Break CDK2/Cyclin E1 Interface Allosterically with Small Peptides

Hao Chen², Yunjie Zhao², Haotian Li¹, Dongyan Zhang¹, Yanzhao Huang¹, Qi Shen³, Rachel Van Duyne⁴,⁵, Fatah Kashanchi³, Chen Zeng¹,², Shi Yong Liu¹,∗

¹Department of Physics, Huazhong University of Science and Technology, Wuhan, Hubei, China, ²Department of Physics, The George Washington University, Washington, D. C., United States of America, ³BNLMS, Center for Quantitative Biology, Peking University, Beijing, China, ⁴George Mason University, National Center for Biodefense & Infectious Diseases, Manassas, Virginia, United States of America, ⁵The George Washington University Medical Center, Department of Microbiology, Immunology, and Tropical Medicine, Washington, D. C., United States of America

Abstract

Most inhibitors of Cyclin-dependent kinase 2 (CDK2) target its ATP-binding pocket. It is difficult, however, to use this pocket to design very specific inhibitors because this catalytic pocket is highly conserved in the protein family of CDKs. Here we report some short peptides targeting a noncatalytic pocket near the interface of the CDK2/Cyclin complex. Docking and molecular dynamics simulations were used to select the peptides, and detailed dynamical network analysis revealed that these peptides weaken the complex formation via allosteric interactions. Our experiments showed that upon binding to the noncatalytic pocket, these peptides break the CDK2/Cyclin complex partially and diminish its kinase activity in vitro. The binding affinity of these peptides measured by Surface Plasmon Resonance can reach as low as 0.5 μM.

Citation: Chen H, Zhao Y, Li H, Zhang D, Huang Y, et al. (2014) Break CDK2/Cyclin E1 Interface Allosterically with Small Peptides. PLoS ONE 9(10): e109154. doi:10.1371/journal.pone.0109154

Editor: Chandra Verma, Bioinformatics Institute, Singapore

Received March 31, 2014; Accepted September 3, 2014; Published October 7, 2014

Copyright: © 2014 Chen et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: SYL is supported by the National Natural Science Foundation of China [31100522] and the National High Technology Research and Development Program of China [2012AA020402]; and Specialized Research Fund for the Doctoral Program of Higher Education [20110142120038]; and the Fundamental Research Funds for the Central Universities, HUST, 2013QN019. YZH is supported by NSFC [11174093]. FK is supported by George Mason University funds and NIH grant AI043894. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Co-author Dr. Fatah Kashanchi is a PLOS ONE Editorial Board member. This does not alter the authors’ adherence to PLOS ONE Editorial policies and criteria.

* Email: lushyong@gmail.com

These authors contributed equally to this work.

Introduction

Protein-protein interactions play critical roles in many biological processes, and therefore may become the targets for drug design [1–4]. In this approach, functional proteins [5,6] and small inhibitors [7–10] are successfully designed by grafting, docking and high-throughput NMR screening.

The main strategies for designing effective peptide inhibitors fall into three categories: 1) Cutting peptide sequence [11,12] from native protein-protein interface; 2) Phage display [13–16]; and 3) Computational design, including docking [17–20], molecular dynamics simulation [21–23], normal mode analysis [24], template-based searching [25] and sequence design [26–28]. Peptide inhibitors derived from natural protein-protein interfaces are found to disrupt protein-protein interaction [11,12,29,30]. For example, Schon et al. [11] cut parts of P53 (sequence 15–29) and tested their binding with MDM2. They found that the peptide PMD2 (ETFSDLWKLL, Kᵢᵣ = 46 nM) bound MDM2 stronger than peptide PMD1 (SQETFSDLWKILPEN, Kᵢᵣ = 580 nM). However, sometimes this cutting strategy does not work. For example, Gondeau et al. [12] found the peptide C4 derived from Cyclin A with HCD₉₀ = 1.8 μM does not disrupt CDK2/Cyclin A complex. Besides this “cutting” strategy, Hu et al. [13] found a peptide pDI (LTFEHYWAQLTS) with the ability to disrupt P53-MDM2 interaction by phage display. And then, using the same technology, Pazzier et al. [16] found a novel peptide PMI (TSFAEYWNLLISP, Kᵢᵣ = 3.4 nM) bound with MDM2 stronger than the wild p53 peptide (ETFSDLWKLLPE). Later, Li et al. [15] reported that systematic alanine scanning on PMI resulted in a mutant NIA that is the strongest binder with MDM2 (Kᵢᵣ = 490 pM). Phage display is a useful method for designing peptide inhibitor of protein-protein interaction, but it is limited to the size of the random library. It cannot cover the entire sequence space. Though the alanine scanning could make up for a number of shortcomings of the phage display technology, the optimized peptide sequence may still not be found without the help of theoretical computational method.

Structure-based computational design of inhibitor has been studied for many years. Protein-peptide docking is one such method [31,32]. London et al. [19] cut the peptide from protein-protein interface in protein-protein docking benchmark 3.0 and CAPRI targets, and docked the peptide to the protein by FlexPepDock [17]. They showed that the derived peptides contributed dominantly to binding free energy, however, it is necessary to validate experimentally if such peptides actually bind their targets. In 2008, Fu et al. [24] successfully designed a 26-mer peptide by modeling backbone flexibility with NMA (normal mode analysis) from Bcl-XL/Bim-BH3 complex structure. 8 of their 17 designed peptides are validated experimentally to bind well with...
Peptide selection according to frequency analysis

We have analyzed the structural occurrence probabilities from the top 1000 protein-peptide decoys with lowest energy calculated by AutoDock. The results show that the top 3 occurrence number of SET2_06, SET3_07, SET3_09 are 528, 110, 92, respectively. So the protein conformations SET2_06, SET3_07 and SET3_09 are favorite conformations to be used to select peptides from top peptide list. Finally, 5 peptides were selected, which are RAALF, RAALQ, RAALQ, FAALA, and GAALY, respectively (see Table 1).

Peptide selection according to binding energy calculation

The binding energy describes the strength of the intermolecular interactions. The ranking results show that the peptides of RAALW, RAALQ, GAALY, PAALA, and RAALM are the top 5 peptides with lowest AutoDock binding energy.

Peptide selection according to a knowledge-based potential

The Pmfscore [37] has been used successfully for protein-protein binding energy prediction. Therefore, we apply this knowledge-based potential to re-rank the protein-peptide docking decoy to get more candidate structures. According to this new ranking result, top 3 peptides are KAASE, DAAAT, YAAL, YAALQ and TAAL, respectively.

Considering all results of the three methods above, 13 peptides were finally selected for further MD simulations as shown in Table 2.

MD simulations

There may be some conformational changes of CDK2/Cyclin complex induced by peptide binding that may render the conformations obtained from docking simulations unstable since the protein is held rigid in the simulations. In order to observe the dynamical behavior, we have done MD simulations using two different sets of Van der Waals cut-off parameters to analyze the stabilities of peptides and the correlated motions of the CDK2/Cyclin interface.

First, we used a sensitive cut-off 14 Å to analyze the stabilities of the 13 CDK2-peptides (shown in Table 2). As a control, we also checked the stabilities of the peptide-CDK2 complexes of TAALD, TAALQ, and LAALS. The three peptides have been investigated computationally and experimentally in previous work [20,38,39]. TAALD and LAALS as inhibitor are found experimentally to be effective; TAALD, while having the highest predicted binding affinity, however, does not show any inhibitory effect [38]. After 5 ns MD simulations, the conformations of CDK2-peptide complex for LAALS, TAALS, DAAAT, YAALQ, RAALW, RAALQ, FAALA and GAALY, respectively (see peptide list. Finally, 5 peptides were selected, which are RAALF, RAALQ, GAALY, PAALA, and RAALM were stable with the peptides remaining in the binding pockets. Peptide TAALD was less stable. Moreover, the peptides RAALF, YAAL, and TAAL were moving away. The MD simulations of all CDK2-peptide decoys are summarized in Table 1. For example, TAALS stayed in the binding pocket (Figure 1), however, RAALF moved away from the binding pocket (Figure 2). Finally, we selected six peptides based on these MD simulation results as summarized in Table 3.

It is known that the ATP-binding sites of CDK2 are modified and regulated by Cyclin binding. A stable interface of CDK2/Cyclin complex is required for ATP binding and thus its enzymatic activity. In order to analyze the dynamical motions of the CDK2/Cyclin interface, we applied a method of dynamical
correlation analysis to the CDK2/Cyclin interface based on the MD simulations with Van der Waals cut-off 10 Å.

If any two heavy atoms of two residues were less than 4.5 Å apart for 75% of the snapshots taken at the interval of 100 ps during 20 ns trajectories, the two residues were said to be correlated and the correlation value was computed, otherwise the correlation value was set to zero. If the residues move in the same (opposite) direction in most snapshots, the motions are defined as correlated (anti-correlated) with positive (negative) correlation values. A correlation value close to zero indicates uncorrelated motion. We focused on the residues at the CDK2/Cyclin interface. The average correlation value of the interface residues

### Table 1. MD simulations of CDK2-peptide docking decoys.

| RANK | Protein-peptide models | AutoDock Energy (Kcal/mol) | Selected | Methods | MD simulation |
|------|-------------------------|-----------------------------|----------|---------|---------------|
| 49   | SET2_RAALF              | -12.84                      | RAALF    | Frequency | Swam away |
| 23   | SET2_RAALG              | -13.11                      | RAALG    | Frequency | Stay        |
| 3    | SET3_RAALQ              | -14.67                      | RAALQ    | Frequency | Blowing up |
| 16   | SET2_FAAA               | -13.3                       | FAAA     | Frequency | Stay        |
| 4    | SET2_GAALY              | -14.33                      | GAALY    | Frequency | Stay        |

| RANK | Protein-peptide models | Pmfscore (Kcal/mol) | Selected |
|------|-------------------------|---------------------|----------|
| 7483 | SET2_KAALE              | -11.34              | KAALE    |
| 26490| SET2_DAAALT             | -10.37              | DAALT    |
| 73048| SET1_YAALE              | -10.34              | YAALE    |
| 73571| SET1_YAALQ              | -9.99               | YAALQ    |
| 40624| SET2_TAALL              | -9.87               | TAALL    |

| RANK | Protein-peptide models | AutoDock Energy (Kcal/mol) | Selected |
|------|-------------------------|-----------------------------|----------|
| 1    | SET2_RAALW              | -15.89                      | RAALW    |
| 3    | SET3_RAALQ              | -14.67                      | RAALQ    |
| 4    | SET2_GAALY              | -14.33                      | GAALY    |
| 5    | SET2_PAALA              | -13.86                      | PAALA    |
| 6    | SET3_RAALM              | -13.82                      | RAALM    |

| CONTROL | Protein-peptide models | AutoDock Energy (Kcal/mol) | Selected |
|---------|-------------------------|-----------------------------|----------|
|         | SET2_TAALS              | -11.28                      | Stay     |
|         | SET2_LAALS              | -10.98                      | Stay     |
|         | SET2_TAALD              | -11.58                      | Stay     |

RANK: The rank of the protein-peptide model sorted by AutoDock binding energy. Methods: Frequency, Pmfscore and AutoDock (details see table 2). SET1, SET2 and SET3 have been defined as CDK2 with different T-loop conformation (see text).

CONTROL: The previous experimental result [20] shows that TAALS and LAALS bound to unphosphorylated form of CDK2, but TAALD not.

Stay: That means that the peptide is staying in the pocket during the MD simulation.

doi:10.1371/journal.pone.0109154.t001

### Table 2. Designed peptides based on three scoring methods.

| Frequency1 | AutoDock2 | Pmfscore3 |
|------------|-----------|-----------|
| FAAA       | RAALM     | KAALE     |
| RAALF      | RAALQ     | DAALT     |
| RAALE      | RAALW     | YAALE     |
| RAALQ      | GAALY     | YAALQ     |
| GAALY      | PAALA     | TAALL     |

1 Frequency: Top 5 was selected according to the number of the peptide sequence in the top 1000 lowest energy docking decoys.
2 AutoDock: Top 5 was selected according to the calculated binding energy by AutoDock.
3 Pmfscore is a statistical potential developed by Jiang et al. [37]. Top 5 was selected according to the Pmfscore.

doi:10.1371/journal.pone.0109154.t002
in the absence of peptide is 0.38. Figure 3 shows the correlation analysis results of the six selected peptides. The interface regions displaying high degree of correlation are marked in white rectangles. The correlation values for the cases of DAALT, YAALQ, RAALG, FAALA, KAALG, and RAALW are 0.31, 0.27, 0.44, 0.39, 0.33, 0.38, respectively. The correlation values reflect the coupled motions between CDK2 and Cyclin in the interface regions, and thus larger correlation values indicate more stable interface.

Therefore, the order of stability of the CDK2/Cyclin interface is YAALQ, DAALT, KAALE, RAALW, FAALA, RAALG. These computational results suggest that the interface regions become less stable if the peptides YAALQ and DAALT bind to CDK2. This prediction is consistent with the experimental results described in the next section. While longer MD simulations would undoubtedly provide a more pronounced correlation map, the short simulations performed here could nonetheless provide an estimate on which peptides may break up the CDK2/Cyclin interface.

**Dissociation of CDK2/Cyclin E in vitro in the presence of six designed peptides**

To visualize and verify the dissociation of CDK2/Cyclin complex by each of the six designed peptides, immunoprecipitations against Cyclin and IgG, with the latter being a negative control for nonspecific background signal, were performed and followed by Western blot for CDK2 as shown in Figure 4(A). Comparing the band intensity on Lane 2 for Cyclin pulldown to that on Lane 3 for IgG pulldown, we clearly observed a weaker intensity indicating the dissociation of CDK2 from the CDK2/Cyclin complex in the presence of peptide DAALT. Upon closer inspection, the left band (lane 4) of YAALQ appears slightly wider and darker than the right band (lane 5) indicating a weak complex dissociation. However, additional evidence is needed to differentiate peptide YAALQ from other four peptides (lanes 6–13) that failed to break up the complex. This is discussed below by the kinase activity experiment.

Figure 4(B) further illustrates how the dissociation of CDK2 inhibits the kinase activity of CDK2/Cyclin complex. Here an immunoprecipitation of the CDK2/Cyclin complex was performed as previously described, followed by the kinase reaction with H1 histone being added as the substrate. The levels of phosphorylation of H1 are shown in the presence of the six designed peptides. Again, the two peptides DAALT and YAALQ exhibit a clear loss of kinase activity. To figure out how strong these two effective peptides bind to CDK2, we measured their binding affinities via Surface Plasmon Resonance as described below.

---

**Figure 1. MD simulation of TAALS-CDK2 docking decoy.** Left: the docked TAALS and CDK2 complex structure, as an initial structure for MD simulation; Right, after 5 ns MD simulation, the TAALS and CDK2 complex structure is shown. The green represent peptide TAALS, and the purple balls are atoms from the key residues: K178, Y180, and the red is the T-loop of CDK2. The MD simulation shows that after 5 ns, the peptide TAALS (Green) induced the conformational change of the CDK2 and moved to the gap between purple and red.

doi:10.1371/journal.pone.0109154.g001

**Figure 2. MD simulation of RAALF-CDK2 docking decoy.** Left: the docked RAALF and CDK2 complex structure, as an initial structure for MD simulation; Right, after 5 ns MD simulation, the RAALF and CDK2 complex structure is shown. The green represent peptide RAALF, and the purple balls are atoms from the key residues: K178, Y180, and the red is the T-loop of CDK2. The MD simulation shows that after 5 ns, the peptide RAALF (Green) swam away from the key pocket sites of CDK2.

doi:10.1371/journal.pone.0109154.g002
### Table 3. Selection based on MD simulation results.

| RANK | Protein-peptide models | AutoDock Energy (Kcal/mol) | Selected peptide | Methods | MD simulation |
|------|-------------------------|-----------------------------|------------------|---------|---------------|
| 23   | SET2_RAALG              | -13.11                      | RAALG            | Frequency | Stay[^1]     |
| 16   | SET2_FAALA              | -13.30                      | FAALA            | Frequency | Stay between key residues and T-loop[^2] |
| 7483 | SET2_KAALE              | -9.35(-11.34)^[^3]          | KAALE            | Pmfscore | Stay between key residues and T-loop[^2] |
| 26490| SET2_DAALT              | -7.90(-10.37)^[^3]          | DAALT            | Pmfscore | Stay[^1]     |
| 73571| SET1_YAALQ              | -6.05(-9.99)^[^3]           | YAALQ            | Pmfscore | Stay[^1]     |
| 1    | SET2_RAALW              | -15.89                      | RAALW            | AutoDock | Stay[^1]     |

RANK: The rank of the protein-peptide model sorted by AutoDock binding energy. Methods: Frequency, Pmfscore and AutoDock (details see table 2). SET1 and SET2 have been defined as CDK2 with different T-loop conformation (see text).[^1]Stay: That means that the peptide is staying in the pocket during the MD simulation.[^2]Key residue and T-loop: Key residues are Y180, K178 of CDK2.[^3]The value in brackets is calculated by Pmfscore.

doi:10.1371/journal.pone.0109154.t003

---

**Figure 3.** Correlation analysis of the motion during a 20-ns MD simulation of the CDK2/Cyclin/peptide complex structures. Monomers with highly (anti)correlated motion are orange or red (blue). Interface regions displaying high degree of (anti)correlation are marked in white rectangles.

doi:10.1371/journal.pone.0109154.g003
Peptide binding measurement by a Surface Plasmon Resonance (SPR) assay

Two peptides (DAALT and YAALQ) and two positive controls (TAALS and LAALS) are tested in the same condition with CDK2 by T200 (all binding data see Table 4).

The response curves of various analyte concentrations were globally fitted to the two-step binding model described by the following equation [40],

\[
A + B \rightleftharpoons \frac{K_{a1}}{K_{d1}} \frac{[AB]^*}{K_{a2}} \rightleftharpoons AB
\]

Where the equilibrium constants of each binding step are \(K_1 = \frac{K_{a1}}{K_{d1}}\) and \(K_2 = \frac{K_{a2}}{K_{d2}}\), and the overall equilibrium binding constant is calculated as \(K_A = K_1(1 + K_2)\) and \(K_D = \frac{1}{K_A}\).

In this model, the analyte (A) binds to the ligand (B) to form an initial complex [AB]* and then undergoes subsequent binding or conformational change to form a more stable complex AB. Data were fitted globally by using the standard two state models provided by Biacore T200 Software v2.0. The binding affinities \(K_D\) to CDK2 for peptides DAALT and YAALQ were measured to be 0.47 \(\mu\)M and 98 \(\mu\)M, respectively. After ATP with a concentration of 60 \(\mu\)M was added, the binding affinities \(K_D\) for peptides DAALT and YAALQ were changed to 37 \(\mu\)M and 61 \(\mu\)M, respectively. Given the large uncertainty of the fitting in SPR kinetic assays, we consider a 10-fold change in binding affinity not very significant. For the three peptides, TAALS, LAALS and YAALQ, the changes in \(K_D\) in the presence/absence of ATP are all within 10-fold. Thus, ATP does not have a significant effect on the binding of these peptides to CDK2.

Therefore, we conclude that YAALQ does not compete directly with ATP for the ATP binding pocket. For DAALT, however, a larger-than-10-fold decrease in the presence of ATP was observed. While it is possible for DAALT to compete directly with ATP by occupying the ATP binding pocket on CDK2, it is more likely that it competes indirectly with ATP. For example, binding of ATP to

Figure 4. Dissociation of CDK2/Cyclin E in the presence of designed peptides. A) C81 fractionated cell extracts containing cdk2/Cyclin E complex were incubated with \(\gamma\)-Cyclin E antibody in the presence of six designed peptides at 10 \(\mu\)M concentration. Following immunoprecipitation of Cyclin E, Western blot for CDK2 was shown here. \(\gamma\)-IgG is included as a negative control. B) Immunoprecipitated Cyclin E samples in the presence of peptides were assessed for kinase activity. Histone H1 (1 \(\mu\)g/reaction) was added to each reaction tube along with 2 \(\mu\)l of \((\gamma\-32P)\) ATP (3000 Ci/\(\mu\)mol). Reactions were incubated at 37°C for 30 min and stopped by the addition Laemmli buffer. The samples were separated on a 4–20% Tris–Glycine gel. Samples were ran on a gel, dried, and exposed to a PhosphorImager cassette and analyzed using Molecular Dynamic’s ImageQuant Software.

doi:10.1371/journal.pone.0109154.g004

Table 4. SPR-derived binding affinities of CDK2 for four peptides with and without 60 \(\mu\)M ATP.

| Peptides | \(K_{a1}(1/Ms)\) | \(K_{d1}(1/s)\) | \(K_{a2}(1/Ms)\) | \(K_{d2}(1/s)\) | \(K_D(\mu\)M\) |
|----------|-----------------|-----------------|-----------------|-----------------|-----------------|
| TAALS    | 11.3 ± 0.1      | 26.0 ± 0.1 E-3  | 11.1 ± 0.2 E-3  | 6.8 ± 2.7 E-6   | 1.4 ± 0.6 E-6   |
| TALLS*   | 3.7 ± 0.1       | 28.2 ± 1.4 E-3  | 9.1 ± 0.2 E-3   | 3.8 ± 1.0 E-6   | 3.3 ± 0.8 E-6   |
| LAALS    | 498.0 ± 8.8     | 11.9 ± 0.2 E-3  | 5.8 ± 2.0 E-5   | 2.9 ± 0.8 E-5   | 8.0 ± 2.9 E-6   |
| LAALS*   | 237.4 ± 6.9     | 51.8 ± 2.3 E-3  | 7.9 ± 0.5 E-3   | 3.1 ± 0.3 E-3   | 6.1 ± 0.6 E-5   |
| DAALT    | 434.1 ± 6.6     | 23.2 ± 0.4 E-3  | 14.1 ± 0.4 E-4  | 1.3 ± 0.6 E-5   | 4.7 ± 2.0 E-7   |
| DAALT*   | 612.9 ± 13.0    | 101.2 ± 4.3 E-3 | 34.6 ± 1.0 E-3  | 10.1 ± 0.1 E-3  | 3.7 ± 0.3 E-5   |
| YAALQ    | 165.7 ± 4.0     | 84.0 ± 2.7 E-3  | 9.0 ± 0.3 E-3   | 2.2 ± 0.1 E-3   | 9.8 ± 0.7 E-5   |
| YAALQ*   | 100.9 ± 1.5     | 53.9 ± 1.4 E-3  | 12.3 ± 0.3 E-3  | 1.6 ± 0.1 E-3   | 6.1 ± 0.3 E-5   |

*With 60 \(\mu\)M ATP.

doi:10.1371/journal.pone.0109154.t004
CDK2 stabilizes a certain CDK2 conformation that is less favorable for DAALT binding. The detailed binding mode between DAALT and CDK2 needs to be resolved by other means beyond SPR experiments. From Figure 5, we can see that the peptide TAALS used a binding mechanism different from that of three other peptides. More precisely, upon injection of peptide, the response curve for TAALS shows a slower increase before reaching a steady value or a horizontal curve as well as a slower decrease after the peak than those for the other three peptides where a sharp jump and a dramatic drop are seen. This indicates that the mechanism of binding of TAALS represents slow binding and slow dissociation. On the contrary, LAALS displays fast binding and fast dissociation, similar to the other two peptides, DAALT and YAALQ. The SPR results confirmed a direct interaction between the peptides and CDK2. The two-state model was a better fit, suggesting that there are two different states in peptide-CDK2 binding processes. We hypothesize that the second state is the induced conformation of CDK2 by peptide binding. This is consistent with our MD simulations. The binding affinities predicted by computational docking simulations and measured by SPR between peptides and CDK2 fall into the same range (0.1 μM—40 μM). Compared to other peptides, the association and dissociation processes of TAALS are very slow.

Discussion

We have performed a series of computational simulations to design and select the effective peptide inhibitors against CDK2/ Cyclin complex. In the structural modeling and docking selection steps, all the T-loops of the selected CDK2 were built by Rosetta loop modeling algorithm from inactive (1E1X [41]) and active (1FIN [42], Chain A) conformations. These results suggest that Rosetta loop modeling algorithm may be better for sampling flexible loop conformation than the Morph Server. In the MD simulation step, we have used two sets of Van der Waals cut-off parameters. The larger Van der Waals cut-off value considers more non-bounded interactions and is more sensitive for MD simulations. In the 5 ns MD simulations, we wanted to speed up the observation of potential instabilities of the peptides binding to CDK2/Cyclin complexes, and thus used a larger cut-off value of 14 Å. However, in the subsequent correlation analysis, we wanted to analyze the dynamical motions in detail and used the default cut-off value of 10 Å. It is generally difficult to design peptide or small molecule inhibitor to target the interface directly since typical protein-protein interface is rather diffusive. Most of the known CDK2 inhibitors target the catalytic ATP-binding pocket of CDK2 [43]. However, this pocket for the family of CDK proteins is so conserved that it is difficult to design specific CDK inhibitors for this pocket. It is thus highly desirable to discover non-ATP competitive inhibitors that function through allosteric interactions. The temperature was set at 300 K. The force field G53a6 was used in all simulations.

Flexible CDK2-peptide docking based on an ensemble of CDK2

Docking Protocol. The peptides and CDK2 docking were performed using the Lamarckian genetic algorithm with the default parameters by AutoDock [48] (version 4.2). AutoDock Tools (http://autodock.scripps.edu/) was used to prepare the ligands and the receptor. The center of grid is set to be in the local peptide binding pocket of inactive (1E1X) and active (1FIN, Chain A) conformations, respectively. The grid points in xyz are set to be 60. The key residues (ARG150, LYS178, TYR180) in the binding pocket of CDK2, which are determined experimentally by point mutation, are set to be flexible when docking. The flexible residues of the receptor are treated in a similar way as the ligand. Hydrogen atoms were added by REDUCE (version 3.14) [49]. The results were clustered using a tolerance of 2.0 Å. Finally, 10 conformations with the lowest binding free energy were kept for further analysis.

MD simulation Protocol. In this study, two separate sets of MD simulations were undertaken by using the software GROMACS [50]. One is to focus on the CDK2 structures with different conformation of its T-loop; the other is for the docked CDK2 peptide structures. Each system was solvated in a cubic box with 10 Å SPC water. Then, each system was first minimized. The protein was constrained with all-bonds and the solvent molecules with counter ions were allowed to move during a 1,000-step minimization and a 100 ps long MD simulation. After relaxation, the system was simulated for 5 ns in total for all studied systems. The temperature was set at 300 K. The force field G53a6 was used in all simulations.
Figure 5. SPR binding assay results of CDK2 and peptides with and without ATP. The blue lines are experimental data, and the red lines are fitted results.

- The binding affinities (K_d) between CDK2 and TAALS (A) with and (B) without 60 μM ATP are 3.3 μM, 1.4 μM, respectively.
- The binding affinities (K_d) between CDK2 and LAALS (C) with and (D) without 60 μM ATP are 61 μM, 8.0 μM, respectively.
- The binding affinities (K_d) between CDK2 and DAALT (E) with and (F) without 60 μM ATP are 37 μM, 0.47 μM, respectively.
- The binding affinities (K_d) between CDK2 and YAALQ (G) with and (H) without 60 μM ATP are 61 μM, 98 μM, respectively.

doi:10.1371/journal.pone.0109154.g005
Correlation Analysis

In the protein network, a node is defined as one single amino acid. If the distance of any two heavy atoms of a pair of different nodes is less than 4.5 Å for at least 75% of the snapshots, then this pair of nodes were said to form an edge [51]. The neighboring nodes in sequence are not considered to be in contact. We have done 30 ns MD simulations for each different state. The dynamical network is constructed with the final 20 ns of the 30 ns trajectories sampled every 100 ps. Then, we define the pairwise correlations \( C_{ij} \) as
\[
C_{ij} = \langle \Delta R_{ij}(t) \Delta R_{ij}(0) \rangle / \langle \Delta R_{ij}^2(t) \rangle^{1/2} \cdot \langle \Delta R_{ij}(t) \rangle^{1/2},
\]
where \( \Delta R_{ij} = r_{ij} - \langle r_{ij} \rangle \) is the position of the atom corresponding to the \( i \)-th node. We calculated the correlations from the MD simulation trajectories using the program Carma [52].

Cell culture

C81 is an HTLV-1-infected T-cell line that expresses Tax protein established from patients with T-cell leukemia. These cells are available through AIDS reagent catalog [53–55]. Cells were cultured in RPMI-1640 containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine (Quality Biological) and were incubated in a 5% CO\(_2\) incubator at 37°C. Cells were cultured to confluence and pelleted at 4°C for 15 min at 3,000 rpm. The cell pellets were washed twice with 25 ml of phosphate-buffered saline (PBS) without Ca\(^{2+}\) and Mg\(^{2+}\) (Quality Biological) and centrifuged once more. Cell pellets were determined using Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Every 5 min. Cell lysates were transferred to Eppendorf tubes and centrifuged once more. Cell pellets were resuspended in lysis buffer (50 mM Tris–HCl, pH 7.5, 120 mM NaCl, 0.5% NP-40, 0.2 mM EDTA, 50 mM NaF, 0.2 mM Na\(_3\)VO\(_4\) and one complete kinase buffer (50 mM HEPES, 10 mM MgCl\(_2\), 5 mM MnCl\(_2\), 1 mM DTT, 50 mM NaF, 0.2 mM Na\(_3\)VO\(_4\), 1 mM DTT, one complete protease cocktail tablet/50 ml) and incubated on ice for 20 min, with a gentle vortexing every 5 min. Cell lysates were transferred to Eppendorf tubes and centrifuged at 10,000 rpm for 10 min. Supernatants were transferred to a fresh tube where protein concentrations were determined using Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Peptide synthesis

All peptides used for this study were commercially synthesized (RS Synthesis, Louisville, KY) with the following sequences:

- NH2-R-A-A-L-G-OH
- NH2-R-A-A-L-W-OH
- NH2-R-A-A-L-Q-OH
- NH2-R-A-A-L-E-OH
- NH2-R-A-A-L-W-OH
- NH2-R-A-A-L-G-OH
- NH2-R-A-A-L-E-OH
- NH2-Y-A-A-L-Q-OH
- NH2-Y-A-A-L-W-OH

The purity of each peptide was analyzed by HPLC to greater than 98% and they were stored at -80°C. Peptides were only thawed once prior to use for biochemical experiments.

Size-exclusion chromatography

C81 whole cell lysate (3 mg) was fractionated on a Superose 6 HR 10/30 column (Amersham Biosciences, Piscataway, NJ) in Buffer D (20 mM HEPES [pH 7.9], 0.05 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.05 DTT, and 20% Glycerol). Flow-through was collected at 0.5 ml for 50 fractions. Every 10th fraction was resuspended in dH\(_2\)O to a concentration of 1 mg/ml and stored at -80°C. Peptides were only thawed once prior to use for biochemical experiments.

Immunoprecipitation

Cdk2 containing chromatography fractions (28–31) were pooled together for immunoprecipitation. The pooled C81 extracts (250 µg each) were combined with 10 µM of each respective peptide. Cyclin E antibody (Santa Cruz, sc-198) was added to each reaction tube (10 µl, 2 µg), the reaction mixture was brought up to 500 µl with TNE\(_{50}\)+0.1% NP-40 (100 mM Tris, pH 8.0; 50 mM NaCl; 1 mM EDTA, 0.1% Nonidet P-40) and was allowed to incubate while rotating overnight at 4°C. α-IgG was added to extract as a negative control, and an IP was performed in the absence of competing peptide, acting as a positive control. The following day, 30 µl of a 30% Protein A & G bead slurry (CalBioChem, La Jolla, CA) was added to each reaction tube and allowed to incubate while rotating for 2 h at 4°C. Samples were spun and washed 2 × with TNE\(_{50}\)+0.1% NP-40 (100 mM Tris, pH 8.0; 300 mM NaCl; 1 mM EDTA, 0.1% Nonidet P-40) and 1 × with TNE\(_{50}\)+0.1% NP-40 to remove non-specifically bound proteins. 2 × Laemmli buffer was added to each sample and heated at 95°C for 3 min. Samples were loaded and run on a 4–20% Tris–Glycine SDS/PAGE gel to be used for both Western blots and kinase assays.

Western Blot

Immunoprecipitated samples were separated on SDS/PAGE gels and were transferred to a nitrocellulose membrane via a constant current of 70 mA overnight. The membrane was blocked with a 3% BSA solution in PBS containing 0.1% Tween-20, rocking for 2 h at 4°C. A 1:1000 dilution of α-cdk2 antibody (Santa Cruz, sc-163) was added to the blocking solution and incubated rocking overnight at 4°C. The membrane was washed with a fresh PBS+0.1% Tween-20 solution in order to wash off any residual primary antibody solution. A 1:1000 dilution of α-rabbit secondary antibody was added to a fresh 3% BSA solution in PBS+0.1% Tween-20 and incubated with the membrane, rocking for 2 h at 4°C. The membrane was washed 2 × with PBS+0.1% Tween-20 and 1 × with PBS to remove any residual antibody. The membrane was exposed to chemiluminescence reagent (Fierce) in the dark for 5 min., and was developed using a BioRad Imager.

Kinase assay

Immunoprecipitated samples were assessed for kinase activity. After the final TNE\(_{50}\)+0.1% NP-40 wash, beads were washed with kinase buffer (50 mM HEPES, 10 mM MgCl\(_2\), 5 mM MnCl\(_2\), 1 mM DTT, 50 mM NaF, 0.2 mM Na\(_3\)VO\(_4\), and one complete tablet of protease cocktail inhibitor/30 ml buffer) to equilibrate the reaction. Histone H1 (1 µg) was added to each reaction tube along with the γ-32P ATP (2 µl at 3000 Ci/mmol). Reactions were incubated at 37°C for 30 min and stopped by the addition of 15 µl Laemmli buffer. The samples were separated by reducing SDS-PAGE on a 4–20% Tris–Glycine gel. Gels were stained with Coomassie blue, destained, and then dried for 2 hours. Following drying, the gels were exposed to a PhosphorImager cassette and analyzed utilizing Molecular Dynamic’s ImageQuant Software.

Peptide binding measurement by SPR

The peptide TAAALS, LAALS, DAALT, and YAALQ2 were bought from Sangon Biotech. The purity of the four peptides was greater than 98% and they were stored at -20°C. When they were used for experiments, they are dissolved at 25°C.

Single-cycle kinetics experiments were performed with a T200 apparatus. The experiments were done on S-CM5 sensor chips coated with 6000 RU of CDK2. A flow cell left blank was used for double-referencing of the sensorgrams. Binding experiments were performed in standard PBS-P buffer with 60 µM ATP as running buffer (10 mM NaH\(_2\)PO\(_4\)/Na\(_2\)HPO\(_4\), 150 mM NaCl, 60 µM ATP and 0.05% surfactant P20, pH 7.4) at 25°C with a flow rate
of 30 µl/min. The peptide samples were prepared in the running buffer, and were injected. The regeneration of the surface was achieved with a 30 second pulse of 20 mM NaOH. Single-cycle kinetics assay were performed using the standard SCK method implemented by the T200 Control Software. In single-cycle analysis, the analyte is injected with increasing concentrations in a single cycle. The surface is not regenerated between injections. A blank injection of buffer only was subtracted from each curve, and reference sensorgrams were subtracted from experimental sensorgrams to yield curves representing specific binding. Data were fitted globally by using the standard two-state model provided by T200 Software.

References

1. Wells JA, McLendon CL. (2007) Reaching for high-hanging fruit in drug discovery at protein-protein interfaces. Nature 450: 1081–1009.
2. Arkir MR, Wells JA (2004) Small-molecule inhibitors of protein-protein interactions: progressing towards the dream. Nat Rev Drug Discov 3: 301–317.
3. Arkir M (2005) Protein-protein interactions and cancer: small molecules going for in the kill. Curr Opin Chem Biol 9: 317–324.
4. Bourgeas R, Basse MJ, March M, Sigardardorff A, Pit WR, et al. (2012) Biophysical and computational fragment-based approaches to targeting protein-protein interactions: applications in structure-guided drug discovery. Q Rev Biophys 45: 1–44.
5. Vassilev LT, Vu BT, Graves B, Carvajal D, Podell F, et al. (2004) In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science 303: 414–418.
6. Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, et al. (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumors. Nature 435: 677–681.
7. Betul S, Alam R, Martin M, Libbers DJ, Han H, et al. (2011) Discovery of a potential allosteric ligand binding site in CDK2. ACS Chem Biol 6: 492–501.
8. Schou O, Frierdich A, Bycroft M, Freund SM, Fersht AR (2002) Molecular mechanism of the interaction between MDM2 and p53. J Mol Biol 323: 491–501.
9. Querzini M, Bertoni A, Casini C, Perugini M, Birnbaum D, et al. (2005) Design of a novel class of peptide inhibitors of cyclin-dependent kinase-2 and its evolutionary conservation. Protein Sci 14: 2268–2277.
10. Verschueren E, Vanhee P, Rousseau F, Serrano L (2013) A unified conformational selection and induced fit approach to protein-peptide docking. PLoS One 8: e58769.
11. Zhang C, Shen Q, Tang B, Lai L (2013) Computational design of hecalic peptides targeting TNFα. Angew Chem Int Ed Engl 52: 11059–11062.
12. Sattler M, Liang H, Nettleship D, Meadows RP, Harlan JE, et al. (1997) Structure of Reβc4-ΔL35 ribonucleoprotein complex: recognition between regulators of apoptosis. Science 275: 983–986.
13. Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, et al. (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumors. Nature 435: 677–681.
14. Pumfery A, Wade JD, Agbottah E, Zhang N, Dadgar S, et al. (2006) Inhibition of HIV-1 replication by selective cyclin-dependent kinase-2 and its evolutionary conservation. Protein Sci 15: 2268–2280.
15. Agbottah E, Zhang N, Dadgar S, Paisfery A, Wade JD, et al. (2006) Inhibition of HIV-1 virus replication using small soluble Tat peptides. Virology 354: 373–381.
16. Smith CA, Kortemme T (2010) Structure-based prediction of the peptide binding pocket of cyclin-dependent kinase-2. Structure 18: 138–149.
17. Brownford SA, Watson D, Rounds J, Taitt CR, Williams A, et al. (2005) Structure-based characterization of peptide binding: from knowledge to know-how. Curr Opin Struct Biol 13: 894–902.
18. Slivka PF, Shridhar M, Lee GI, Sammond DW, Hutchinson MR, et al. (2008) A new class of peptide antagonists of the TLR4-MD2 interaction. Chemicochem 9: 645–649.
19. Davidse NA, Viana M, Kellner J, Schiel S, et al. (2013) Computational design of peptide ligands. Trends Biotechnol 31: 231–239.
20. Lewis N, Raveh B, Schaufler-Furman O (2013) Peptide docking and structure-based characterization of peptide binding: from knowledge to know-how. Curr Opin Struct Biol 23: 894–902.
21. Slivka PF, Shridhar M, Lee GI, Sammond DW, Hutchinson MR, et al. (2008) A new class of peptide antagonists of the TLR4-MD2 interaction. Chemicochem 9: 645–649.
22. Smith CA, Kortemme T (2010) Structure-based prediction of the peptide binding pocket of cyclin-dependent kinase-2. Structure 18: 138–149.
23. Bartova I, Otyepka M, Kuzi, Koca J (2004) Activation and inhibition of cyclin-dependent kinase-2 by phosphorylation: a molecular dynamics study reveals the functional importance of the glycine-rich loop. Proc Natl Acad Sci U S A 101: 5330–5335.
24. Verschueren E, Cardenas J, Easley R, Wu W, Kehn-Hall K, et al. (2008) Effect of transcription peptide inhibitors on HIV-1 replication. Virology 376: 308–322.
25. Agbottah E, Zhang N, Dadgar S, Paesfery A, Wade JD, et al. (2006) Inhibition of HIV-1 virus replicaition using small soluble Tat peptides. Virology 354: 373–381.
26. Moris GM, Husey R, Lindstrom W, Sanner MF, Belew RK, et al. (2009) AutoDock and AutoDockTools: Automated docking with selective receptor flexibility. J Comput Chem 30: 2785–2791.

Acknowledgments

We are grateful to Professor Houjin Zhang for providing CDK2 and Professor Luhua Lai for SPR testing.

Author Contributions

Conceived and designed the experiments: CZ SL. Performed the experiments: QS FK. Analyzed the data: HC YZ HI SL YH. Contributed reagents/materials/analysis tools: SL QS FK. Wrote the paper: YZ CZ SL.
49. Word JM, Lovell SC, Richardson JS, Richardson DC (1999) Asparagine and glutamine: using hydrogen atom contacts in the choice of side-chain amide orientation. J Mol Biol 293: 1715–1747.
50. Van Der Spoel D, Lindahl E, Hess B, Groenhof G, Mark AE, et al. (2005) GROMACS: fast, flexible, and free. J Comput Chem 26: 1701–1718.
51. Sethi A, Eargle J, Black AA, Luthey-Schulten Z (2009) Dynamical networks in tRNA: protein complexes. Proc Natl Acad Sci U S A 106: 6620–6625.
52. Glykos NM (2006) Software news and updates. Carma: a molecular dynamics analysis program. J Comput Chem 27: 1765–1768.
53. Easley R, Carpio L, Guendel I, Klase Z, Choi S, et al. (2010) Human T-lymphotropic virus type 1 transcription and chromatin-remodeling complexes. J Virol 84: 4755–4768.
54. Kehn K, Fuente Cde L, Strouss K, Berro R, Jiang H, et al. (2005) The HTLV-I Tax oncoprotein targets the retinoblastoma protein for proteasomal degradation. Oncogene 24: 523–540.
55. Wu K, Bottazzi ME, de la Fuente C, Deng L, Gitlin SD, et al. (2004) Protein profile of tax-associated complexes. J Biol Chem 279: 495–508.