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Stepwise Combinatorial Evolution of Akt Bisubstrate Inhibitors

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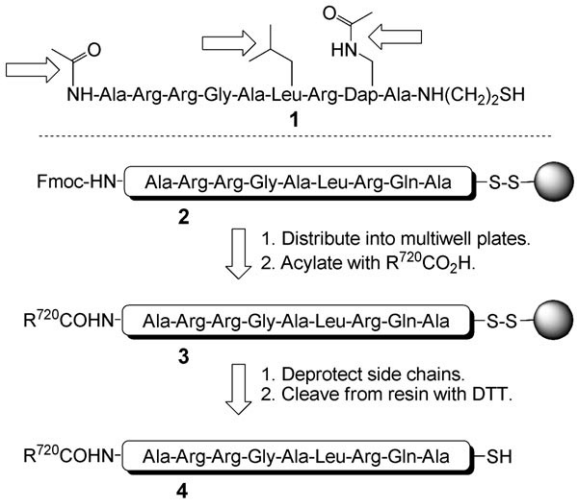
Protein kinases are key enzymatic participants in the signal transduction pathways that control nearly every aspect of normal cell function. In addition, many of these enzymes are often found to be aberrantly expressed or abnormally active in a diverse array of human ailments. For example, the Akt protein kinase is hyperactive in a variety of cancers, where it promotes tumor cell survival by blocking cell death.[1] Not surprisingly, there is considerable interest in acquiring inhibitors for Akt. Like other members of the protein kinase family, Akt employs ATP as the phosphoryl donor for the phosphorylation of Ser residues in protein substrates. Several ATP analogues and nonphosphorylatable peptides have been reported that block the catalytic activity of Akt.[2] Recently, bisubstrate analogues have been championed as useful structural, mechanistic, and biological probes for protein kinases.[3] These inhibitory agents simultaneously block the binding of both substrates in a two substrate enzyme-catalyzed reaction. We report herein the directed molecular evolution of a potent Akt bisubstrate inhibitor using a stepwise, combinatorial library-based strategy.

Bisubstrate inhibitors for protein kinases have been constructed by assembling three component parts into a single unimolecular species. Generally, a tether, which links an ATP surrogate to a peptide-based species, is finely tuned to ensure that both the ATP and the protein binding sites can be comfortably occupied by the inhibitor. However, there is no compelling structural or enzymatic requirement that limits the bisubstrate strategy to previously described components (for example, ATP analogues such as adenosine or H-7). Rather, we felt that it might be possible to use the bisubstrate concept as a means to identify a new functionality that prevents ATP from binding to the kinase under study. We anticipated using a peptide, which targets the protein-binding region, as a framework upon which the ATP-blocking site moiety could be created.

Three parent peptides (Ac-Ala-Arg-Arg-Gly-Ala-Leu-Arg-Gln-Ala-HN$(CH_2)_2$SH; Ac-Ala-Arg-Arg-Gly-Dap(Ac)-Leu-Arg-Gln-Ala-HN$(CH_2)_2$SH; Ac-Ala-Arg-Arg-Gly-Ala-Leu-Arg-Dap(Ac)-Ala-HN-$(CH_2)_2$SH) were prepared so that, upon subsequent combinatorial expansion, a bisubstrate-like species could be identified by utilizing an appropriate screening protocol. The amino acid sequence contained in these parent peptides was based on known Akt preferences.[4] Detailed analysis of one of these peptides (**1**) revealed competitive behavior versus ATP (Supporting Information; Scheme 1). However, given the absence of aromatic moieties, it is unlikely that **1** significantly encroaches on the ATP binding region (thereby providing a potential opportunity to identify new functional groups that block ATP binding). Although the three-dimensional structure of Akt has been solved[5] it was simply not clear which site on the peptide might be responsible for the observed competitive inhibition pattern versus ATP. Nonetheless, based on known Akt protein binding site recognition preferences, we ruled out certain residues (vide infra) as likely culprits and chose three sites (highlighted with arrows) upon which to focus our attention. Although peptide **1** represented an exciting starting point, it is an extraordinarily poor Akt inhibitor, exhibiting a K_i value of 3.2 ± 1.1 mM. Consequently, we required a synthetic strategy that would allow us to simultaneously evolve this derivative into a more potent inhibitor while preserving bisubstrate inhibitory behavior.



Scheme 1. Peptide **1** and potential sites that could promote competitive inhibition behavior versus ATP. A combinatorial resin-based strategy (**2** to **3** to **4**) that introduces molecular diversity at specific sites on a "bisubstrate" site-directed peptide.

We initially examined whether the N terminus acts as the component that precludes ATP binding. A close analogue of **1** (that is, **2**) was synthesized on a Tentagel resin that contains a disulfide linker between peptide and solid support (Scheme 1).[6] Peptide **2** contains a "placeholder" Gln at the acetylated Dap site in **1**, with the former being more synthetically convenient to work with (vide infra). The N-terminal Fmoc group of the peptide-resin **2** was removed and the resin-ap-

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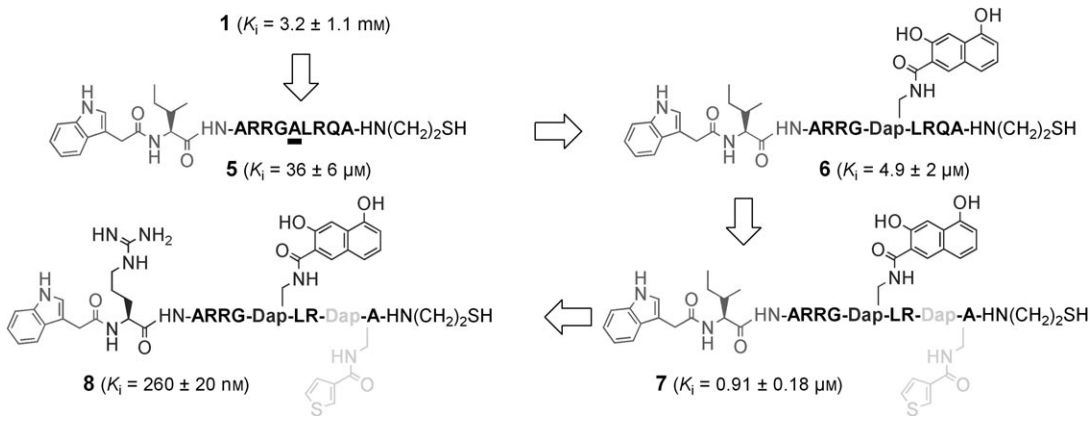

Scheme 2. Combinatorial evolution of a weak Akt inhibitor into a potent analogue with retention of the bisubstrate phenotype.

pended peptide introduced, in equal quantities, into individual wells of multiwell synthesis plates. One of 720 structurally diverse carboxylic acids was added to each well, along with appropriate reagents to activate the acid moiety. Following N-terminal modification, the side-chain protecting groups were removed and the peptide subsequently cleaved from the resin with assay buffer, which contained dithiothreitol. The library of 720 peptide derivatives was then screened for inhibitory potency. The lead derivative, compound **5**, contains an indole-derivatized (L)-Ile moiety (Scheme 2). **5** is a 100-fold more powerful inhibitor than the parent peptide **1** and retains the competitive inhibition pattern versus ATP. In addition, compound **5** exhibits a predominantly competitive inhibition pattern versus the peptide substrate (Supporting Information). Although it is tempting to presume that the newly identified aromatic indole moiety of **5** targets the ATP binding pocket, it could very well associate with some other region of Akt. We decided to address this issue by introducing modifications at other sites on the peptide that might be culpable as substituents that interfere with ATP binding. We excluded as likely culprits, the Ala, Gly, and Arg residues as the first two do not possess notable side-chain moieties that could engage the enzyme and the Arg residues are known to be required for the binding of protein substrates.[4] The remaining possible perpetrators in **5**, Leu and Gln, were replaced with Ala. The Gln-to-Ala substitution furnished a species (**9**) in which the competitive pattern versus ATP was eliminated (Figure 1 B). Both the Dap residue in **1** and the Gln moiety in **5** share a common structural motif, namely an amide moiety. The Ala-for-Gln substitution strongly suggests that the side-chain amide of Gln (or the acetyl-Dap) is responsible for the desired ATP competitive behavior.

With the identification of the peptidic component that compromises ATP recognition, we examined whether inhibitory potency could be augmented even further without interfering with the bisubstrate phenotype. The Ala residue in **5** was replaced with a Dap moiety and its amine side chain modified with 720 different carboxylic acids in a fashion analogous to that outlined in Scheme 1. The lead derivative obtained from the Ala replacement was **6** (Scheme 2), which possesses a dihydroxynaphthalene moiety and a K_i that is nearly 1000-fold

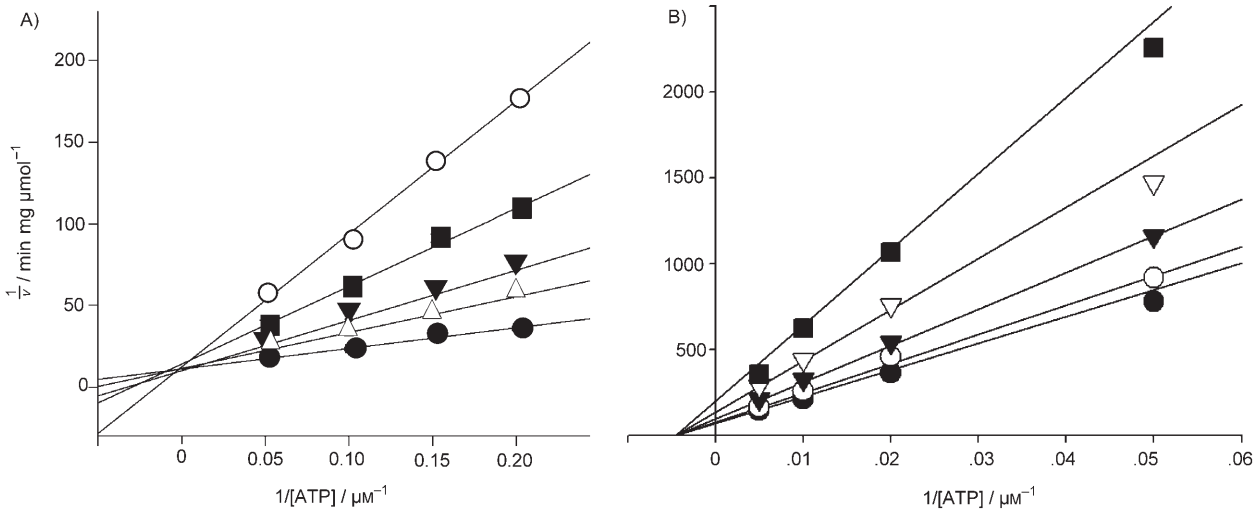

Figure 1. A) Inhibition pattern of compound **8** (3.2, 1.6, 0.8, 0.4, 0 μM) versus variable ATP and B) inhibition pattern of compound **9** (100, 50, 20, 5, 0 μM) versus variable ATP where [Akt] = 2 ng μL^{-1} and [peptide substrate] = 37.5 μM.

better than that exhibited by **1**. The free hydroxyl groups on the naphthalene appear to be important for inhibitory activity as the corresponding dimethoxy derivative (a member of the library) is a tenfold poorer inhibitor than **6**.

The Gln-to-Ala substitution transformed peptide **5** into a species that fails to serve as a competitive inhibitor versus ATP, suggesting that the side chain amide carbonyl of Gln is the key moiety that generates the bisubstrate character of this, and related, inhibitors of Scheme 1. We wondered whether it would be feasible to introduce molecular diversity at the critical Gln side chain and retain the bisubstrate phenotype. Indeed, the Scheme 1 strategy furnished the even more potent inhibitor **7** (Scheme 2), which retains competitive inhibition patterns versus both variable peptide and ATP substrates (Supporting Information).

Although **7** is a reasonably effective inhibitory agent, a previous Akt substrate specificity study revealed a preference for positively charged residues at the N terminus of active site-directed peptides.[4] By contrast, the indole-substituted Ile residue positioned at the periphery of the peptide is decidedly lipophilic. Although this N-terminal substituent was identified as the primary lead in the initial library screen to furnish **5**, it is possible that either the indole ring or the Ile side chain may actually be embedded in a less than favorable hydrophilic environment. We chose to replace the Ile residue with 50 different amino acid analogues, several of which were positively or negatively charged (Supporting Information). Interestingly, the most potent analogue (**8**) from this library contains an Arg residue at the former Ile site. Compound **8** ($K_i = 260 \pm 20$ nM) exhibits a greater than 10000-fold improved inhibitory efficacy for Akt relative to the parent peptide **1** ($K_i = 3.2 \pm 1.1$ mM). As with all the other derivatives described in this study, **8** serves as a competitive inhibitor versus variable ATP (Figure 1A) and variable peptide substrate (Supporting Information).

Many protein kinase-catalyzed reactions proceed by an ordered mechanism in which ATP binds first, followed by peptide (or protein) substrate. Bisubstrate inhibitors for these enzymes exhibit a competitive pattern versus ATP, but a noncompetitive pattern versus peptide substrate as the substrate and inhibitor bind to different forms of the enzyme (that is, the substrate to the enzyme-ATP complex and the inhibitor to the free enzyme).[3a] By contrast, **8** acts in a competitive capacity versus both ATP and associate with the active site. Under these circumstances, compound **8** directly competes with both substrates for the same free form of the enzyme; thus, the dual competitive pattern. Finally, we note that Livnah et al. describe the conjugation of isoquinoline derivatives (ATP surrogates) with peptides to furnish Akt inhibitors in a recently disclosed patent.[7]

In summary, we have converted an extraordinarily weak peptide-based Akt inhibitor into a 10000-fold more potent derivative that exhibits a classic bisubstrate inhibition pattern versus ATP and a peptide substrate. Structural characterization of the Akt–inhibitor complex should prove helpful is assessing the enzyme functionality involved in inhibitor recognition. Nevertheless, we have identified the specific functional group on the inhibitor responsible for the competitive behavior versus ATP. The stepwise library strategy offers a means to retain desirable properties during the directed evolution of inhibitory species.

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Keywords: bisubstrate inhibitor · combinatorial chemistry · phosphorylation · protein kinase · signal transduction

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