Tethering Small Molecules to a Phage Display Library: Discovery of a Selective Bivalent Inhibitor of Protein Kinase A

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There is much current interest in developing fragment-based strategies for targeting protein surfaces and active sites.[1] Toward this goal, we describe a new fragment-based bivalent ligand selection methodology that allows for the discovery of protein surface-targeted cyclic peptides that are steered by an active site binding small-molecule ligand (Figure 1). This approach allows for coupling high-affinity but promiscuous ligands to the large chemical space encompassed by biological libraries. Here, we demonstrate the feasibility of this approach by converting the nonselective kinase inhibitor, staurosporine, into a selective inhibitor of the cAMP-dependent protein kinase (PKA).

With over 500 members in the human genome, protein kinases comprise an important class of enzymes, and their deregulation is often implicated in various disease states.[2] Thus, selective inhibition of protein kinases has the potential to provide useful reagents and therapeutic leads.[3] Most small-molecule inhibitors of kinases target the conserved ATP binding pocket, often displaying high affinity. While this has produced therapeutically useful inhibitors, they are often promiscuous.[4]

An elegant strategy for increasing affinity and selectivity is the development of bisubstrate analogue inhibitors,[5a] where a peptide analogue of the kinase's protein substrate is covalently attached to an ATP-competitive small molecule.[5b−f] Cole and co-workers demonstrated the feasibility of this approach against the insulin receptor protein tyrosine kinase (IRK).[5c] In another example, Schepartz and co-workers[5f] improved selectivity by utilizing structurally constrained peptide substrates conjugated to the staurosporine-like natural product, K252a. Though elegant, a potential drawback of the aforementioned bisubstrate design approaches is that they necessarily rely on structural information as well as prior knowledge of a specific peptide substrate for the targeted kinase. Many kinases of biological or pharmacological interest do not satisfy the above criteria and therefore lie outside of the scope of these design strategies. We envisioned that an in vitro bivalent selection approach that combined small-molecule targeting with biological selection would have the potential to complement design strategies and provide selective bivalent inhibitors without prior structural or peptide substrate information.

Two previous selection efforts have coupled large biologically derived peptide libraries to synthetic molecules utilizing the orthogonal chemistry of cysteine residues.[6] This chemistry, though very promising, can have low yields and potentially suffers from nonspecific reactivity in the presence of the library platform (such as phage particles or ribosomes) as well as cysteine residues within the library being interrogated. With this in mind, we describe a noncovalent tethering strategy that would select for cyclic peptides from a phage display library[7] directed toward the active site of a protein kinase by an ATP-competitive small molecule. The small molecule would be functionally tethered to the library by noncovalent self-assembly mediated by a coiled-coil heterodimer that has been previously utilized for numerous designs[8] (Figure 1). This

(B) (2)–βAGG–RIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVA

(C) C(X)₆C–GGGGAAALTDTLQAETDQLEDEKSALQTEIANLLKEKEKLEFILA–Phage
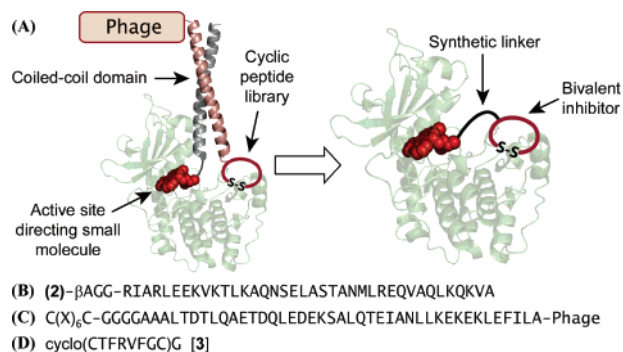
(D) cyclo(CTFRVFGC)G [3]

Figure 1. Bivalent inhibitor selection strategy: (A) Noncovalent tethering of staurosporine with a phage-display peptide library through a coiled-coil heterodimer for targeting a kinase, PKA. The selected peptide can be subsequently conjugated to staurosporine to provide a bivalent kinase inhibitor. (B) Staurosporine derivative **2** conjugated to the Jun. (C) Phage-displayed cyclic peptide library attached to Fos (X = any of the 20 natural amino acids). (D) Selected cyclic peptide **3**.

warhead-guided phage display selection would potentially afford cyclic peptide motifs with affinity for the kinase surface adjacent to the active site. The selected peptides could be subsequently conjugated to the small-molecule warhead to afford a bivalent inhibitor with the potential for increased affinity and enhanced selectivity for the targeted kinase.

To test this new approach, we chose the much studied ATP-competitive kinase inhibitor, staurosporine **1**, as our promiscuous kinase targeting warhead. The carboxylated derivative of staurosporine **2** (Figure 2A) with a reported IC₅₀ value 47-fold weaker than staurosporine[9] **1** was tethered to Jun (Figure 1B) and retained its ability to inhibit PKA (Supporting Information, Figure S1). Next, a phage-displayed peptide library[7] was constructed adjacent to the Fos domain, which heterodimerizes with Jun. The library incorporated two conserved Cys residues flanking the six positions of diversity, each incorporating all 20 amino acids (Figure 1C).[7b] With the library in hand, selection was carried out against PKA. In each round of selection, the **2**−Jun conjugate was mixed with the phage-displayed library conjugated to Fos and exposed to immobilized PKA. After six rounds of selection, several peptides were isolated and characterized for inhibiting PKA. The cyclic peptide CTFRVFGC was the most abundant sequence identified and was also found to be the most active in initial assays against PKA, with IC₅₀ values in the mid-micromolar regime (Supporting Information, Figure S5). The successful selection of the cyclic peptides highlights the important distinction from previous approaches in that our bivalent tethering approach required no prior knowledge of the structure of the kinase or the chemical structure of the protein substrate.[5]

Having established that active-site-directed selection was indeed possible, we turned toward testing our central hypothesis: that our strategy could be used to discover covalently linked bivalent
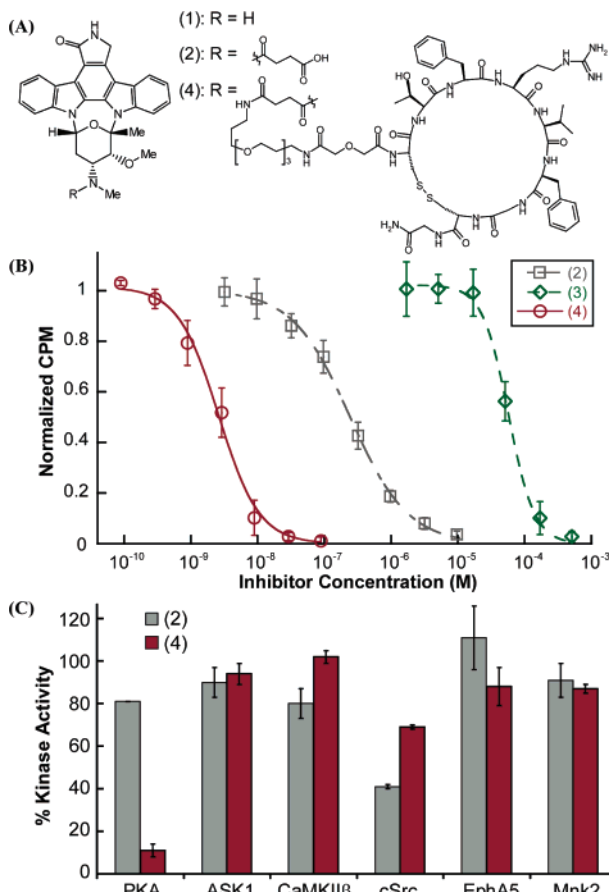
Figure 2. Inhibitory activity and selectivity of bivalent PKA inhibitor: (A) Chemical structures of warhead **2**, selected cyclic peptide **3** conjugated to **2** to afford bivalent inhibitor **4**. (B) Inhibition of PKA (2 nM) with **2**, **3**, and **4**. (C) An activity screen (PKA, ASK1, CaMKIIβ, cSrc, EphA5, and Mnk2) shows significantly increased inhibition at 100 nM by the selected bivalent inhibitor **4** compared to **2** only for PKA.

inhibitors with significantly higher affinity and increased selectivity compared to the starting warhead **2**. Toward this goal, the bivalent molecule **4** was synthesized by covalently coupling **2** to the selected cyclic peptide cyclo(CTFRVFGC)G, **3**, through a 30 Å PEG linker (Figure 2A). This linker was chosen to span an intermediate distance within the range of the calculated distances (11−42 Å) accessible to the Fos/Jun-tethered complex. With the molecules in hand, PKA inhibition was interrogated (Figure 2B).

The cyclic peptide **3** showed modest inhibition (IC$_{50}$ = 57 ± 3 μM), while the modified staurosporine derivative **2** alone displayed high nanomolar inhibition (IC$_{50}$ = 243 ± 16 nM). Importantly, the bivalent inhibitor **4** showed considerably increased inhibition (IC$_{50}$ = 2.6 ± 0.3 nM), with >90-fold and >21 000-fold relative increases when compared to the staurosporine derivative **2** and the cyclic peptide **3**, respectively. These inhibition studies strongly indicate a synergistic binding mode for **4** comparable to designed bisubstrate analogue inhibitors reported by Parang et al.[5c]

With a potent bivalent inhibitor in hand, we set out to address the important question: does this approach enhance the selectivity of the inhibitor? Hence, the selectivity of the bivalent inhibitor **4** was compared to the starting warhead **2** via a kinase activity screen (Kinase Profiler, Millipore), against a panel of five distinct kinases chosen specifically for their ability to bind staurosporine at concentrations comparable to PKA[4b] (K_{dapp} values for stauro-

sporine: ASK1 = 120 nM, CaMKIIβ = 3 nM, cSrc = 100 nM, EphA5 = 150 nM, and Mnk2 = 22 nM), their availability, and their wide distribution across the human kinome.[2] Selectivity assays were carried out for **2** and **4** at a fixed concentration of 100 nM. The inhibition results clearly demonstrate that the bivalent inhibitor **4** significantly reduces kinase activity only for PKA (Figure 2C) when compared to the parent inhibitor **2**. Structural alignments of the five kinases mapped onto the crystal structure of PKA indicate that, though the active sites are quite similar, there are a number of dissimilar surface sites that could be responsible for imparting specificity to the bivalent inhibitor (Supporting Information, Figure S7). Interestingly, the attachment of our cyclic peptide was observed to decrease the activity of the parent inhibitor **2** in the case of CaMKIIβ and cSrc.

Thus, our results confirm that the phage-display-selected peptide **3** can impart increased affinity and selectivity to the small-molecule ligand **2**. This tethering strategy therefore holds the potential to identify inhibitors of protein kinases with increased selectivity, while relying minimally on structural or substrate information for a given target. Future studies will aim to clarify mode of inhibition, obtain structural information, study methods for cellular delivery, and apply this approach to pharmacologically relevant kinase targets. More generally, the ability to incorporate user defined synthetic warheads during in vitro selection may extend this bivalent ligand discovery strategy to numerous biological targets as well as selection platforms for increasing ligand affinity, and more importantly, selectivity.

Supporting Information Available: Experimental details and the complete citations for refs 1a and 4b. This material is available free of charge via the Internet at http://pubs.acs.org.

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