Point of VIEW

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Fragment-Based Ligand Discovery Meets Phage Display

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ABSTRACT In a powerful complement to traditional ligand discovery methods such as high-throughput screening, fragment-based ligand discovery methods identify ligands piece by piece. A recent advance combines the concepts of fragment-based ligand discovery with phage-display technology to yield bivalent kinase inhibitors with high potency and specificity.

Chemical biology currently lacks sufficient tools to selectively modulate the activity of any desired protein target in real time. Genetic manipulation can selectively turn off a single gene, and small interfering RNA can, with perhaps somewhat less specificity, down-regulate the production of a selected protein. Still, to rapidly shut down the activity of a protein, small molecules have many advantages (*1*). In a recent paper, Ghosh and colleagues (*2*) describe a clever new method to discover such molecular probes.

Drug developers face many of the same challenges as chemical biologists, and both apply some of the same techniques. For example, high-throughput screening, in which thousands to millions of compounds are tested to discover those that have a desired biological effect, originated in the pharmaceutical industry but has now become widespread in academic laboratories. But high-throughput screening has limitations. The number of possible small molecules is many orders of magnitude larger than those that can be gathered into a chemical library. Moreover, just as there are more six-letter words than three-letter words, the number of possible molecules grows rapidly with the size of the molecule. To illustrate the challenge, suppose that you are trying to guess a six-letter password. You could test all six-letter combinations, but this would require a considerable effort (Figure 1, panel a). However, if you could search for shorter strings, and then put the strings together, the process could be completed with fewer, shorter word fragments (Figure 1, panel b).

The same philosophy can be applied to discovering small molecules, and over the past decade a concept called fragment-based ligand discovery has developed rapidly (Figure 1, panel c) (*3*, *4*). Rather than requiring millions of compounds to discover a hit, fragment-based screening methods generally require only hundreds or thousands of small molecule fragments. However, the endeavor is stymied by two significant challenges. First, because the fragments are usually quite small, they are likely to bind to the target weakly, and so discovering them may require unconventional approaches. Second, once the fragments are discovered, it is not always clear how to combine them productively.

Nonetheless, fragment-based techniques are proving fruitful for one of the hottest target families in drug discovery, the protein kinases. These enzymes modify proteins' activity by transferring a phosphate from ATP to the target protein and are central players in cellular signal transduction. To develop small-molecule inhibitors against kinases, the most obvious place to start is with the ATP binding site. However, with >500 kinases in the human genome, all of which bind ATP, developing a selective kinase inhibitor is challenging (*5*).

Ghosh and colleagues have developed a fragment-inspired method that starts with a small molecule that binds in the ATP-binding site and selects a second molecule that binds elsewhere on a particular kinase

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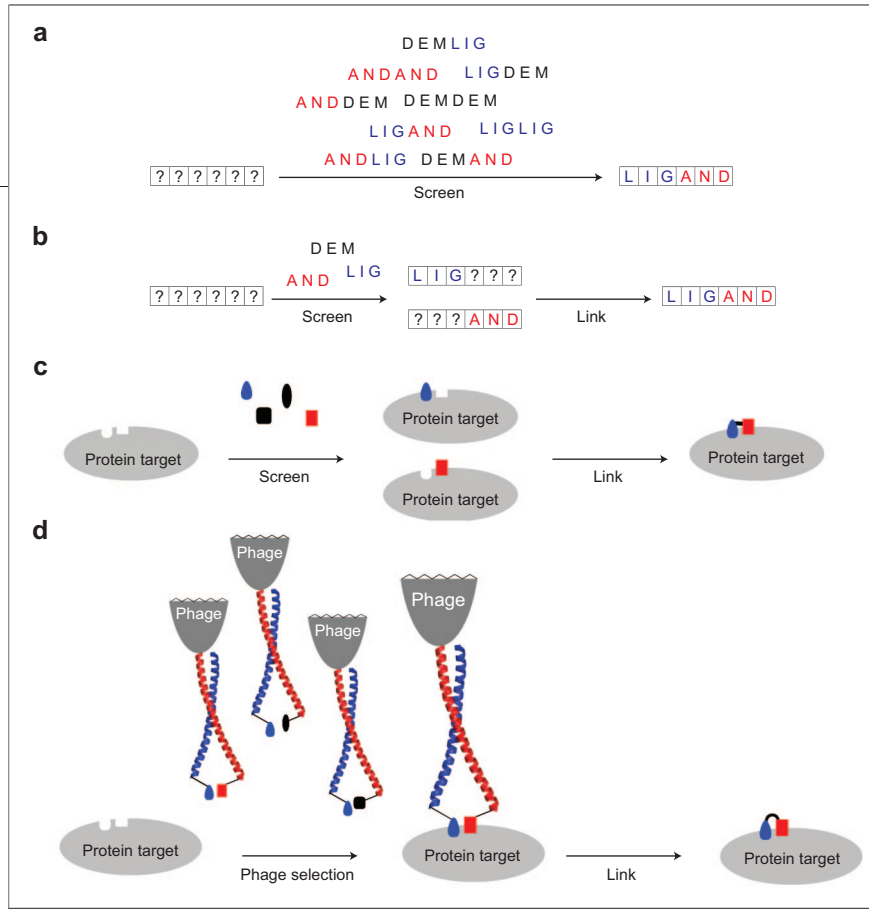

Figure 1. Fragment-based ligand discovery. a) Trying to guess a password by testing every possible string of letters is time-consuming and inefficient. Moreover, even one or two "wrong" letters is enough to prevent a near-hit from being discovered. b) If small "word fragments" can be tested individually, the process could be more efficient. c) In fragment-based ligand discovery, small-molecule fragments that bind to a protein are first identified, and then either grown or linked together to generate high-affinity ligands. d) Ghosh and colleagues have developed a new method of fragment-based ligand discovery in which a high-affinity ligand is tethered to one member of a coiled-coil protein dimer, while the other member carries a library of peptides generated through phage display. This allows the selection of peptides that can subsequently be combined with the small molecule to yield highly potent and specific inhibitors. (Fos-Jun cartoon made using the crystallographic coordinates 1FOS and the program PyMOL (http://pymol.sourceforge.net) (*15*).)

(Figure 1, panel d). The ATP-mimetics are either an adenosine analogue (AdoC) or an analogue of a high-affinity nonselective kinase inhibitor, staurosporine (compound **1**, Figure 2). Either of these small molecules is coupled to the "zipper" domain of the protein Jun.

Next, a library of small cyclic peptides is synthesized through phage display (*6*). This library is attached to the zipper domain of the protein Fos, which can form a heterodimer with Jun. The Fos-attached peptides can vary in six positions flanked by two cysteine residues that can form a disulfide, cyclizing the peptide, and are separated from the zipper domain of Fos by a spacer consisting of several additional amino acids. The six variable positions can accommodate any of the common amino acids, and Ghosh and colleagues constructed a library of >1 billion members, far exceeding the size of any small-molecule collection. When Fos and Jun come together, the cyclic peptides are in close proximity to the small-molecule ATP analogues, and the conjugates, still bound to phage, can be screened against the target kinase.

A batch of small-molecule-conjugated Jun was mixed with the Fos library and exposed to immobilized cAMP-dependent protein kinase A (PKA), a well-characterized kinase. After unbound phage was rinsed away, bound phage was eluted and amplified, and the process was repeated for several cycles. In the case of the AdoC-derivatized Jun libraries, despite precautions, the process yielded only peptides that bound to the matrix holding PKA. However, when selection was performed using staurosporine-conjugated Jun, the researchers discovered a number of peptides after six rounds of selection. The most abundant was CTFRVFGC (compound **2**, Figure 2). Of importance is that the affinity of the identified peptide is likely too low to have been identified without the help of the staurosporine-conjugated Jun.

The researchers synthesized this cyclic peptide and tested its affinity along with the staurosporine derivative used for selection (Figure 2). They then synthesized a bivalent inhibitor consisting of the two molecules joined by a flexible linker. Happily, the bivalent molecule **3** has a potency greater than either of the two components, with an IC_{50} of 2.6 nM compared to 243 nM for the staurosporine derivative **1** (or 78 nM for the Jun-staurosporine conjugate) and 57 μM for the cyclic peptide **2**. Moreover, the bivalent inhibitor is much less active against five diverse kinases than against PKA. Although five kinases represent a very small portion of the kinome, it is a nonetheless an encouraging start.

One conceptual precedent for this work is the creation of bisubstrate analogue inhibitors, which involves attaching an ATP-mimetic to a peptide derived from a kinase substrate (*7–9*). One strength of the new technique is that nothing need be known about the structure of the kinase or about its preferred substrate since the peptide is selected empirically by phage display. In fact, in the current example, it is not clear where on PKA the selected cyclic peptide binds, and the observation that none of the selected peptides have sequence identity to known physiological substrates suggests they may bind outside of the substrate binding site (*2*). Though not mentioned by the

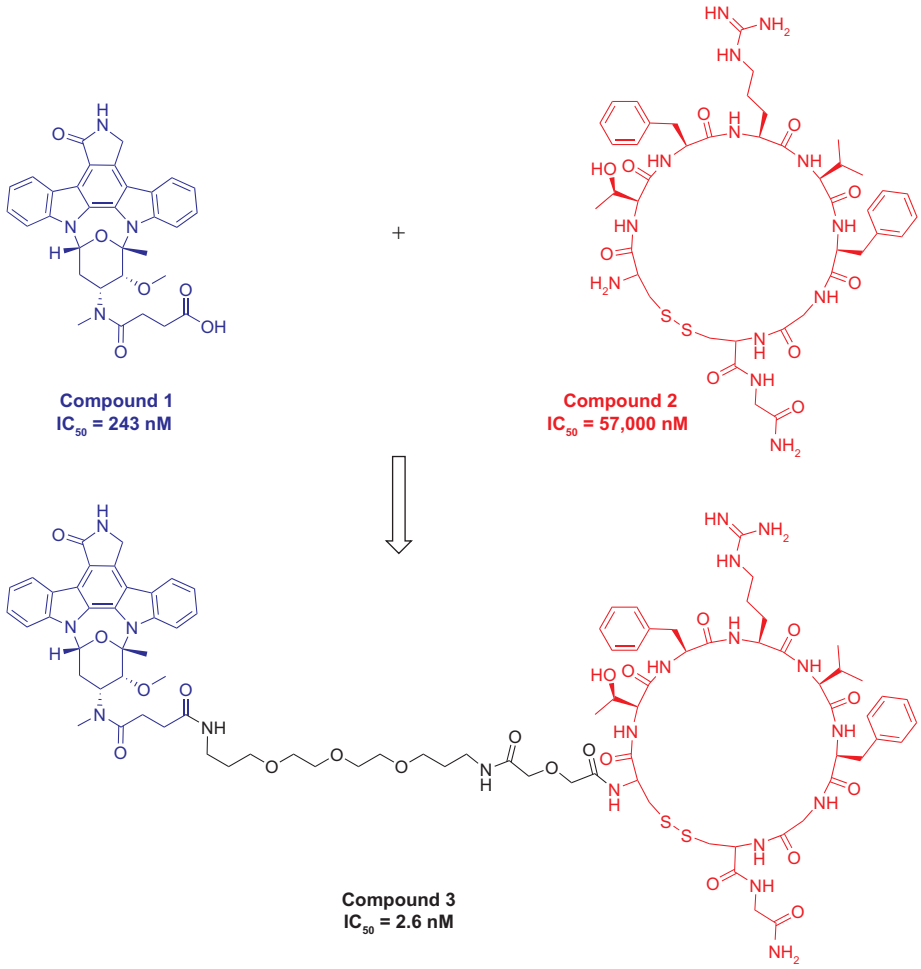

Figure 2. Structures of the small-molecule ligand 1, the selected peptide 2, and the bivalent inhibitor 3.

authors, another precedent is the concept of self-assembling chemical libraries, in which small-molecule fragments are attached to DNA. Small molecules that bind a target protein can be identified by their DNA tags and subsequently linked together (*10*). In some formats, this technique can draw on libraries comparable in size to those achieved by phage display (*11*). Finally, Tethering with Extenders also starts with a fragment already known to bind to a protein. A covalent linker (such as a disulfide bond) on that fragment is used to capture another fragment, thereby both finding a second fragment with affinity for the target and establishing the connectivity between the two fragments (*12*).

As with any proof of concept, there are limitations with the current study. First, although inspired by fragment-based ligand discovery, the molecules involved are too large to be considered fragments. The identified peptide **2** has a molecular weight of 986.17, nearly twice that recommended by Lipinski *et al.* for an oral drug (*13*). Even the succinoylated staurosporine derivative **1** has a molecular weight of 566.6, which is larger than most of the clinically approved kinase inhibitors such as imatinib (molecular weight 493.6), erlotinib (molecular weight 393.4), sunitinib (molecular weight 398.5), dasatinib (molecular weight 488.0), and sorafenib (molecular weight 464.8).

A second complication is the linker, which is ~30 Å long. The benefits of fragment assembly are eroded by the entropic costs of freezing rotatable bonds in linkers, so ideally the linker should be as short as possible (*14*). The bivalent compound **3** binds to PKA with low nanomolar affinity, which is impressive. However, if the linker were more ideal, the energies of binding of the two components **1** and **2** should be roughly additive, which would yield an inhibitor with a potency in the low picomolar range.

In the current example, the binding site of the cyclic peptide is unknown, so the authors chose a long linker to maximize the likelihood that both portions of the bivalent inhibitor would be able to bind productively. Hopefully, a better understanding of the binding mode would allow future compounds to have a shorter linker. There is, however, a darker possibility: Perhaps the cyclic peptide binds far away from the ATP-binding site. Indeed, the geometry of the Fos-Jun dimer could very well prefer compounds that bind at some distance from one another. The α-carbons at the end of the zipper region are >10 Å apart (*15*), and with three or more amino acids as spacers between the ends of the proteins and the small molecules, the binding sites could be >40 Å apart from one another (*2*).

Finally, given the very nature of the phage-display methodology employed, one of the components must be a peptide. While the number of peptide drugs is increasing (*6*), peptides often suffer from poor absorp-

tion, distribution, metabolism, excretion, and pharmacokinetic properties. Indeed, the low cell permeability of most peptides suggests that they are unlikely to be broadly effective against intracellular targets, and most kinases are intracellular.

Thus, the technique may prove more useful with extracellular targets, of which there are an abundance of intriguing choices. For example, it has potential to target protein–protein interactions. Small-molecule ligands are known for the cytokine interleukin-2, which is involved in T-cell proliferation, and such ligands could serve the same function as the staurosporine derivative served for PKA (*16, 17*). Moreover, in theory one need not even be limited to small-molecule ligands. One intriguing approach might be to start with a small-molecule ligand attached to Jun, use the technique to a identify a peptide ligand, and then apply the technique again, this time using the newly identified peptide as the known binder to find a second peptide ligand that could be linked to the first to yield a fully peptidic bivalent inhibitor. Whatever the direction, it is likely that the marriage of phage display with concepts from fragment-based ligand discovery will produce many exciting discoveries.

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