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# Selection of inhibitory peptides for Aurora-A kinase from a phage-displayed library of helix–loop–helix peptides

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ABSTRACT

Conformationally constrained peptide libraries have been made by grafting randomized amino acid sequences onto a rigid scaffold derived from natural proteins. Here, as a library scaffold, we propose a de novo designed helix–loop–helix motif. We constructed a peptide library of the loop region and screened against Aurora-A, which is a member of the Aurora family of serine/threonine protein kinases, to successfully isolate the inhibitory peptides. A semi-rational strategy, which combines phage-displayed libraries and de novo designed peptides, would provide a new way to generate selective peptide inhibitors for the protein kinase family.



Combinatorial peptide libraries have been broadly recognized as useful resources for screening bioactive ligands that bind to receptors and enzymes. However, the identified peptides generally possess considerable conformational flexibility, so they show provide no 3D information of the pharmacophores in the interaction between the peptides and the targeted proteins. These facts have accelerated attempts to construct conformationally restricted peptide libraries by grafting randomized amino acid sequences onto a rigid scaffold derived from natural proteins, or incorporating them into stable secondary structural motifs.[1] Previously, as a library scaffold, we proposed a de novo designed helix–loop–helix motif, which is characterized by two α-helices connected by a loop, and constructed a phage-displayed peptide library of the α-helix region to screen against a cytokine (G-CSF) receptor or a ganglioside (GM1).[2–4] In the present work, we have constructed a peptide library of the loop region from the helix–loop–helix and have screened against Aurora-A, which is a member of the Aurora family of serine/threonine protein kinases. Aurora-A is critical for the proper regulation of mitosis and is often targeted in anti-cancer drug discovery.[5,6] Although the peptide substrates and activators of Aurora-A were obtained by modification of Kemptide and TPX2[7–10], this report is the first example of de novo peptides recognizing Aurora-A.

We used the intramolecular antiparallel helix–loop–helix peptide **YT1** (AELAALEAELAALE-$G_7$-KLAALKAKLAALKA) as a library scaffold (Fig. 1).[2,3] The N-terminal and C-terminal segments associate with each other by virtue of the hydrophobic interactions of leucine residues positioned at *i* and *i* + 4 to stabilize the α-helical structures. Based on peptide **YT1**, we constructed two peptide libraries with different length of the loop. Randomizations of 5 in 7 amino acids of the loop and 9 in 11 amino acids of the loop gave the libraries, **L-lib7** and **L-lib11**, respectively (Table S1). Randomized oligonucleotides encoding the libraries were overlapped by annealing, and then amplified by polymerase chain reactions (Table S2). The amplified

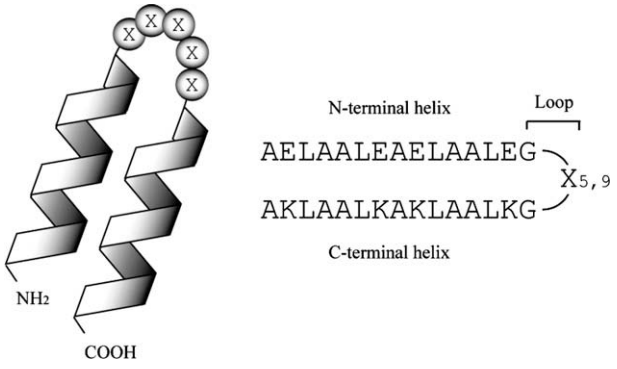


**Figure 1.** The structure of novel peptide library. An illustration of the libraries is shown at the left. X represents a position of randomized amino acids in the loop region. The constructed libraries were named as **L-lib7** and **L-lib11,** according to the overall length of the loop regions.

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oligonucleotides were digested with restriction enzymes and ligated with phagemid vector pComb8.[11] The N-terminal of the helix–loop–helix peptide was connected with the *pel*B leader sequence and the C-terminal was connected with a spacer (GGGSGGGS), an E-tag epitope (GAPVPYPDPLEPR), and pVIII phage coat protein. The phagemid vector was electroporated into competent *Escherichia coli*. XL1-blue cells and amplified with helper phage VCSM13 to give two libraries, **L-lib7**, and **L-lib11**. The library sizes were $2.7 \times 10^7$ and $2.0 \times 10^7$, respectively.

Prior to the affinity selection, we examined kinase activity of immobilized Aurora-A on microtiter plates (Fig. S1), because immobilization sometimes disrupts protein structures and the protein loses activity. A recombinant Aurora-A carrying a GST-tag was immobilized onto an anti-GST antibody-coated plate or onto a glutathione-coated plate, then the plates were blocked with skim milk. To detect kinase activity, we used immobilized metal ion affinity-based fluorescence polarization (IMAP), in which fluorescent substrates phosphorylated by kinases are captured with metal ion beads to change the polarization.[12] The immobilized Aurora-A showed kinase activity, suggesting that the original protein structure was maintained.

The phage-displayed peptide libraries, **L-lib7** and **L-lib11**, were pre-incubated with GST and skim milk to eliminate phages non-specifically adhered to GST and the blocking reagent, then the libraries were used for biopanning against Aurora-A. The repeated selections were implemented in 4 rounds: the ratio of the output relative to the input phages was monitored at each round to indicate enrichment of the binding phages. As shown in Figure 2, phage enrichment was observed during selection with **L-lib11**, whereas no phage enrichment is apparent in the same selection against skim milk. These results suggested that the biopanning of **L-lib11** isolated peptides that specifically bind to Aurora-A. On the other hand, unfortunately, no phage enrichment was observed during selection with library **L-lib7**.

To confirm the binding affinity of the selected phages (52 clones), we examined phage ELISA for Aurora-A (GST-tagged) and reference proteins (GST, anti-GST antibody, and skim milk) used for the immobilization on ELISA plates. All the selected phage clones bound only to Aurora-A and not to the other proteins (Fig. S3). In addition, phage clones displaying the scaffold peptide **YT1** and helper phage (VCSM13) showed no binding to Aurora-A or to the other proteins. These results suggest that the amino acid sequence of the loop region plays an important role in binding to Aurora-A. Of 52 clones, 5 clones were randomly chosen and examined in detail.

We synthesized five peptide candidates, **1**, **3**, **54**, **128**, and **161**, by solid-phase methodology using Fmoc chemistry (Table S4) and examined the conformations of the peptides by circular dichroism (CD). All of the peptides showed CD spectra typical for α-helical structure, and their α-helical contents (%) were comparable to that of the parent peptide **YT1** (71% α-helical content). Thus, the selected peptides retained their secondary structure in buffer, TBS, used for the biopanning (Table 1).

Next, we characterized the inhibition activity of the peptides against Auroa-A. The inhibition assay was performed by IMAP. In this assay, a change in fluorescence polarization of phosphorylated FITC-tagged Kemptide was monitored in the presence of the peptide.[12] Peptides **1**, **54**, and **128** showed inhibitory activity against Aurora-A, whereas peptides **3** and **161** showed no activity (Table 1). It was found that peptide **54** was the most active, showed 35% inhibition at a concentration of 100 μM (Fig. 3). However, we suspected that peptide **54** could act as a substrate for Aurora-A due to a serine residue (Ser21) in the loop region: thus, phosphorylation of **54** could compete with that of the FITC-tagged substrate. To eliminate this possibility, we synthesized a mutant peptide **54-S21A**, in which

**Table 1**
Structure and inhibitory activity of synthetic peptides

| Peptide | The amino acid sequences in the loop region of the helix–loop–helix peptide | α-Helical content (%) | %Inhibition at 100 μM peptides[b] |
|---|---|---|---|
| YT1 | GGGGGGG | 71 | ni |
| 1 | GERRLIIFNSG | 65 | 20 ± 5 |
| 3 | GARHFYVQSVG | 61 | ni |
| 54 | GRRVVVSFAWD | 67 | 35 ± 2 |
| 128 | GSSERKLVLLG | 76 | 17 ± 2 |
| 161 | GVGEFSVSLLG | 77 | ni |
| 54-S21A | GRRVVVAFAWD | 63 | 42 ± 4[c] |
| L-54-S21A[a] | GRRVVVAFAWD | — | ni |

[a] Peptide **L-54-S21A** is the 11-mer peptide derived from peptide **54-S21A**.
[b] Inhibitory activity of Aurora-A was measured with IMAP technology (ni = no inhibition).
[c] %Inhibition at 10 μM peptide **54-S21A**.

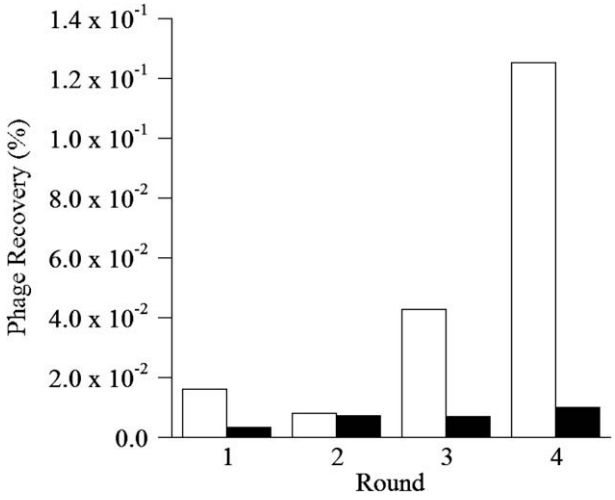


**Figure 2.** Biopanning against Aurora-A with a phage-displayed peptide library **L-lib11** (AELAALEAELAALE-GX$_9$G-KLAALKAKLAALKA). Recovery rates (%) of phage were estimated for Aurora-A (□) and for skim milk (■).

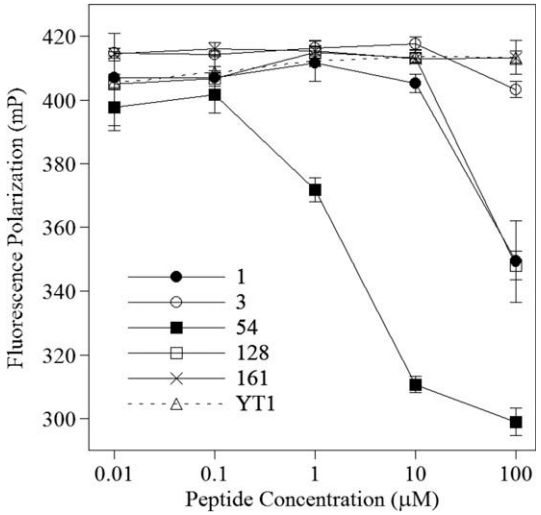


**Figure 3.** Inhibitory activity of selected peptides for Aurora-A, studied using IMAP. 100 nM fluorescent substrate was incubated with the synthesized peptides for 60 min in 20 mM HEPES (pH 7.4), 0.01% Tween 20, 2 mM DTT, 30 μM ATP, 1% DMSO, and 5 mM $MgCl_2$ with 0.23 μg/mL of Aurora-A. Phosphorylated fluorescent substrate was monitored using IMAP technology.

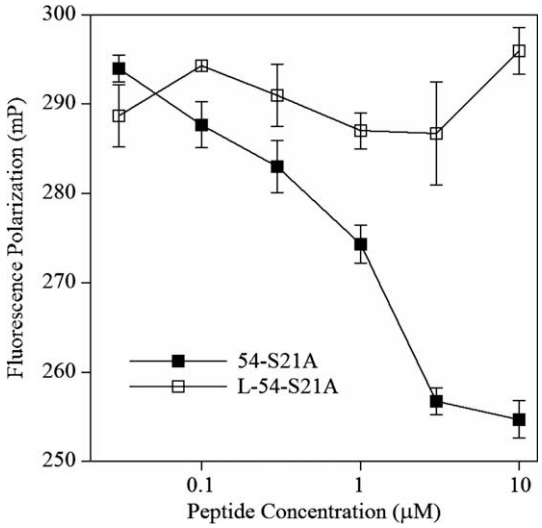


**Figure 4.** Inhibitory activity of peptide **54-S21A** and **L-54-S21A** for Aurora-A. 100 nM fluorescent substrate was incubated with the synthesized peptides for 30 min in 20 mM HEPES (pH 7.4), 0.01% Tween 20, 2 mM DTT, 30 μM ATP, and 5 mM $MgCl_2$ with 0.23 μg/mL of Aurora-A. Phosphorylated fluorescent substrate was monitored using IMAP technology.

the serine was replaced with alanine, and examined its inhibitory activity. As shown in Figure 4, peptide **54-S21A** (63% α-helical content) also showed inhibitory activity against Aurora-A. Finally, we synthesized a linear peptide of the loop region of **54-S21A** (**L-54-S21A**: GRRVVVAFAWD) and examined its structure–activity relationship. The loop peptide **L-54-S21A** showed no inhibitory activity, suggesting that helix–loop–helix structure was essential for inhibitory activity against Aurora-A.

In this work, we successfully constructed a phage-displayed peptide library of the loop region of a de novo designed helix–loop–helix that provided inhibitory peptides against Aurora-A. The constrained conformation of the peptide was found to be essential for recognizing the target protein. Further directed evolution or fragment-based design of the selected peptides will provide improved binding affinity and specificity.[13–15] Selective discrimination of protein kinases is of intense interest because there are more than 500 members of this enzyme family in the human genome, and aberrant protein kinases are often implicated in various diseases and are therefore targeted proteins in drug discovery.[15–18] A semi-rational strategy, which combines phage-displayed libraries with de novo designed peptides, would provide a new way to generate selective peptide inhibitors for the protein kinase family.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.01.015.

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