A Highly Potent and Selective PKCα Inhibitor Generated via Combinatorial Modification of a Peptide Scaffold

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Signal transduction is the biochemical mechanism by which information is transmitted between distinct cellular sites. Signaling pathways differ from their classical biochemical counterparts in a number of ways. For example, the enzymes of glycolysis and the TCA cycle catalyze the conversion of small molecules into products, which are then passed onto the next enzymatic member of the pathway. By contrast, the protein participants of signaling pathways primarily associate with and act upon one another. The amino acid sequences ("consensus recognition sequences") that drive these critical protein−protein interactions are readily identified using combinatorial peptide-based libraries.[1] Consensus sequence information has proven to be helpful in piecing together signaling pathways. In addition, peptides containing these sequences are potentially useful inhibitory reagents that could furnish information about the biological role of signaling proteins. Unfortunately, consensus sequence peptides tend to display modest affinities (K_D or K_i > low μM) for their protein targets. We,[2] as well as others,[3] have shown that consensus sequences for signaling proteins can be converted into higher affinity ligands using the three-dimensional structure of the protein target as a guide. Nevertheless, the tertiary structure for only a small minority of all signaling proteins has been assigned, thereby limiting the generality of this approach. We describe herein a library-based strategy that transforms consensus sequences into high-affinity ligands *in the absence of any tertiary structural information of the protein target.*

We chose PKCα for our initial studies, an enzyme that is a recognized chemotherapeutic target for several malignant disorders.[4] The structure of PKCα is not known. A variety of peptide-based inhibitors have been described, the very best of which display IC_{50} or K_i values in the high nM to low μM range.[5] The consensus substrate sequence for PKCα is −Arg−Arg−Lys−Gly−Ser−Hyd− Arg− (where Hyd = Phe/Leu/Ile/).[6] We designed the closely analogous nonphosphorylatable peptide Ala−Arg−Arg−Gly−*Ala*− Leu−Arg−Gln−Ala, in which the Ser residue is replaced by *Ala*. Previous studies have demonstrated that the Arg residues and the hydrophobic amino acid at P-1 promote PKCα recognition.[6] Consequently, these critical residues were retained and we sought to identify high-affinity replacements for presumed nonessential residues or regions on the consensus peptide. In the absence of the three-dimensional structure of the target protein, three distinct sites on the peptide framework were chosen for the introduction of molecular diversity (libraries **I**−**III**). For example, a peptide containing (L)-2,3-diaminopropionic acid (Dap) at the former *Ala* position was synthesized, distributed in equal amounts to individual wells of eight 96 well plates, and then acylated with one of 720 different carboxylic acids to create library **II**. Analogous libraries **I** and **III** were constructed as well. Following Dap acylation, the side chain protecting groups were removed with trifluoroacetic acid and the peptide was then cleaved from the resin with assay buffer (which contains dithiothreitol). The peptide solutions were filtered
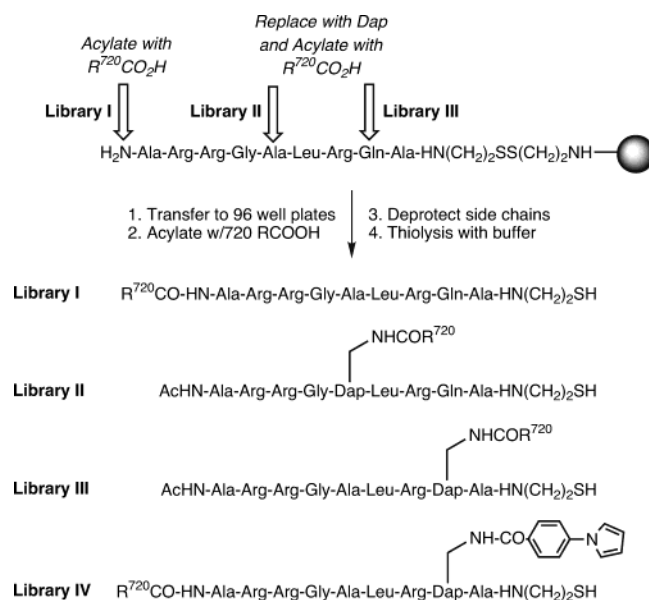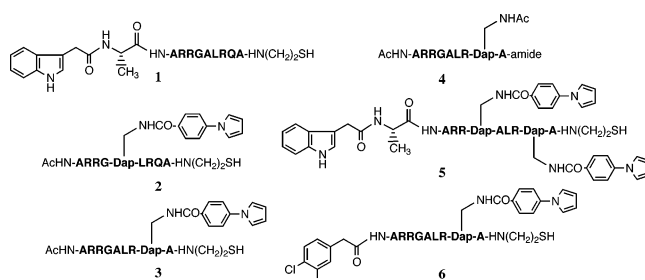
**Scheme 1**

Chart 1

into deep-well plates, stored, and subsequently evaluated for inhibitory potency using a previously described radioactive assay (Supporting Information).

Leads (**1**−**3**) from the three libraries are depicted in Chart 1. All three compounds display several orders of magnitude improvement in inhibitory efficacy relative to the diacetylated control peptide **4** (Table 1). Interestingly, the best leads from libraries **II** (**2**) and **III** (**3**) contain the same substituent, a 4-pyrrole phenylacyl moiety. The latter result suggests that PKCα possesses a binding pocket that displays a special affinity for this substituent. Given the weak inhibitory activity displayed by peptide **4**, it is likely that the peptide backbones of **2** and **3** are not rigidly held by the PKCα surface but rather assume unique enzyme-bound conformations that promote insertion of the 4-pyrrole phenylacyl into a high affinity pocket. Indeed, peptide **5**, which contains the three substituents identified from libraries **I**−**III**, displays an inhibitory potency

Table 1. PKCα Inhibitory Potencies of Compounds **1**−**6**[a]

compound	IC$_{50}$ (μM)	K_i (μM)
1	10.4 ± 2.1	not determined
2	5.7 ± 0.4	not determined
3	4.7 ± 0.8	0.55 ± 0.07
4	1100 ± 210	350 ± 80
5	3.1 ± 0.7	not determined
6	0.0019 ± 0.0002	0.00080 ± 0.00025

[a] K_i values were obtained by varying peptide substrate concentration.
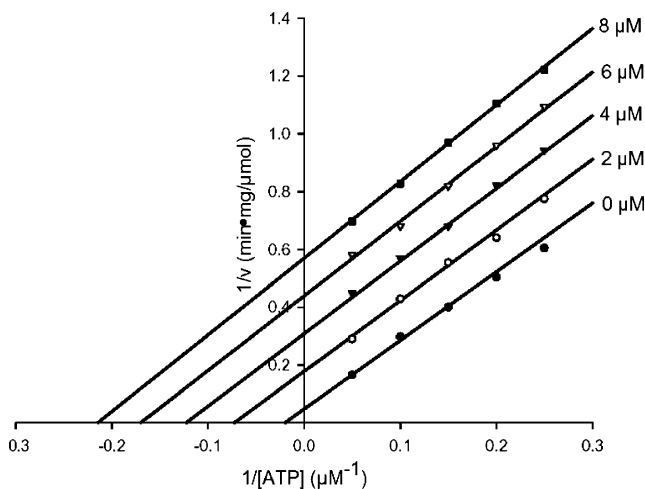
Figure 1. Inhibition Pattern of Compound **3** versus Variable [ATP].

similar to that of the individual peptide leads **2** and **3**. This result is consistent with the notion that there exists a *single* 4-pyrrole phenylacyl docking site within the substrate-binding region of PKCα. This result also highlights one of the potential pitfalls associated with combining, in a single molecule, lead substituents obtained independently of one another.

The ATP binding pocket of PKCα is known to accommodate an array of hydrophobic heterocyclic compounds and could very well serve as the binding site for the pyrrole phenylacyl moiety. We examined this possibility by obtaining the inhibition patterns for peptide **3** (and the diacetylated control peptide **4**). Compound **3** is a competitive inhibitor versus variable peptide substrate (data not shown) but serves as an uncompetitive inhibitor with respect to ATP (Figure 1). Since ATP and **3** do not act on PKCα in a mutually exclusive fashion, this suggests that the 4-pyrrole phenylacyl moiety binds to a subsite other than the ATP pocket. The advantage associated with this behavior is that the high intracellular levels of ATP will not curtail the inhibitory potency of **3**.[7]

The 4-pyrrole phenylacyl group in **3** enhances inhibitory activity by 3 orders of magnitude relative to **4**. Furthermore, peptide **3** surpasses the inhibitory potency displayed by some of the most powerful peptide-based active site-directed inhibitors of PKC, including the 33 amino acid-containing defensins.[5f] Nevertheless, we decided to explore whether an even more potent inhibitor of PKCα could be identified by taking advantage of one of the features inherent within the strategy outlined in Scheme 1. With the acquisition of a lead substituent at one position in the active site-directed inhibitor (e.g., **3**), it should be possible to employ this substituent as a biasing element in the search for affinity-enhancing moieties at other sites on the peptide chain. We chose the 4-pyrrole phenylacyl moiety from peptide **3** as the biasing substituent and prepared sublibrary **IV**, which contains diversity elements positioned at the N-terminus. The primary lead **6** was identified from library

IV and, as with leads **1**−**3**, resynthesized and enzymologically characterized. Compound **6** displays a K_i of 800 pM, approximately 3 orders of magnitude more potent than compound **3** and 6 orders of magnitude more potent than the starting parent peptide **4**. To the best of our knowledge, compound **6** is the most powerful protein binding site-directed inhibitor ever reported for a protein kinase.

PKCα belongs to a family of closely related protein kinases (PKCs).[9] The high sequence homology displayed by the PKC family members has rendered acquisition of isoform-selective inhibitory agents exceedingly difficult.[8] Indeed, as far as we are aware, a potent PKCα-selective inhibitor has not been reported. Although the leads identified in libraries **I**−**III** display a less than 3-fold selectivity for PKCα versus other PKC isoforms (data not shown), extraordinary selectivity is observed with the secondary library lead **6**. The latter exhibits a profound preference for PKCα versus its closely related conventional PKCβ (385-fold) and PKCγ (580-fold) counterparts. Higher selectivities are observed versus the more distantly related novel (PKCδ, 2730-fold; PKCε, 600-fold; PKCη, 1310-fold; PKCθ, 1210-fold) and atypical (PKCι, 940-fold; PKCζ, 640-fold) subfamilies. These results suggest that the N-terminal substituent in **6** accesses a structurally distinct subsite unique to PKCα.

In summary, we have identified an extraordinarily potent and highly selective PKCα inhibitor via the stepwise combinatorial modification of a consensus sequence scaffold. The inhibitory agent exhibits an uncompetitive inhibition versus ATP, thereby suggesting that the intracellular effectiveness of **3** (or **6**) will not be curtailed by the high levels of ATP present in living cells.

Supporting Information Available: Experimental details of the synthesis, characterization, and enzymology of the libraries and identified lead compounds (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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