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Design, synthesis, and characterization of an ATP-peptide conjugate inhibitor of protein kinase A

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Abstract—An ATP-peptide conjugate was synthesized as a bisubstrate analogue inhibitor of the serine/threonine kinase protein kinase A. The compound was found to be a linear, competitive inhibitor with respect to ATP substrate, exhibiting a K_i of 3.8 μM. The compound was noncompetitive with respect to peptide substrate. The inhibitor was shown to be selective for protein kinase A versus the closely related protein kinase C as well as tyrosine kinase Csk. This analysis provides new evidence for the dissociative transition state of protein serine/threonine kinases and illustrates a simple method to convert a low affinity peptide substrate to a selective and moderately potent inhibitor for these enzymes.

Protein kinases are critical catalysts for cell signal transduction,[1] initiating the transfer of the gamma-phosphoryl group of ATP to serine, threonine, and tyrosine hydroxyls in proteins. Their wide importance in biology and medicine has led to the intense study of their enzyme mechanisms. Indeed, several selective inhibitors of specific protein tyrosine and threonine kinases have proved valuable as therapeutics.[2,3] Despite much progress, there is still an incomplete understanding of the mechanisms used by protein kinases. Furthermore, highly selective inhibitors have been difficult to develop. Most approaches have centered on developing nucleotide analogues.[4,5] However, since all kinases have a conserved ATP binding pocket, nucleotide analogue inhibitors are inherently challenging to develop. In contrast, protein kinases show more diversity in peptide and protein substrate recognition[6] and in principle, peptide substrate site blockers with high specificity might be easier to discover. In an effort to bridge these two approaches, we and others have been attempting to develop bisubstrate analogues as potent and selective inhibitors of protein kinases.[7] In an initial report, it was found that a potent and selective inhibitor of the insulin receptor kinase (IRK) could be generated by linking ATPγS via a short acetyl spacer to a peptide derivatized with aminophenylalanine (Scheme 1, **1**).[8] Design of the molecule was based on previous work indicating that the distance between the entering nucleophilic atom and the attacked phosphorus should approach 5 Å, mimicking the reaction coordinate of a dissociative transition state.[9] This work has recently been extended to another tyrosine kinase, Csk, with potential applications for proteomic and X-ray crystallographic analysis.[10]

These encouraging findings led to the question of whether a related approach might also be applicable to protein serine/threonine kinases. Previously reported ATP-peptide conjugates like **2**, with ATP directly linked to a peptide substrate, had been shown to be weak inhibitors of protein kinase A.[11] In this manuscript, we examine the effect of introducing an acetyl spacer and try to extend use of this bisubstrate analogue design to protein kinase A inhibition.

For this work, we selected aminoalanine as the serine analogue, which would be subsequently linked to ATPγS via an acetyl bridge (Scheme 1, **3**). Kemptide, a well defined substrate for protein kinase A,[12,13] was chosen as the substrate peptide. This sequence had also been used in the series of bisubstrate analogue inhibitors containing a direct link between ATP and peptide.[11] The kemptide peptide was built on a solid-phase Wang resin using standard Fmoc chemistry (Scheme 2). Serine was replaced with Alloc-protected α-Fmoc-aminoalanine, which was then selectively deprotected with tetrakis-triphenyl phosphine palladium(0).[14] The peptide was

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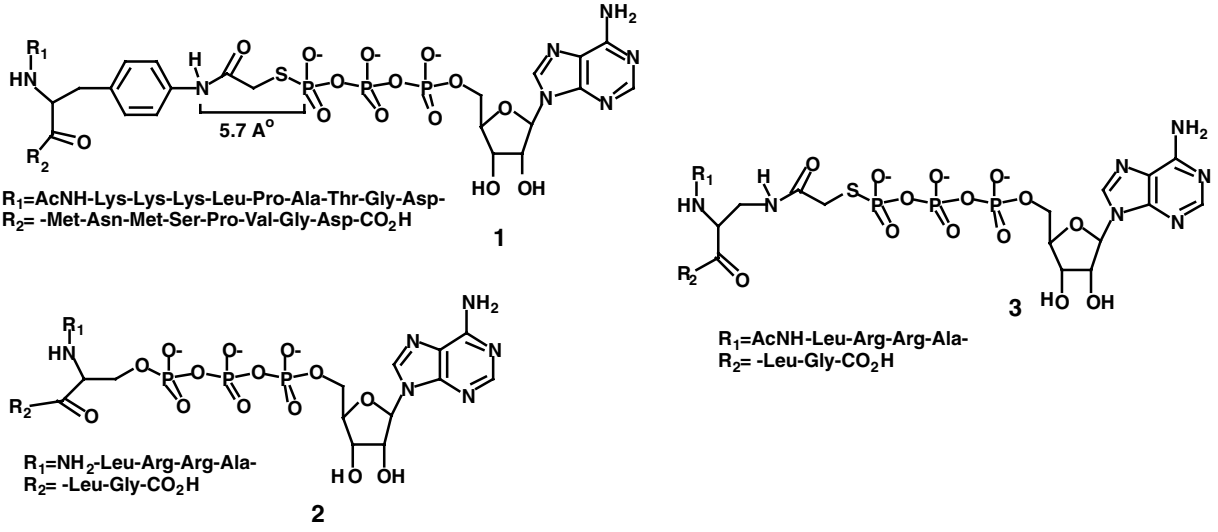
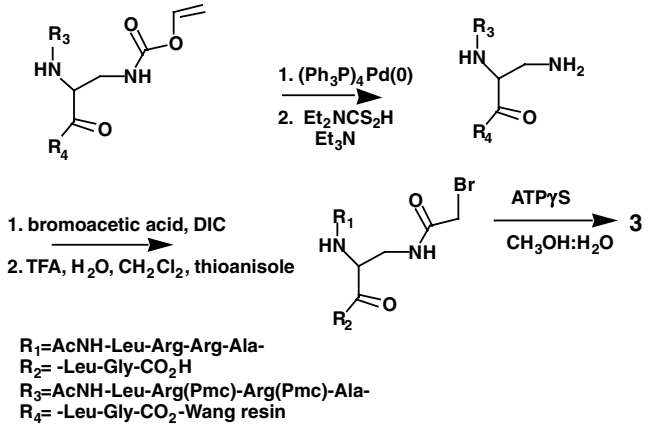


Scheme 1. Bisubstrate analogue inhibitor structures.



Scheme 2. Synthetic scheme for bisubstrate analogue inhibitor of protein kinase A.

bromoacetylated, cleaved from the resin and purified using reversed-phase HPLC in the presence of 0.05% trifluoroacetic acid. ATPγS was coupled to the bromoacetylated peptide to produce the desired compound (**3**). Like compound **1**, compound **3** was found to be acid labile,[8] therefore the final reversed-phase HPLC purification (Fig. 1) was performed with neutral solvents. The structure and purity of the desired compound was verified using ^{1}H NMR and negative ion electrospray mass spectrometry (Fig. 1). Compound **3** was found to be resistant to decomposition at −80 °C as well as in the presence of thiols (data not shown).

The bisubstrate analogue (**3**) was evaluated as a protein kinase A inhibitor using recombinant protein kinase A expressed from *E. coli*.[15] It was found to be a linear competitive inhibitor versus ATP with a K_i of 3.8 μM (Fig. 2). While not as potent as IRK inhibition by compound **1**, the K_i of compound **3** is approximately 30-fold lower than the K_i (120 μM, derived from the published IC_{50}) of ATP linked directly to the kemptide sequence in compound **2**. Interestingly, **3** was non-

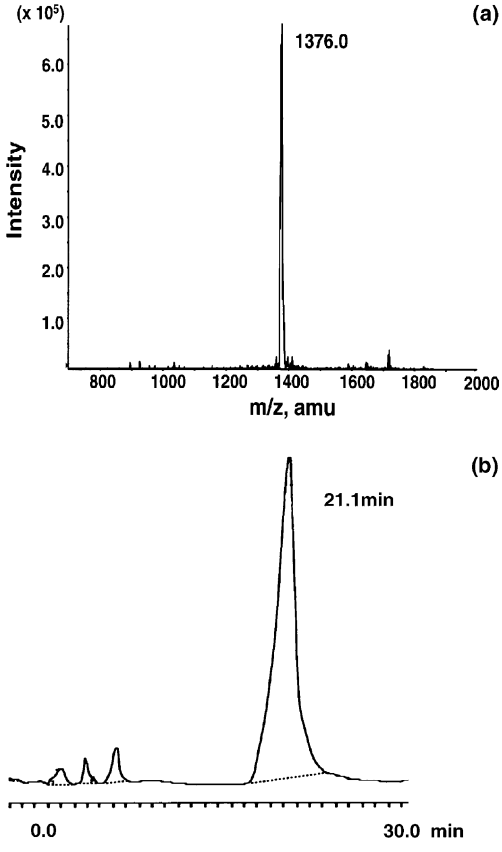


Figure 1. Characterization of bisubstrate analogue compound. (a) Mass of compound was verified using electrospray mass spectrometry (negative ion mode). (b) Purity of compound was established using reverse phase analytical HPLC. HPLC analysis carried out on a Varian C-18 chromatography column, using solvents A, 100% water and B, 100% acetonitrile. The following gradient was used: 0–5 min 0% B; 5–30 min 0–20% B; 30–75 min 20–35% B, 1 mL/min.

competitive versus peptide substrate (Fig. 2). Such a kinetic pattern is typical of potent bisubstrate analogues, which inhibit enzymes that show ordered binding of

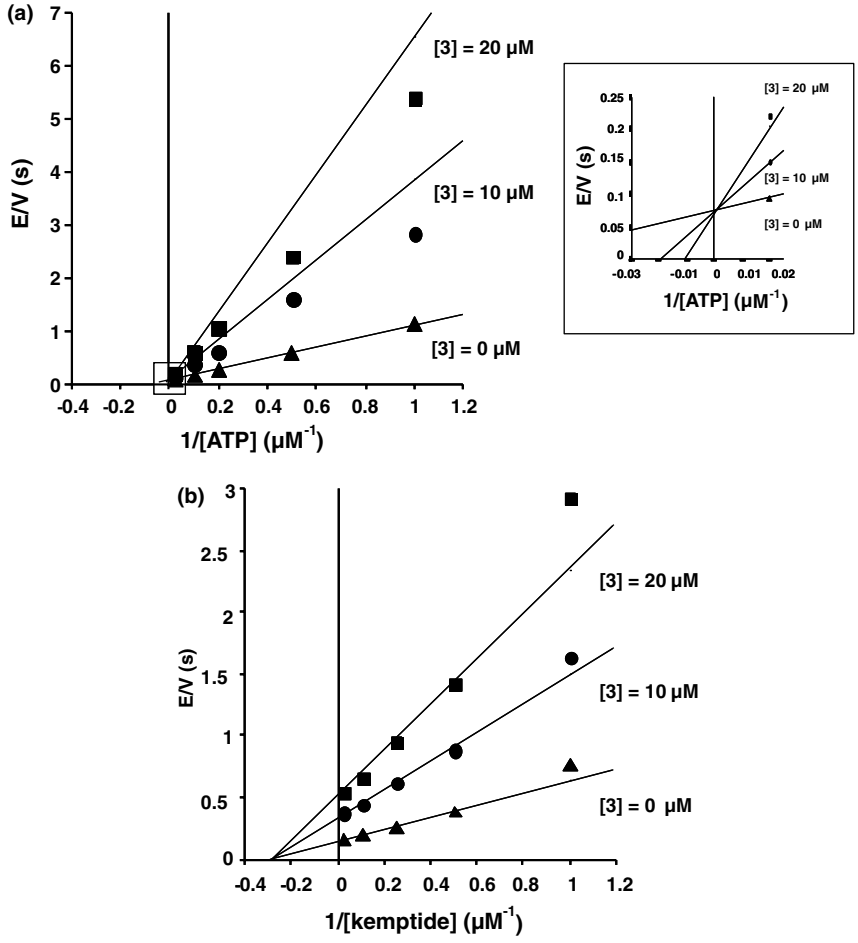


Figure 2. Kinetic analysis of PKA inhibition by bisubstrate analogue inhibitor. (a) E/V versus 1/[ATP] evaluated at varying inhibitor concentrations ($K_i = 3.8 \pm 0.3\,\mu M$, $K_m(ATP) = 14.1 \pm 1\,\mu M$). (Inset) expansion of the plot near the y-intercept. Kemptide concentration was held fixed at 15 μM. (b) E/V versus 1/[kemptide] evaluated at varying inhibitor concentrations. ATP concentration was held fixed at 10 μM. All phosphorylation assays were performed in 40 mM Tris–HCl buffer (pH 7.5), 10 mM $MgCl_2$, and 100 μg/mL bovine serum albumin at an enzyme concentration of 0.7 nM. Assays performed using materials and modified protocol from Calbiochem Protein Kinase A Assay Kit.

their two substrates.[16,17] While protein kinase A does not show a strictly ordered kinetic mechanism, it does show a clear preference for nucleotide followed by peptide binding.[18] Indeed, inhibition by the peptide inhibitor PKI is stimulated about 50-fold by the presence of ATP, consistent with an ordered binding mechanism.[18]

Importantly, the bisubstrate analogue described here was found to be more than 20-fold selective versus the related protein kinase, protein kinase C and the less similar protein tyrosine kinase Csk (Fig. 3). This information points to ability of the peptide moiety to contribute affinity and specificity toward the inhibition. The question arises as to why the inhibition by **3** is about 10-fold weaker than the analogous strategy for IRK. One known feature of protein kinase A is that a second Mg^{2+} ion confers high affinity for ATP binding, and under these conditions the K_m for ATP drops roughly 10-fold.[19] The bisubstrate analogue (**3**) would be unlikely to readily accommodate two metals since the gamma phosphate is in this case a diester, therefore, the loss of second metal ion binding may be detrimental to a high affinity interaction of **3** with protein kinase A. In addition, it is uncertain whether the acetyl carbonyl in **3** creates unfavorable interactions that are avoided in the IRK inhibitor by Mg^{2+} chelation.[8]

While the bisubstrate analogue (**3**) is not an exceedingly potent protein kinase A inhibitor, the improved affinity of **3** versus the directly linked compound **2** supports a dissociative transition state for protein kinase A and by extension, other serine/threonine kinases. Determining the degree of associative versus dissociative character for phosphoryl transfer reactions catalyzed by enzymes continues to be a vexing problem in mechanistic biochemistry.[20–25] An associative model was previously proposed for protein kinases because the X-ray structure of protein kinase A showed a cluster of positively charged groups coordinating the gamma phosphate of ATP, making the phosphate a better electrophile.[26] More recently, linear free energy relationship studies and other work have indicated a dissociative model for the tyrosine kinase subset of this superfamily of enzymes.[23] The findings shown here with protein kinase A can be taken to support a dissociative mechanism for serine/threonine kinases. Recent theoretical and experimental studies support this mechanism as well for serine/threonine kinases.[27]

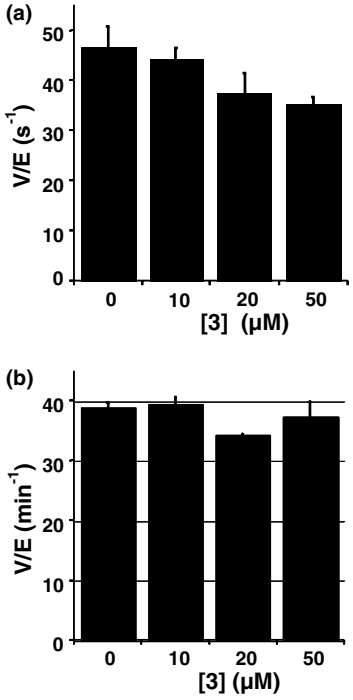


Figure 3. Specificity of bisubstrate analogue inhibitor (**3**). (a) PKC velocity versus inhibitor concentration. PKC assays performed in 20 mM Tris–HCl buffer (pH 7.5), 500 μM $CaCl_2$, 10 mM $MgCl_2$, 25 μM peptide, 15 μM ATP, 100 μg/mL bovine serum albumin at an enzyme concentration of 0.8 nM. Assays performed using materials and modified protocol from Calbiochem Protein Kinase C Assay Kit. (b) Csk velocity versus inhibitor concentration. Csk assays performed in 60 mM Tris–HCl buffer (pH 7.4), 12 mM $MgCl_2$, 200 μM ATP, 700 μg/mL poly(Glu, Tyr) (K_m(ATP) = 195 μM, K_m(poly(Glu, Tyr)) = 654 μM),[29] 1 mM DTT, 100 μg/mL bovine serum albumin at an enzyme concentration of 30 nM.

The finding that an acetyl linked peptide-ATP conjugate can be a selective serine/threonine kinase inhibitor suggests applications for similar compounds in structural genomics and proteomics. Since protein serine/threonine kinases make-up roughly three-fourths of the kinome,[28] the finding with protein kinase A could be useful for a large array of important enzymes. In general, the affinity of peptide substrates for kinases are in the millimolar range, including kemptide for protein kinase A, and the strategy described here along with related findings with tyrosine kinases allows straightforward transformation of peptide affinities to the low micromolar–high nanomolar range. Thus, pull-down experiments to identify kinases responsible for a particular phosphorylation[10] as well as enhanced X-ray crystallographic analysis of serine/threonine kinases[8] can now be viewed as realistic applications of these bisubstrate analogues.

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