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Enhanced interrogation: emerging strategies for cell signaling inhibition

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Abstract

Here we summarize recent and developing chemical approaches for modulating signaling pathways. In particular, we discuss targeting mutant signaling proteins, disrupting protein-protein interactions in cellular signaling networks, designing bivalent inhibitors of signaling proteins and identifying allosteric regulators of signaling enzymes. Over the past decade, great progress in the harvesting of chemical tools for basic research and clinical medicine has been made, but many challenges remain, and examples of exciting future targets are highlighted.

Attacking signaling pathways with chemical inhibitors has provided a wealth of cellular functional and kinetic information and an increasing number of therapeutic agents. Earlier studies with natural products galvanized this field^{1,2}. For example, mechanistic analysis of the natural cyclic peptide cyclosporine revealed that targeting the protein phosphatase calcineurin through a cyclophilin complex could have profound effects on dampening immune signaling¹. The natural product staurosporine has served as the paradigm for a nonselective, ATP site-directed protein kinase inhibitor². More recently, high-throughput screening of synthetic chemical libraries has led to the identification of a number of selective protein kinase inhibitors³. Despite significant progress, a large number of interesting signaling targets remain without high-quality chemical modulators. The value of such chemical probes depends heavily on their relative specificity and pharmacokinetic properties. Below we describe progress and challenges in four fertile areas of signaling-inhibitor development: (i) targeting mutant signaling proteins; (ii) disrupting protein-protein interactions in cellular signaling networks; (iii) designing bivalent inhibitors of signaling proteins; and (iv) identifying allosteric regulators of signaling enzymes (Fig. 1). We believe that these strategies will likely prove increasingly important in the pharmacology of signaling.

Targeting mutant signaling proteins

A wide range of genes are mutated in human cancers, some resulting in hyperactivity (oncogenes) and others in loss of function (tumor suppressor genes). Many of the oncogenic mutations occur in protein kinases, leading to upregulation of their catalytic activities. Important clinical examples of this include the tyrosine kinases Abl, epidermal growth factor receptor (EGFR), c-Kit and the serine/threonine kinase B-raf^{4,5}. A major advance in the care of patients with chronic myeloid leukemia (CML) was the observation that such patients show substantial responses to treatment with the synthetic kinase inhibitor imatinib⁴. These

neoplasms express a dysregulated Bcr-Abl tyrosine kinase fusion. By binding selectively to the ATP-pocket of the Bcr-Abl kinase domain, accessing an unusual conformational state of the enzyme, imatinib induces remission in the vast majority of early- and advanced-stage CML patients that are given this potent and selective ATP site-directed inhibitor⁴. Activating mutations in EGFR are responsible for a significant subset of patients with non-small cell lung cancer⁶. The ATP-site small-molecule inhibitor erlotinib preferentially binds the kinase domain in these constitutively active receptors and prolongs disease-free survival in many patients bearing these mutations⁶. Imatinib can also potently inhibit the kinase domain of mutant, activated c-Kit, in a fashion presumed similar to Abl inhibition, and is very useful in the treatment of gastrointestinal stromal tumors. Several ATP-site inhibitors in development are apparently selective for mutant B-raf, showing promise for treating melanoma^{4,5}.

Despite these successes, increasing numbers of patients receiving protein kinase inhibitors with CML and non-small cell lung cancer are developing resistance mutations, many in a so-called ‘gatekeeper’ residue⁷. The gatekeeper residue in Abl is a conserved threonine in the ATP binding pocket whose mutation weakens the binding of imatinib to the kinase domain without reducing the catalytic activity of Bcr-Abl. Such resistance mutations are reminiscent of the HIV-1 mutants that arise in patients during treatment with HIV reverse transcriptase and protease inhibitors⁸. Analogous to anti-infective drug development, oncology investigators are combating such secondary mutations with next-generation therapies that maintain inhibitory potency against such altered kinases. An elegant example of this is the application of dasatinib for imatinib-resistant CML⁹. Compared to imatinib, dasatinib binds a different conformation of the Abl kinase domain and is not affected by the gatekeeper mutation.

Loss-of-function mutations are in principle more challenging to approach pharmacologically because it is often difficult to find molecules that can complement these defective proteins. However, signs of progress in mutant protein rescue are emerging. The tumor suppressor transcription factor p53 is commonly mutated in human cancer at a number of distinct sites, which leads to the destabilization of the p53 fold^{10,11}. High-throughput screening efforts to find cell-permeable ligands that can stabilize mutant p53 have been pursued with some success^{10,11}; several heterocyclic small-molecule scaffolds show low micromolar potency in stabilizing wild-type and several distinct mutant p53 proteins. At first thought, it might seem nearly impossible to identify a small molecule that can correct a range of scattered destabilizing point mutations in a protein. However, it should be understood from thermodynamic considerations that any small molecule that binds preferentially to a conserved folded state over a denatured form of p53 would be expected to stabilize p53 (wild type or mutant) regardless of binding site. In fact, a variety of small-molecule scaffolds have been found that have this property, and some of these show promise in *in vivo* anti-tumor activity in p53-deficient cancer xenograft models^{10,11}. The tumor suppressor lipid phosphatase PTEN, which is also subject to destabilizing mutations in human cancer, would appear to be a worthy future candidate for similar screening efforts¹².

Perhaps the most challenging inactivating mutations to complement are those of active-site residues in enzymes. Enzymes typically possess an intricate architecture that is fine tuned for transition-state stabilization. Nevertheless, it has been shown that some inactivating point mutations in tyrosine kinase active sites can be rescued by small molecules^{13,14}; imidazole, in place of the natural arginine, orients the binding of the substrate tyrosine on mutated c-Src. In particular, the absolutely conserved active-site arginine residue, when mutated to alanine or glycine, can be complemented successfully in cells with nontoxic imidazole derivatives^{13,14}. It is noteworthy that the corresponding mutation in c-Kit is found in some people with inherited piebaldism (a rare disorder characterized by distinct patches of skin

and hair that contain no pigment), which would suggest a therapeutic approach for such patients¹⁵. One of the most common mutations in human cancers occurs in the active site of the small G protein Ras, disabling the GTPase activity of this enzyme¹⁶. The GTP-bound form of Ras activates downstream signaling pathways, and thus, small molecules that can reactivate the GTPase of mutant Ras would likely be very attractive anti-neoplastic agents.

Disrupting protein-protein interactions in signaling networks

Dynamic changes in protein-protein interactions are a hallmark of cell signaling. Such interactions can convey highly specific information regarding activation or inhibition of particular pathways (Fig. 1b). For example, defined phosphotyrosine sites on the C-terminal tail of the activated EGFR are believed to recruit specific Src homology 2 (SH2) domain-containing proteins that trigger individual cascades, resulting in cellular proliferation¹⁷. In contrast, binding events involving some BH3 domain-containing proteins in the Bcl-2 family can block programmed cell death signaling pathways¹⁸. Inhibitors of such protein-recruitment events can act as molecular surgical tools for research as well as therapeutic agents. However, identifying highly efficacious protein-protein interaction disruptors has often proved elusive to pharmacologic investigators.

In many cases, the protein-protein binding interfaces extend over a broad area and are shallow, which makes them poorly suited to small-molecule ligand recognition. On the other hand, some protein-protein interactions are characterized by hot spots that lend themselves to pharmacological intervention. Pioneering studies on integrin molecular recognition identified the importance of the Arg-Gly-Asp (RGD) motif in mediating receptor binding¹⁹. Antibody (abciximab), peptide (eptifibatide) and small-molecule (tirofiban) inhibitors of RGD-containing complexes with glycoprotein IIb/IIIa receptor have been developed as valuable anti-platelet aggregation drugs used to prevent restenosis after coronary artery angioplasty¹⁹. X-ray crystal structures have confirmed the molecular basis for inhibition by these agents²⁰, which all target the RGD binding pocket.

Intracellular protein-protein interactions have been especially challenging to target because of the need for cell penetration by potential inhibitory compound. Nevertheless, exciting progress has been made disrupting Mdm2-p53 interactions, BH3 binding events, SMAC-XIAP interactions, CBP- β -catenin binding events and Notch receptor complexes²¹⁻²⁵. With p53-Mdm2 disruptors, a key hydrophobic surface in Mdm2 can be targeted by small molecules like the nutlins²¹. Nutlins, which are a series of aryl-imidazolines, prevent the proteasomal destruction of p53 and can thus promote cancer-cell death and have been highly useful research tools²¹. Abt-737 is a high-affinity ligand that binds to the BH3 domain of anti-apoptotic proteins Bcl-xL and Bcl-2, preventing them from antagonizing pro-apoptotic proteins such as Bak and Bax and thereby enhancing programmed cell death of cancer cells²². Abt-737 is an aryl-sulfonamide that was identified by a combination of NMR screening and structure-based design and blocks BH3-mediated protein-protein interactions²².

SMAC binding to XIAP involves a short N-terminal epitope of SMAC that is favorably mimicked by uncomplicated synthetic compounds²³. Small-molecule inhibitors of the SMAC-XIAP protein-protein interaction induce apoptosis in cancer cells²³. A series of conformationally restrained diazabicyclic SMAC mimetics prevent XIAP binding to SMAC, thereby stimulating cellular caspase activation²³. The β -catenin-CBP protein-protein interaction appears to block neuronal differentiation. The designed synthetic small molecule ICG-001 appears to interfere with the β -catenin-CBP interaction but not the closely related β -catenin-p300 interaction and thus fosters neuronal differentiation²⁴. Conformationally restrained α -helix peptides have recently gained currency in disrupting protein-protein

interactions²⁵. A notable example is a 'hydrocarbon-stapled' peptide SAHM1 that blocks Notch activation and shows promise as an anti-cancer agent²⁵. The 16-residue α -helical stretch of MAML1 (the transcriptional coactivator Mastermind), which forms a ternary complex with the proteins Notch1 and CSL, was used to design the stapled peptide SAHM1. The stapling process involves olefin-containing unnatural residues, present at the i and i+4 positions of one face of the MAML1 α -helix, that are cross-linked by a ring-closing olefin metathesis reaction. The preorganized α -helix is also believed to facilitate cell-membrane penetration. A plethora of clinically relevant protein-protein interaction targets remain undrugged. For example, a peptide segment of the mitogen-inducible gene 6 (Mig6) protein disrupts the asymmetric dimer interface of EGFR and prevents EGFR kinase activation²⁶.

Bivalent inhibitors of signaling proteins

Many receptor-ligand reactions are governed by multivalency. A classic example of this occurs in antibody-antigen recognition²⁷. The concept that tethering two or more small ligands together to afford a more potent and selective pharmacologic agent has been recognized for decades²⁸ (Fig. 1c). Such bivalent inhibitory compounds show increased avidity by combining the free energies of binding of the individual interactions without suffering the entropic penalty associated with forming a ternary complex²⁸. Bisubstrate analog inhibitors for enzymes that catalyze reactions between two substrates exemplify such properties²⁹. In a seminal study, it was shown that the bisubstrate analog S-carboxymethyl-CoA(-)-carnitine ester can inhibit carnitine acetyltransferase rapidly and tightly. One key to success with bivalent strategies is the incorporation of an effective linker between the ligands. Ideally, the linker needs to be sufficiently rigid that it orients the ligands in favorable locations for protein interaction, and it must be unobtrusive (or even enhancing) toward receptor molecular recognition²⁸. With protein kinases, consideration of the dissociative phosphoryl transfer transition state was helpful in designing an effective linker for potent bisubstrate analogs³⁰. In this case, the bisubstrate inhibitor possessed a two-carbon spacer linking a modified ATP moiety to a peptide substrate analog. This design fits the geometry of a more expanded transition state in a dissociative kinase reaction mechanism and results in a potent inhibitor.

Fragment-based discovery approaches offer promise for finding potent bivalent signaling inhibitors^{31–36}. Biophysically based approaches involving NMR, MS or X-ray crystallography have been used to find very small, high-potency ligands that bind to independent sites on proteins^{31–36}. Efforts have been made to design linkers that preserve the individual ligand affinities that lead to additive binding energies. Several highly potent ATP-site protein kinase inhibitors have been generated in this fashion³³.

The use of chemoselective reactions such as click chemistry, which involves a specific cycloaddition between azide and alkyne substrates, with small-molecule libraries of these compounds has led to the efficient production of bivalent signaling inhibitors³⁴. Success in selectively targeting protein tyrosine phosphatases with such triazole derivatives exemplifies such methodology³⁴. The potential for such fragment assembly using dynamic and reversible reactions that can be templated by the target are also being explored³⁵. Structure-based approaches have been effective as well in identifying bivalent inhibitors for protein tyrosine phosphatases³⁶. Based on studies showing pockets for multiple tyrosine phosphate residues present in the active site of protein tyrosine phosphatase 1B, a bivalent scaffold was assembled that showed high affinity and specificity for its target³⁶.

Allosteric regulators of signaling enzymes

Allostery in protein regulation is a ubiquitous feature of cell signaling. Protein kinase activation at locations remote from the active site by interaction with small molecules (for

example, cyclic AMP with protein kinase A) or a macromolecule (for example, a cyclin with cyclin-dependent kinase) is one such paradigm. Allosteric regulation represents an attractive approach for drug discovery (Fig. 1d). This is since allosteric sites are often more weakly conserved within protein families, affording opportunities for high selectivity. Non-nucleotide reverse transcriptase inhibitors serve as a practical example of allosteric agents in the clinic³⁷. In addition, a number of G protein-coupled receptors have been modulated by allosteric compounds³⁸. Two GPCR allosteric modulators approved for clinical use are cinacalcet and maraviroc. Cinacalcet is a positive allosteric modulator of the calcium-sensing receptor and is prescribed for the treatment of secondary hyperparathyroidism and parathyroid carcinoma. Maraviroc is an anti-HIV drug and an allosteric antagonist of the chemokine receptor CCR5 to prevent HIV from binding to the cell surface³⁹.

As described above, Bcr-Abl is a key target in CML. Using a differential cytotoxicity screen of a 50,000 heterocyclic compound library, GNF-2 was identified to be a highly selective allosteric Bcr-Abl inhibitor which showed no effect on the kinase domain alone⁴⁰. Subsequent structural and biochemical studies verified that GNF-2 binds to the myristate-binding site of Abl, which leads to changes in the conformational dynamics of the ATP binding site^{40,41}. Based on the structural information of the Abl-imatinib-GNF-2 complex, GNF-5, an analog of GNF-2, was synthesized with improved pharmacologic properties. Moreover, GNF-2/5 can be used in combination with imatinib to act cooperatively⁴¹.

Success has also been achieved in the identification of allosteric regulators of apoptotic pathway caspases⁴². The BIR3 domain of XIAP binds to an inactive caspase-9 subunit at a site that prevents its allosteric activation through homodimerization⁴². Two hydrophobic small molecules, DICA and FICA, were identified that bind to an allosteric site at the dimerization interfaces of caspase-3 and caspase-7 (refs. 43,44). A cocrystal structure of caspase-7 bound to FICA and DICA showed different binding modes for the two small molecules in the allosteric site^{43,44}. Both compounds trigger conformational rearrangement in the protease domain and irreversibly trap the inactive conformation^{43,44}. FICA bound to one chain displaces Tyr223 on the opposite chain using *trans* inhibition. DICA displaces Tyr223 on the same chain using *cis* inhibition⁴⁵. Studies using chemical probes and mutations suggest that only a small set of hydrogen bonding networks is critical for mediating the transition from active to inactive conformational states^{43,44}. This helps explain how small molecules can trigger relatively significant structural transitions^{43,44}. Caspase-1 has a cysteine binding site close to the dimerization cavity⁴². This cysteine can be used as an anchor for covalent tethering, which is a site-directed disulfide trapping technology. With this approach, a pyrazole-thiophene compound was identified as a caspase-1 inhibitor⁴². Structural analysis revealed a similar allosteric inhibition mode by conformational switching, so that the substrate-binding loop is prevented from productive interaction with a protein substrate⁴².

Summary and future directions

The increasing power of structural, computational, synthetic and screening technologies has facilitated the identification of selective ligands for what were heretofore considered undruggable signaling targets. In this piece, we have highlighted some exciting new strategies for modulating signaling pathways. It is noteworthy that several of these inhibitory approaches exploit features of classical signaling mechanisms: bivalency, allostery and dynamic protein-protein interactions. As our knowledge of what constitutes signaling expands to include a wider range of protein post-translational modifications including reversible lysine acetylation, methylation and ubiquitylation, opportunities for inhibitor intervention should also multiply. Indeed, we have already begun to see the development of increasingly sophisticated and selective protein deacetylase, acetyltransferase,

methyltransferase, and demethylase inhibitors⁴⁶. Enhanced interrogation of these and other signaling targets pharmacologically combined with advances in genetics, imaging and proteomics promises to take our understanding of cellular networks and pathways to ever increasing heights.

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Figure 1.

Strategies for targeting signaling pathways. **(a)** Hyperactive mutant proteins can be inhibited by small molecules that target the active site. Loss-of-function mutations can be rescued by small molecules that complement the mutant protein. **(b)** The downstream effects of certain protein-protein interactions can be interrupted by synthetic molecules. A and B are two interacting proteins. **(c)** Inhibition of signaling proteins by bivalent ligands. **(d)** Allosteric inhibitor induces conformational changes remotely that disrupt ligand binding.