-genetic studies relied on the transient or stable

overexpression of the engineered kinase instead of its endogenous expression. However, overexpression systems can disturb kinase signaling pathways[86] and potentially produce misleading results. In a recent work, CRISPR/Cas9 was used to mutate a serine residue to cysteine at the DFG-1 position in the ATP-binding pocket of the non-receptor tyrosine kinase feline sarcoma oncogene (FES), a potential target for cancer and immune disorder therapies.[87] A complementary fluorescent covalent probe was also developed to specifically target and label the mutant kinase. With a small change at the genome and protein level, this strategy showed that regulation at transcriptional and (post)-translational level activity were minimally affected when CRISPR/Cas9-generated mutant cells behaved comparably to WT cells in downstream signaling assays. This combined chemical-genetic strategy was developed as a target validation method for FES kinase, but the rapid advancements in gene editing technologies will considerably accelerate the application of chemical-genetic approaches to a larger number of protein kinases.

Conflict of Interest

There is no conflict of interest to declare.

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