

# Optimizing the Amino Acid Sequences of Peptides and Improving Their Specificity of Binding to SH3 Domains of Target Proteins

Yanrong Ren<sup>1</sup>, Qiang Wang<sup>1</sup>, Xiaobo Li<sup>2</sup>

<sup>1</sup>School of Biological and Chemical Engineering, Chongqing University of Education, Chongqing 400067, P. R. China

<sup>2</sup>Human Resources Office, Chongqing University of Education, Chongqing 400067, P. R. China

## ABSTRACT

**Introduction:** It is always a crucial challenge in biotechnology to avoid promiscuous binding between an anticancer peptide and multiple SH3 domains, thus reducing potential toxic effects. In spite of a great deal of experimental efforts, the association between amino acid sequence and binding specificity of peptide remained largely unknown. **Aim:** The purpose of this study was to optimize the amino acid sequence of peptide ligands and render high specificity towards designated therapeutic targets. **Results:** By exploring peptide ligands in MINT database and utilizing SH3Peplnt tool for *in silico* peptide-target binding, here we investigated how the amino acid sequence of a peptide determined its specificity of binding to the SH3 domain of c-Src protein. We found that the 5<sup>th</sup> and the 6<sup>th</sup> residues of proline-rich motif had large influence on peptide-target binding. By purposely modifying the amino acid at these two key positions, the overall level of binding promiscuity was significantly reduced. **Conclusion:** Taken together, these findings corroborated that the SH3 domain of c-Src protein can discern subtle differences in the amino acid sequence of ligands, which provided a unique opportunity for rational design of therapeutic peptides.

**Key words:** SH3 domain; c-Src; promiscuity; specificity; peptide rational design

## INTRODUCTION

As one of the major causes of death worldwide<sup>1</sup>, cancer is characterized by uncontrolled division of tumor cells and invasion into other tissues.<sup>2</sup> Chemotherapy is adopted as one of the major approaches to treat cancer, by which a cytotoxic agent is delivered to the cancer cells. However, traditional chemotherapeutic drugs target tumor cells by disrupting necessary cellular functions of normal cells, thus leading to a variety of adverse effects.<sup>3</sup> In addition, multidrug resistance in patients can also cause failure of chemotherapy.<sup>4</sup> Because of that, cancer treatment using peptides is emerging as a more targeted to circumvent the problems of conventional chemotherapy.<sup>5</sup> As molecules formed by combinations of amino acids linked by peptide bonds through the dehydration-condensation reaction, peptides are

characterized by many pharmacological advantages, such as smaller size, ease of synthesis and modification, high tumor-penetrating ability, and favorable biocompatibility.<sup>6</sup> In recent years, a number of peptide-based therapies have been tested in both *in vitro* and *in vivo* experimental models and applied to treat various types of cancer.<sup>7</sup> During the process of developing peptide-based drugs, many proteins have been selected as potential drug targets due to their critical roles in the pathogenesis of cancers. Of the various protein targets chosen, c-Src is an extensively studied kinase oncogene in academia and industry.<sup>8</sup> c-Src is a non-receptor tyrosine kinase that involved in intracellular signaling and

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**Correspondence:**

**Yanrong Ren,**

Address: 9 Xuefu Avenue,  
Nan'an District, Chongqing  
University of Education,  
Chongqing 400067,  
P. R. China

Ph.no: +86(023)86380026

Fax: +86(023) 62658983

E-mail: ren\_sci@sina.com



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regulates the phosphorylation of multiple proteins.<sup>9</sup> Aberrant activation of c-Src is found to be correlated with transformation, proliferation, tumor angiogenesis, and malignant progression of a wide variety of human cancers.<sup>10,11</sup> These properties render c-Src a target for a series of chemical anticancer drugs. While c-Src inhibitors effectively arrest the cycle progression of tumor cells<sup>12,13</sup>, they also induce serious adverse reactions as most conventional chemotherapy agents.<sup>14,15</sup>

The c-Src protein is composed of an N-terminal myristoylation sequence attached to the SH4 domain, a unique region followed by SH3 and SH2 domains, a linker region, a kinase domain SH1 domain, and a C-terminal regulatory domain.<sup>16,17</sup> While the kinase domain serves as the target for many chemical anticancer drugs, the SH3 domain is receiving increasing attention in recent years for its involvement in multiple important cellular processes, including signal transduction, cytoskeleton regulation, and membrane trafficking.<sup>18</sup> In the meantime, SH3 domains generally mediate peptide-protein interactions through the recognition of proline-rich motifs in the amino acid sequence of peptide.<sup>19,20</sup> Therefore, the SH3 domain of c-Src protein is considered as a potential target of therapeutic peptides with antitumor activity.<sup>21</sup>

However, it must be noticed that the SH3 domains may, in a sense, be highly versatile in interacting with peptide ligands. For example, SH3 domains of different proteins may commonly favor a given consensus motif. As a result, one peptide ligand may accidentally bind to multiple protein targets via SH3 domains, thus playing diverse and unpredictable roles in the cell.<sup>22</sup> Therefore, it is always crucial to avoid promiscuous binding between a candidate peptide and multiple SH3 domains, in order to reduce potential toxic effects. Although the consensus amino acid sequence of proline-rich motif serves as an anchor for interacting with most SH3 domains, the specificity may vary between individual peptides, which is profoundly influenced by residues in the core motif.<sup>20</sup> Because of that, the amino acid sequence of a candidate peptide can be elaborately designed, so as to render high affinity and specificity against designated therapeutic targets.<sup>23</sup>

In spite of a great deal of experimental efforts,<sup>20</sup> the association between amino acid sequence and binding specificity of peptide remained largely unknown. This situation motivated us to make efforts on this issue from a computational perspective. By exploring online peptide database and utilizing bioinformatics tool, we investigated how the amino acid sequence of a peptide may influence its binding specificity towards the SH3 domain of c-Src protein. We primarily retrieved a set

of prototype peptides that have already been experimentally validated for binding to the SH3 domain of c-Src protein, among which we identified several peptides binding to fewer targets. These peptides with better binding specificity exhibited some common features in amino acid sequence. Based on that, we purposely optimized the amino acid sequence of all prototype peptides. The results showed that such optimization effectively hindered promiscuous peptide-target binding, which provided a practical way of reducing the safety risks of therapeutic peptides.

## MATERIALS AND METHODS

### Preparation of prototype peptides

We primarily searched the Molecular INTeraction (MINT) database (<http://mint.bio.uniroma2.it/mint/Welcome.do>) for experimentally validated molecules interacting with the SH3 domain of human c-Src protein, including proteins, drugs and peptides. Among these molecules, only peptides were retained for further analysis. The peptides with class I canonical proline-rich motifs were recognized with 'stringr' package (<https://cran.r-project.org/web/packages/stringr/>) in the statistical environment R. The consensus sequence for class I proline-rich motifs was denoted as +xΦPxΦP, where x represented any naturally occurring amino acid, Φ represented a hydrophobic amino acid (i.e., alanine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, valine or glycine) and + represented a positively charged amino acid (normally arginine or lysine). The information of amino acid properties (i.e., hydrophobic and positively charged) was queried in TP53 Database of International Agency for Research on Cancer (<http://p53.iarc.fr/AAProperties.aspx>).

### Prediction of peptide-target interactions

The prototype peptides were submitted to MoDPepInt (Modular Domain Peptide Interaction, <http://modpepint.informatik.uni-freiburg.de/>), an interactive web server with multiple bioinformatics tools for the prediction of domain-peptide binding. In this study, we utilized the SH3PepInt tool of MoDPepInt server, which was based on efficient and sophisticated graph kernel technique and did not require pre-alignment of the peptides. Trained on published peptide-protein interaction data with support vector machines, SH3PepInt can predict the SH3 domains potentially interacting with the peptide of interest. The query sequences of all prototype peptides were supplied in a FASTA format. Then, the output results, as a downloadable table, presented the potential interactions between prototype peptide and 69 human

SH3 domains (including c-Src protein). The specificity/promiscuity of domain-peptide binding was measured by the number of SH3 domains predicted to interact with a certain prototype peptide. The more domains except for c-Src a prototype peptide was predicted to bind to, the lower binding specificity (i.e., the higher binding promiscuity) it indicated.

### Domain-peptide docking

The interaction between prototype peptide and target SH3 domain was modeled using the CABS-dock web server (<http://biocomp.chem.uw.edu.pl/CABSdock>), a highly efficient tool for the flexible docking of peptides to proteins. Peptide sequence was entered in single-letter amino acid code. Protein domain structure was provided as Protein Data Bank (PDB) code along with the chain identifier. Then, possible structures of the peptide were generated and randomly placed on the surface of the target domain. Within the set of resulting docking models, the top 10 selected models with the highest accuracy were presented by CABS-dock in detail. The accuracy of docking models was assessed with the root-mean-square deviation (RMSD) between predicted and experimental peptide structures, i.e., lower RMSD value indicated higher quality of prediction.

## RESULT AND DISCUSSION

### The association between amino acid sequences and domain-peptide binding

To investigate the correlation between amino acid sequence and promiscuous peptide-target binding, we collected a set of prototype peptides for analysis (see Materials and Methods). First of all, we searched the Molecular INTeraction (MINT) database,<sup>24</sup> so as to obtain a list of molecules that have been experimentally validated to interact with the SH3 domain of human c-Src protein. These molecules included proteins, drugs and peptides, among which only 10 peptides with class I canonical proline-rich motifs were retained for analysis. Then, the 10 prototype peptides were uploaded to the MoDPepInt (Modular Domain Peptide Interaction)<sup>25</sup> web server. SH3PepInt was a tool provided by MoDPepInt,<sup>26</sup> which used graph kernel approach to perform alignment-free prediction of domain-peptide interaction. Therefore, by querying the amino acid sequence, we were enabled to identify SH3 domains potentially interacting with the prototype peptides (see Materials and Methods).

The output results showed that all prototype peptides were predicted to bind to the SH3 domain of c-Src protein, suggesting the consistency between SH3PepInt

models and experiments. Besides c-Src, other proteins with SH3 domain were also predicted to interact with some of the prototype peptides. And the number of such promiscuous interactions varied greatly between peptides (Table 1). While some peptides only interacted with a few proteins (e.g., peptide 8 and peptide 5), some other peptides could bind to up to 25 proteins (e.g., peptide 6 and peptide 2). Interestingly, we noticed that those peptides showing relatively higher binding specificity tended to (1) have leucine (with symbol L) as the 5<sup>th</sup> residue of proline-rich motif (e.g., peptides No. 8, No. 5 and No. 4), and (2) have proline (with symbol P) as the 6<sup>th</sup> residue of proline-rich motif (e.g., peptides No. 8 and No. 4). The above patterns (Figure 1) indicated that amino acid sequence of proline-rich motif may be correlated to the degree of promiscuity.

### Optimization of amino acid sequences and improvement of binding specificity

In view of the correlation between the amino acid sequence in proline-rich motif and the promiscuity of domain-peptide binding, we hypothesized that certain amino acid residues of the prototype peptides could be purposely modified to improve binding specificity towards c-Src protein. We tested two parallel schemes of optimization. First, we substituted leucine for the 5<sup>th</sup> residue of proline-rich motif. Second, we replaced the 6<sup>th</sup> residue of proline-rich motif with proline. Except for prototype peptides No. 8, No. 5 and No. 4 that originally had leucine as the 5<sup>th</sup> residue of proline-rich motif, the first scheme applied to the other 7 prototype peptides. For 6 out of these 7 peptides, the number of promiscuous interactions declined after optimization (Figure 2, Table S1). The second scheme applied to 8 prototype peptides, except for peptides No. 8 and No. 4. A decline in the number of promiscuous interactions was observed in 7 out of these 8 peptides (Figure 3, Table S2).

Such observations demonstrated that the 5<sup>th</sup> and the 6<sup>th</sup> residues of proline-rich motif can largely influence on peptide-target binding. Therefore, we combined the above two schemes and modified both the 5<sup>th</sup> and the 6<sup>th</sup> residues. A side-by-side comparison was made between the original and the modified peptides (Figure 4, Table S3). One-tailed paired t-test indicated that the overall level of promiscuity of prototype peptides was significantly reduced after modification (P-value = 0.0075). These findings came together to suggest that c-Src binding specificity of peptide ligands can be greatly determined by the amino acid types at key positions in the proline-rich motif, which provided a unique oppor-

tunity for sequence modification and rational design of therapeutic peptides.

### Validating modified peptides with domain-peptide docking

Molecular docking approach has been widely adopted to understand ligand-protein interaction.<sup>27</sup> So the above results were validated by reproducing conformation of docked peptide in crystal structure of target SH3 domain. As a typical example, peptide No. 5 was originally predicted to bind to the SH3 domain of hematopoietic cell kinase (HCK). According to the calculation of ModPepInt, the probability of such binding significantly decreased after modifying the 5<sup>th</sup> and the 6<sup>th</sup> residues of proline-rich motif. Therefore, the original and modified sequences of peptide No. 5, along with the 3D structure of HCK SH3 domain (PDB ID: 4HCK), were entered into the CABS-dock web server.<sup>28</sup> CABS-dock performed highly efficient and flexible docking simulation to search for possible binding conformations. The accuracy of docking models was measured by the root-mean-square deviation (RMSD). The top 10 models with the highest accuracy (i.e., the lowest RMSD value) were selected as final results (see Materials and Methods). It was shown that the original and modified peptides had different binding positions and orientations on the surface of HCK SH3 domain (Figure 5A and 5B). Regarding the top 10 best models (Figure 5C), the modified peptide exhibited significantly higher RMSD, namely lower quality of binding, than the original peptide (P-value =  $5.99 \times 10^{-5}$ ). These results supported the prediction that modifying both the 5<sup>th</sup> and the 6<sup>th</sup> residues of proline-rich motif may prevent peptide No. 5 from binding to HCK SH3 domain, thus lowering the promiscuity of peptide No. 5.

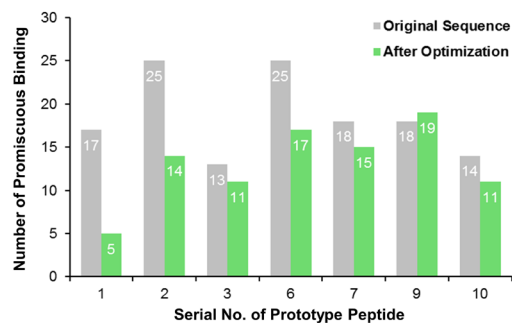
### Implication of the current results

Peptides are ideal molecules for drug development because of low molecular weight and good cellular uptake. Over these years, the application of peptides is rapidly growing in a variety of therapeutic areas, with the number of peptide drugs under clinical trials increasing steadily. The number has climbed from 1.2 per year in the 1970s to 16.8 per year in the 2000s.<sup>6</sup> Currently, more than 60 peptide drugs have been approved for marketing and several hundreds of novel therapeutic peptides are under preclinical or clinical development.<sup>5</sup> The key contributor to the success of these peptides is their potent and specific, yet safe, modes of action.

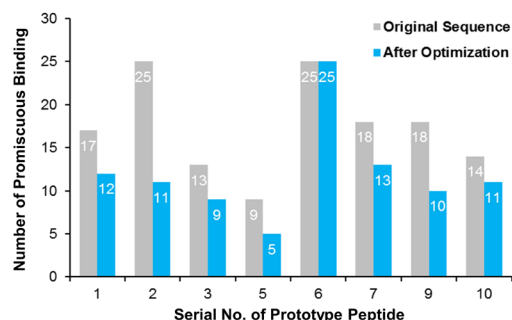
As a class of promising anticancer agents, peptides bind to key proteins in tumor cells with low toxicity to normal tissues.<sup>29</sup> This tumor-targeting ability of peptides is

Serial No.	Proline-Rich Motif	Number of Predicted Interactions
8	R A L P L P P	3
5	R P L P L L P	9
4	R P L P L P P	10
3	R P L P A L P	13
10	R P L P P V P	14
1	R P L P P V P	17
9	R R L P A L P	18
7	R A L P P L P	18
6	R P L P P L P	25
2	R P L P R L P	25

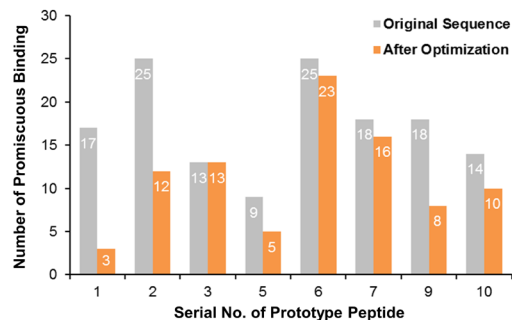
**Figure 1: The association between the 5th and the 6th residues of proline-rich motif and binding specificity of prototype peptides.**



**Figure 2: Most peptides, except for peptide No. 9, exhibited better binding specificity after optimizing the 5th residue of proline-rich motif.**

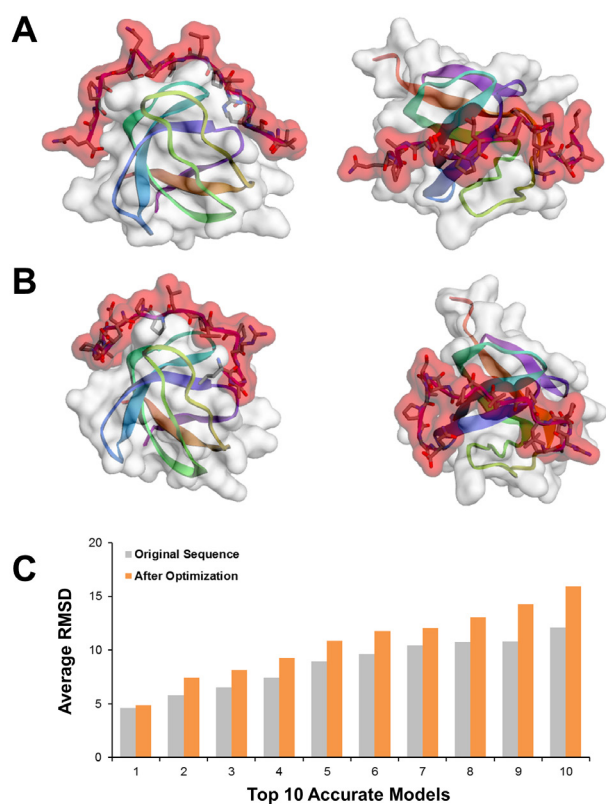


**Figure 3: Most peptides, except for peptide No. 6, exhibited better binding specificity after optimizing the 6th residue of proline-rich motif.**



**Figure 4: Most peptides, except for peptide No. 3, exhibited better binding specificity after optimizing the 5th and the 6th residues of proline-rich motif.**





**Figure 5: Domain-peptide binding validated by molecular docking. (A) The best fitted binding model of original peptide No. 5 and HCK SH3 domain. (B) The best fitted binding model of modified peptide No. 5 and HCK SH3 domain. (C) The docking RMSD values of original and modified sequences of peptide No. 5.**

determined by their complementary binding to a variety of key proteins in tumor cells. For instance, the positively charged arginine and lysine in peptide can selectively form hydrogen bonds with the negatively charged components of target protein domains.<sup>30</sup> Nonetheless, these complementary properties may not always guarantee perfect specificity of peptide-target binding. For example, SH3 domains have become a well-known and promising anti-cancer target of peptide ligands with proline-rich motifs. But SH3 domains are one of the most abundant domain families encoded in eukaryotic genomes. So far, at least 300 SH3 domains have been identified in the human proteome.<sup>31</sup> As a result of highly conserved amino acid sequence and structure of different SH3 domains, one proline-rich peptide can be recognized by multiple proteins with SH3 domain. The promiscuous nature of SH3 domains in binding to proline-rich peptides may lead to unexpected adverse reactions due to impact on various cell-signaling pathways and biological functions.<sup>32</sup> So it is an open challenge in biotechnology to design peptide ligands with a high specificity of binding to the SH3 domain of designated

target (e.g., c-Src) and without interacting with other proteins.

In the present study on c-Src protein, we demonstrated that subtle alterations in the amino acid sequence could significantly change the specificity of binding to the target SH3 domain. We primarily searched MINT database for a set of prototype peptides, which have been experimentally validated for binding to the SH3 domain of c-Src protein. Relying on the SH3PepInt tool, we predicted the interaction between the peptides and various SH3 domains. Then, by comparing the amino acid sequence of prototype peptides with relatively high and low specificity of binding to c-Src protein, we found that leucine as the 5<sup>th</sup> residue and proline as the 6<sup>th</sup> residue of proline-rich motif could render prototype peptide a reduced promiscuity. In the last step, we purposely modified the 5<sup>th</sup> and the 6<sup>th</sup> residues of relevant peptides, which led to significantly better specificity of peptide-target binding. The above results showed that promiscuous binding of peptide molecule can be effectively ameliorated by rational design.

Based on various public crystallographic data,<sup>33-38</sup> the SH3 domain has been thoroughly researched and broadly recognized as one of the best available systems for the examination of ligand-protein interactions. For example, Larson *et al.* constructed a diverse alignment of SH3 domain sequences.<sup>39</sup> By analyzing conservative structural features within this alignment, several positions in the domain were identified for mediating the peptide-binding function. The existence of such key positions implied that the recognition of peptide ligands might be systematically explained,<sup>40,41</sup> which effectively facilitated experiments of rational ligand designing. Alexandropoulos *et al.* found specific proline-rich sequences prone to bind to Fyn, Lyn, and Hck SH3 domains, respectively.<sup>20</sup> Pisabarro *et al.* designed mutations in peptide, so that the affinity for Abl SH3 domain was selectively increased by 20-fold.<sup>42</sup> And Ferguson *et al.* used phage display for ligand optimization and obtained a peptide 1000-fold increased affinity for the SEM-5 SH3 domain.<sup>43</sup> Here we provided a novel clue as to increasing the specificity of binding to SH3 domain. The current results will inspire more subsequent work on optimization of amino acid sequence, so as to improve the safety of therapeutic peptides.

Despite of useful information provided by the present study, more efforts are required to address some limitations of the current results. First, the basis of this study was the prototype peptides retrieved from MINT database, with which we found the important role played by the 5<sup>th</sup> and the 6<sup>th</sup> residues of proline-rich motif. However, the number of available prototype remained

relatively low for higher power of statistical tests. More work, such as literature searching and text mining, will be required to collect more information about prototype peptides proved to bind to the SH3 domain of human c-Src protein. Second, with a larger set of prototype peptides, more patterns of amino acid sequences related to peptide-target binding specificity can be explored. Apart from the 5<sup>th</sup> and the 6<sup>th</sup> residue, other residues in or flanking the proline-rich motif should also be investigated using our method. Third, the current results of peptide-target binding were mainly based on *in silico* prediction, which often led to alternate results in reality. An inconsistent track record of docking-based ligand discovery raised the concern about the reliability. For instance, it was reported that fewer than 20% of docking screens were eventually supported by crystal structure identified with experiments.<sup>44</sup> In particular, those small molecules, such as peptides composed of a few amino acids, are found to be docked less reliably.<sup>45</sup> Therefore, besides calculating the binding orientations and free energies, the optimized peptides need to be artificially synthesized and experimentally assayed for binding affinity against targets of interest.<sup>46</sup>

## CONCLUSION

Taken together, by analyzing the promiscuity of peptide-target binding, we corroborated the ability of the SH3 domain of c-Src protein to discern subtle differences in the amino acid sequence of peptide ligands. Based on that, we virtually optimized the proline-rich motifs of relevant peptides and improved the binding specificity. Further computational and experimental efforts will be required to validate and expand current results, which can be applied to the rational design of peptide-based anticancer drugs.

## ACKNOWLEDGEMENT

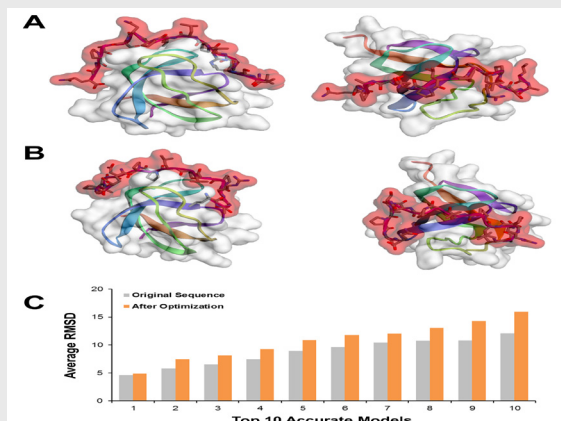
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## Pictorial Abstract



## SUMMARY

- Promiscuous binding between anticancer peptide and multiple off-targets is a crucial challenge in biotechnology.
- Here we explored peptide ligands in MINT database and utilized SH3PepInt tool for in silico peptide-target binding.
- By optimizing the amino acid sequence of peptide ligands, we rendered high specificity towards the SH3 domain of c-Src target.
- These findings provided a unique opportunity for rational design of therapeutic peptides.

## About Authors



**Yanrong Ren:** Professor at Chongqing University of Education, whose major interest is therapeutic peptide design and drug safety assessment.



**Qiang Wang:** Expert on structures of therapeutic peptides at Chongqing University of Education.



**Xiaobo Li:** Expert on human resource at Chongqing University of Education.

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