Binding Mechanism of CDK5 with Roscovitine Derivatives Based on Molecular Dynamics Simulations and MM/PBSA Methods

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ABSTRACT: Roscovitine derivatives are potent inhibitors of cyclin-dependent kinase 5 (CDK5), but they exhibit different activities, which has not been understood clearly up to now. On the other hand, the task of drug design is difficult because of the fuzzy binding mechanism. In this context, the methods of molecular docking, molecular dynamics (MD) simulation, and binding free energy analysis are applied to investigate and reveal the detailed binding mechanism of four roscovitine derivatives with CDK5. The electrostatic and van der Waals interactions of the four inhibitors with CDK5 are analyzed and discussed. The calculated binding free energies in terms of MM-PBSA method are consistent with experimental ranking of inhibitor effectiveness for the four inhibitors. The hydrogen bonds of the inhibitors with Cys83 and Lys33 can stabilize the inhibitors in binding sites. The van der Waals interactions, especially the pivotal contacts with Ile10 and Leu133 have larger contributions to the binding free energy and play critical roles in distinguishing the variant bioactivity of four inhibitors. In terms of binding mechanism of the four inhibitors with CDK5 and energy contribution of fragments of each inhibitor, two new CDK5 inhibitors are designed and have stronger inhibitory potency.

Keywords: Roscovitine derivatives; CDK5; Molecular dynamics simulation; Molecular docking; Binding mechanism

1. Introduction

Cyclin-dependent kinases (CDKs) are a family of heterodimeric serine/threonine protein kinases, each of which includes catalytic CDK subunit and an activating cyclin subunit. They can control the progression of the cell cycle [1]. The cyclin-dependent kinase 5 (CDK5), discovered in 1992 [2-4], is involved in many neuronal diseases, such as traumatic brain injury, stroke, cell migration, Alzheimer diseases (ADs), et al.[5]. In fact, similar to other CDKs, separate CDK5 does not exhibit activity, and can be activated by combining one or two cyclin subunits, p35 or p39 in this system [2]. Some researchers suggested that p35 is an irreplaceable activator comparing with p39 [6]. p25, a proteolytic segment, is truncated from p35, contains the C-terminal portion of p35, and has high concentrations in AD patients [7]. The interaction of CDK5 and p25 can extend to the T-loop of non-phosphorylated CDK5, that is, if the complex of CDK5/p25 forms (higher activity than CDK5/p35 [8]), CDK5/p25 without phosphorylation would be immersed in absolutely active state [9]. Therefore, the CDK5/p25 complex has become an attractive pharmacological target. The design of novel CDK5/p25 inhibitors attracts much interest in recent years [1, 10-19].

Recently, many researchers designed and synthesized several kinds of inhibitors of CDK5/p25 including purine [13], bisindoles [12, 14], aloisines [15], quinolin-one [16], amino-thiazoles [17], paullones [18, 19] and other typical inhibitors [1]. Theoretically, Haq et, al. [20] applied the molecular docking and 3D-QSAR modeling (CoMFA and CoMSIA) to reveal the important interactions between the receptor active site residues and compound's functional groups and to understand the binding orientation. Cavalli et al. [21] investigated protein–ligand interaction in CDK5 in terms of steered molecular dynamics simulations. Ji et al. [22]

studied the selectivity of paullones inhibiting GSK-3 rather than CDK5 based on molecular dynamics simulations and free-energy calculations, revealing that paullones could be used as potent selective inhibitors. One inhibitor of purines, (R)-roscovitine [2-(R)-(1-ethyl-2-hydroxy-ethylamino)-6-benzylamino-9-isopropyl-purine] containing an asymmetric carbon can inhibit CDK5/p25 and other CDKs [12, 23, 24] and is a promising CDK inhibitor currently in clinical trials (CDK5/p25, IC₅₀ = 0.2 μM) [23]. It has been found that the roscovitine could make an unusual collapsed conformation of the glycine-rich loop (an important site of CDK regulation) more stable [12]. The results obtained by Tan et al. [24] demonstrated that it is possible to obtain more efficient roscovitine-like inhibitors through modifying the structure of roscovitine to improve the electrostatic field, leading to more complementary to the protein binding site. In aspect of the drug design, Zhu et al. [25] suggest that inhibitors with larger rotatable side-chain are unfavorable for binding with CDK5 by comparing with inhibition mechanism of three different inhibitors ((R)-roscovitine (RRC), aloisine-A (ALH) and indirubin-3'-oxime (IXM)) with CDK5.

In spite of the above experimental and theoretical studies, so far, the detailed binding mechanism of roscovitine derivatives (see Table 1) with CDK5/p25 (simplified as CDK5 hereunder) has not been clearly understood. Molecular dynamics (MD) simulation [21, 22, 24, 25] can be used to solve the above problem in terms of experimental results. Revealing the reason why the four roscovitine derivatives (see Table 1) with CDK5 have different inhibitory potency can help us gain some insights into the binding modes and binding mechanism of them with CDK5, and provide new ideas for the design of more promising CDK5 inhibitors. Herein, we apply molecular docking, molecular dynamics (MD) simulation, and binding free energy calculation to investigate and

reveal the binding mechanism between four roscovitine derivatives and CDK5. The main findings can be described as follows. First, the computed binding free energies are in agreement with experimental inhibitory potency of four inhibitors. Second, the hydrogen bonds of inhibitors with Cys83 and Lys33 are favorable to binding affinity of CDK5 with inhibitors. Third, the van der Waals energies dominate the binding free energies of CDK5/inhibitor complexes, especially the key contacts with Ile10 and Leu133 play important roles in distinguishing the variant bioactivity of four inhibitors. Fourth, based on the binding mechanism of the four inhibitors with CDK5 and energy contribution of fragments of each inhibitor, the two newly designed CDK5 inhibitors in current work have stronger inhibitory potency.

2. Computational details

2.1 Initial Structure Preparation

The starting geometry of CDK5 for molecular docking is got from the X-ray structure 1UNL of dimeric CDK5 (chains A and B) complex with the activator p25 (chains D and E) and with one inhibitor RRC (called A2 in this paper) in the RCSB Protein Data Bank (PDB: 1UNL, resolution 2.20 Å) [12]. In this work, the four roscovitine derivatives are taken as CDK5 inhibitors constructed by 3D graphical software as shown in [Table 1](#). Geometric optimization and the electrostatic potential calculations are performed at the B3LYP/6-31G(d) level [26] using Gaussian 09 software [27].

2.2 Docking calculations

The crystal structure of CDK5/A2 can be obtained from the RCSB Protein Data Bank (PDB: 1UNL, resolution 2.20 Å) [12]. In order to examine the reliability of docking method of Autodock4.0 software [28], first, the inhibitor RRC (A2) is extracted from the crystal structure CDK5/A2 (PDB ID:1UNL), then it is docked into the ATP binding site of CDK5 [13, 24] by Autodock4.0. The corresponding structure is similar to its crystal structure, suggesting the docking method of Autodock4.0 is relatively reliable. Therefore, other inhibitors are docked to CDK5, respectively, using Autodock4.0. The detailed docking process is shown as follows. The other three inhibitors are also docked to the ATP binding site of CDK5, respectively, based on Autodock4.0 program using the Lamarckian genetic algorithm [28]. Before docking, polar hydrogen atoms are added to CDK5 and Kollman all-atom charges are assigned to this enzyme. During each molecular docking process, CDK5 is set to be rigid, while automatic bond settings are used, allowing the torsion angles of all acyclic, rotatable bonds in the ligand to vary except for amide bonds. The grid maps are centered on the ATP site of CDK5, and the three dimensions of the grid are 90 ×90 ×90 points with a 0.375 Å spacing value, therefore, there is sufficient space to include most of the protein and the active sites. For each of the 200 independent genetic algorithm runs, a maximum 25,000,000 energy evaluations is performed using the default operator weights and a population size of 300. The other docking parameters are set to the default values. Here, the root-mean-square deviation (RMSD) fluctuations of backbone heavy atoms, whose values are all less than 2.0 Å, are clustered together. After clustering analysis, the structures with lower mean binding free energy and the larger number of conformations are chosen as the preferred docking conformations.

2.3 Molecular dynamics simulations

The LEAP module of the Amber10 package [29] is used for the addition of all missing hydrogen atoms of the protein and ligands. The restrained electrostatic potential (RESP) method [26, 30, 31] is used to determine its partial atomic charges at the B3LYP/6-31G(d) level. The standard AMBER force field (ff03) [32] and the general AMBER force field (gaff) [33] are applied to describe the interactions of protein and ligand, respectively. Each system is immersed into an octahedron periodic box with at least 10 Å distance around the complex, containing approximately 7600 TIP3P [34] water molecules, and the minimum distance between each protein and box walls is set as 10 Å. The appropriate numbers of sodium ions are included to maintain each system electro-neutrality. Then, all molecular dynamics simulations are carried out using Amber10 package. First, each system is minimized by two steps: (1) applying harmonic restraints to all protein atoms and the inhibitor and (2) allowing all atoms to move freely in turn. Each energy minimization is performed by the steepest descent method for the first 2000 steps and the conjugate gradient method for the subsequent 2000 steps. Then, a position restrained dynamics simulation is conducted on each system. CDK5, each inhibitor, and water molecules are coupled separately to a temperature bath of 300 K with a coupling time of 0.1 ps. Ultimately, a 20 ns MD simulation is carried out on each system. During MD simulation processes, the Particle Mesh Ewald (PME) [35] is employed to deal with the long-range electrostatic interactions, and all covalent bonds containing hydrogen atoms are constrained using the SHAKE algorithm [36]. The coordinates are saved every 1ps for the subsequent binding free energy calculation and the energy decomposition analysis.

2.4 Binding Free Energy Calculations

The binding free energies (ΔG_{bind}) of the inhibitors (A1-A4) to the CDK5 is analyzed by molecular mechanics Poisson-Boltzmann/Generalized Born solvent accessible surface area MM-PBSA/GBSA [37, 38] procedure, integrated in the Amber10 package. This method has been used successfully to study a wide variety of problems. [39-42] The binding free energy of a protein-ligand complex (ΔG_{bind}) is calculated in terms of the following relations [43, 44]:

$$\Delta G_{\text{bind}} = \Delta G(\text{complex}) - [\Delta G(\text{protein}) + \Delta G(\text{ligand})] \quad (1)$$

$$\Delta G_{\text{bind}} = \Delta E_{\text{gas}} + \Delta G_{\text{sol}} - T\Delta S \quad (2)$$

$$\Delta E_{\text{gas}} = \Delta E_{\text{ele}} + \Delta E_{\text{vdw}} \quad (3)$$

$$\Delta G_{\text{sol}} = \Delta G_{\text{polar}} + \Delta G_{\text{nonpolar}} \quad (4)$$

$$\Delta G_{\text{nonpolar}} = \gamma(\text{SASA}) + \beta \quad (5)$$

The gas-phase energies (ΔE_{gas}) can be divided into electrostatic energies (ΔE_{ele}) and the van der Waals energy (ΔE_{vdw}). The solvation free energy (ΔG_{sol}) is further decomposed into polar and nonpolar contribution. ΔG_{polar} is the polar solvation contribution calculated by solving the Poisson Boltzmann (PB) equations for MM-PBSA method or generalized Born (GB) model for MM-GBSA [45]. $\Delta G_{\text{nonpolar}}$ is the nonpolar solvation contribution and is estimated by the solvent accessible surface area (SASA) determined using a water probe radius of 1.4 Å. The corresponding solvating parameters γ and β are 0.00542 kcal/(mol Å) and 0.92 kcal/mol, respectively. T and S are the temperature and the total solute entropy, respectively. If the ligands have similar structure, the entropy contributions are not considerably different for ligands binding to a same protein site [46]. Therefore, the entropy contribution (ΔS_{bind}) is not included [47] in this study. For the sake of illustrating the interactions between inhibitor and each protein residue, in current work, the MM-GBSA [48] decomposition analysis in Amber10 is performed.

3. Results and discussion

Prior to MD simulations, the four CDK5 inhibitors (Table 1) are docked into the ATP binding site of CDK5, respectively, and the optimal conformations are represented in Fig. 1. Clearly, the four inhibitors adopt similar binding modes in the binding pocket, which is between the N and C terminal lobes of the kinase.

Clustering analysis by first sorting all the docked poses from lowest energy (best docking) to highest is shown in Fig. S1. Detailed clustering analysis data are clearly represented in Table S1. It is not difficult to see that the poses at the first column correspond to reasonable structure. In addition, Fig. S2 shows the superimposing of the experimentally crystal conformation containing the inhibitor R-roscovitine[12] (also called A2 in this work) and the optimized docking conformation. The computing RMSD value between them is 0.54 Å in terms of VMD program[49], which confirms that the obtained docking structure is consistent with the experimental one. Therefore, the above docking method is appropriate and credible for studied systems. The structures with lower binding energy and the larger number of conformations are chosen as the best docking conformations and utilized as the initial structures for subsequent MD simulations.

The detailed binding modes for four CDK5/inhibitor complexes are shown in Fig. S3. The docking details of Ki values, docking energies, and binding energies are listed in Table S2. As shown in Fig. S3, four inhibitors position themselves in the similar hydrophobic pocket consisting of residues Ile10, Val18, Ala31, Val64, Phe80, Glu81, Phe82, Cys83, Gln85, Lys89, Leu133, and Ala143 of CDK5, in which some hydrogen bonds form between the inhibitors and the residue Cys83/Lys89 (allosteric residue). The residue Lys89 locates in the edge of

binding pocket of CDK5. Similar to the docking results of the reference [5], the inhibitor A1 locates in the similar position to interact with CDK5, for example, the hydrogen bond is formed between Lys89 and the nitrogen of the aminoarylic core of A1.

After MD simulations, the conformation of CDK5/A2(R-roscovitine) as displayed Fig. 2(B) is similar to **docking conformation (Fig. S3(B))**, so **it also has similar interaction by comparing with** the experimental crystal structure of CDK5/p25/R-roscovitine complex (PDB code 1UNL)[12]. The above results imply that the method used in current work is reliable. In addition, it is worth noting from Fig. 2(B) that after MD simulations, the residue Lys89 seems to have weak interaction with each inhibitor based on the fading away of the hydrogen bond with the MD time evolution. **It is due to the outward movement of the allosteric Lys89 by comparing the docking and MD results (Fig. S4)**. During MD simulations, the initial conformations (the preferred docking conformations) of CDK5/inhibitor complexes will be changed with the time evolution, leading to the changes of binding sites and interactions.

3.1 System stability and residue flexibility with the time evolution

In order to evaluate the dynamic stability of four complexes, the root-mean square deviations (RMSDs) in relation to the initial structures of the backbone C α atoms for the bound CDK5 are analyzed as displayed in Fig. 3. During initial several nanoseconds, the RMSD values of each system increase sharply, and then after 5 ns (13ns for CDK5/A2), they tend to be stable with the RMSD fluctuation values of ~ 1.5 Å for these complexes, **and corresponding with the results (C α -rmsd ~ 1.6 Å) obtained from the simulation of the CDK5/p25 complex [50]**, it suggests that they have reached stable and equilibrated. Thus, MD trajectories during the

last 5 ns simulations are taken for the following conformational and energetic analyses. It is well known that the gyration radius (R_{gyr}) is a function of molecular size [51]. As shown in Fig. S6, the average values of four complexes are $23.6 \pm 0.08 \text{ \AA}$, which are close to that of the free CDK5. Furthermore, the calculated R_{gyr} is consistent with the experimental value [8] determined by the X-ray diffraction.

In order to examine the reliability of conformations (CDK5/inhibitors), we have computed the experimental residue B-factor values (black curve) (based on X-ray crystal structure (PDB: 1UNL) [12]) and those (red curve) from the last 5ns MD simulation for CDK5/R-roscovitine as shown in Fig. S5. Clearly, the B-factor curve from MD simulation is roughly consistent with the experimental residue B-factor curve [52], confirming that the conformation of CDK5/R-roscovitine obtained from current MD simulation is reliable. Although there are some deviations of B-factor values between experimental data and calculated data in some loops and helices, for example, the calculated B-factor values of conserved glycine rich loop (G-loop) and PSAALRE helix (Lys33-Glu51) [10] are lower than experimental values, and other domains like α -D helix and CMGC kinase domain fluctuate slightly during several frames from MD simulations by comparing the specific experimental data, these do not influence the above inference.

To demonstrate the residue flexibility of the bound CDK5, the root mean square fluctuations (RMSFs) for each system are computed and compared with free CDK5. Fig. 4 denotes flexible and stable loops in the four systems by comparing the values of RMSF, and the flexible loops have large RMSF values. The similar fluctuations are observed in the four systems as shown in Fig. 4. However, there are small differences between bound CDK5 and free CDK5. For the complex of CDK5/A4 (Fig. 4D),

the L1 domain (Glu25-Ile29) of β -sheet N-domain region, L2 (Leu37-His60) of the helix region, L3 (Leu107-Ile134) of α -helix C-domain region, and L4 (Leu248-Pro271) of apple domain obviously depart from free CDK5, revealing the higher flexibility.

3.2 Binding mechanism of CDK5/inhibitor complexes and design of CDK5 inhibitor

In order to examine the binding mechanism between four inhibitors and CDK5 and understand the different biological activities of four inhibitors, the binding free energies and energy components are computed for each CDK5/inhibitor complex in terms of the MM-PBSA method. As shown in [Table 2](#), the calculated binding free energies of CDK5/A1~CDK5/A4 are -34.06, -32.98, -30.14, -24.44 kcal/mol, respectively, confirming the biological activity ordering of A1>A2>A3>A4, which are in agreement with the experimental IC₅₀ ranking [\[5\]](#). It is worthy to note that there is an approximately linear relationship ($r^2 = 0.80$) between the calculated free energy and experimental bioactivity data (pIC₅₀), which implies the reliability of the parameters used to calculate the free energy.

It is easy to note from the structures of inhibitors in [Table 1](#) and binding free energies in [Table 2](#) that the bioactivities (IC₅₀ values) of four inhibitors are very sensitive to the substituted groups (R^1 and R^2). As illustrated in [Table 1](#), the central scaffold (fragment A) is the same for the four inhibitors. In the case of A1, the R^1 group (fragment B) is the 4-pyridine group, the R^2 group (fragment C) is the (R)-1-hydroxy-but-2-ylamino group. A2 and A3 can be obtained via substituting the 4-pyridine group (R^1 group, fragment B) of A1 using benzyl and 2-pyridine groups, respectively. A4 can be generated through substituting the (R)-1-hydroxy-but-2-ylamino group (R^2 group, fragment

C) of A1 using chlorine atom. Thus, in order to conveniently analyze and discuss the difference of binding affinity, hereunder, the four inhibitors are divided into two groups, that is, Group I (A1, A2, and A3) with the same R¹ substituent group and Group II (A1 and A4) with the same R² substituent group.

To distinguish the contribution of each residue of CDK5 to the binding, the binding free energy decomposition is performed. The total energies per inhibitor–residue pair of four complexes (CDK5/A1~CDK5/A4 complexes) are shown in Fig. 5, which demonstrates that the key residues (the $\Delta G_{\text{bind,PB}}$ values lower than -1.0 kcal/mol and higher than 0.5kcal/mol are listed) for binding interactions between CDK5 and inhibitors are as follows: CDK5/A1(I10, V18, K33, E81, F80, C83, Q85, L133); CDK5/A2(I10, G11, V18, F80, F82, C83, L133); CDK5/A3(I10, V18, K33, F80, F82, Q130, L133, N144); CDK5/A4(I10, V18, A31, K33, F80, F82, C83). To obtain the details about the binding mechanism of CDK5/inhibitor complexes, the program of LIGPLOT [53] is applied to generate the 2D schemes of protein-ligand complexes based on MD simulation results and the binding mode including hydrogen bonds and hydrophobic interactions are intuitively shown in Fig. 6, in which the key residues of each bound CDK5 are similar to those obtained from the binding free energy decomposition (see Fig. 5).

In order to examine and understand which energy terms are more favorable to the binding of each CDK5/inhibitor complex, it is necessary to compare the individual energy components (ΔE_{ele} , ΔE_{vdW} , ΔG_{pol} , $\Delta G_{\text{nonpolar}}$) as shown in Table 2, in which the interactions include the contributions from side chains and the backbone of the residues. Table 2 demonstrates that the van der Waals energy term (ΔE_{vdW}) has larger contribution on the binding affinity of each CDK5/inhibitor complex. The

polar solvation energy term (ΔG_{pol}) is significantly unfavorable for the binding in four complexes. The gas phase electrostatic energy term (ΔE_{ele}) encourages the binding, but it still does not completely remedy the negative effect resulted from the polar solvation free energy (ΔG_{pol}). The nonpolar solvation free energies ($\Delta G_{\text{nonpolar}}$) slightly drive the binding. Importantly, the correlation coefficient (r^2) between the ΔG ($\Delta E_{\text{vdW}} + \Delta E_{\text{ele}} + \Delta G_{\text{pol}}$) and total computed free energy (ΔG_{bind}) is about 0.99, which reveals that the van der Waals energy interaction and electrostatic energy interaction are dominant for differentiating the binding affinity of four inhibitors with CDK5. These results are in agreement with the binding mechanism of cyclobutyl-substituted imidazole inhibitors with CDKs [54]. However, for the 2-aminothiazole inhibitors [55], the van der Waals component is the mainly contribution binding.

In order to reveal how the electrostatic interactions affect the binding affinity of A1, A2 and A3 with CDK5, the hydrogen bond interactions are carefully analyzed in terms of Fig. 6. As shown in Fig. 6A, there are three stable hydrogen bonds between A1 and its adjacent residues in the CDK5/A1 complex. One hydrogen bond is established between the nitrogen atom (N7) of thiazole ring (A1) and the backbone nitrogen of Cys83, and the second hydrogen bond forms between N13 of binding pyridine and carbonyl oxygen atom of Cys83, and the third hydrogen bond forms between the oxygen atom (O37) of isobutyl and Lys33. The formed hydrogen bonds and their occupancies are displayed in Table 3. The three hydrogen bonds are stable with the evolution of MD time with average distances of 3.24, 2.97 and 2.76Å for N7 (A1)-N (Cys83), N13 (A1)-OD (Cys83) and O37 (A1)-NZ (Lys33), respectively, as represented in Fig. 6A. The corresponding hydrogen bonds occupancies are 85.16%, 89.96%, and 70.46%, respectively, as shown in Table 3. The above results

reveal that the hydrogen bonds of inhibitors with Cys83 and Lys33 can stabilize the inhibitors in binding sites. In the case of CDK5/A2 (Fig. 6B), three stable hydrogen bonds are developed between A2 and the nitrogen atom of Cys83, A2 and the oxygen atom of Cys83, and A2 and the oxygen atom of Ile10, respectively, with the occupancies of 97.62%, 82.78% and 45.22%, respectively. There is a weak hydrogen bond between A2 and Ile10 as shown in Table 3. As mentioned above, the two stable hydrogen bonds between Cys83 of CDK5 and A2(Fig. S3, Fig. 6, and Table 3) form, which are similar to docking results (Figure 3B) in reference 12. In the results obtained by Zhu et al. [25], they also suggested that there are three stable hydrogen bonds between A2 and CDK5, but the corresponding hydrogen-bond occupancies are different from current results. It is due to the fact that they performed only 10ns MD simulation on CDK5/A2. In the study of Soumya Lipsa Rath, et al.[54], the similar contacts exist in the complex of R-roscovitine with CDK5 by comparing our MD results, for example, the residues Cys83 and Ile10 play an important role in the hydrogen and hydrophobic interactions. In addition, the CDK5 inhibitors aloisine-A and indirubin-3'-oxime with similar activity as R-roscovitine both form hydrogen bonds with Cys83 and there is another hydrogen bond between indirubin-3'-oxime and Glu81[25]. Similar binding mode are found in complexes of quinolin-2(1H)-one derivatives and CDK5 by Wenge Zhong et al. [56].

As shown in Fig. 6C, for CDK5/A3 complex, there is one hydrogen bond between the oxygen atom of A3 and the nitrogen atom of Asn144, whose atom type is ND2 in amber program. Fig. 7 represents the gas phase electrostatic energy, polar desolvation energy and net electrostatic energy for CDK5/A1, CDK5/A2, and CDK5/A3 complexes. As mentioned above, there exist the hydrogen bonds between A1 and

Cys83/Lys33, A2 and Cys83/Ile10, A3 and Asn144. The gas phase electrostatic energy (ΔE_{ele}), polar desolvation energy (ΔG_{pol}), and net electrostatic energy ($\Delta E_{\text{ele}} + \Delta G_{\text{pol}}$) account for the sum of energy terms of the above polar residues. The gas phase electrostatic energies of CDK5 with inhibitors A1, A2, and A3 are -8.16, -3.33, -1.07 kcal/mol, respectively, and the polar desolvation energies are 6.28, 3.43, 1.12 kcal/mol, respectively. As a result, the net electrostatic energies are -1.88, 0.1, 0.05 kcal/mol, respectively, revealing the biological activity ordering of A1>A2 (or A3). For CDK5/A4, A4 forms two stable hydrogen bonds with Cys83 with the occupancies of 97.04%, 92.80%, respectively as shown in Fig. 6D and Table 3. The gas phase electrostatic energies of CDK5 with inhibitors A1 and A4 are -8.16, -3.66 kcal/mol, respectively, and the polar desolvation energies are 6.28, 2.62 kcal/mol, respectively. As a result, the net electrostatic energies are -1.88, -1.04 kcal/mol, respectively, making the biological activity ordering of A1>A4.

As mentioned above, the van der Waals energy terms (ΔE_{vdw}) considerably dominate the binding affinity of four inhibitors with CDK5 as shown in Table 2. The van der Waals interaction energy spectra of inhibitor-residue pair in CDK5/inhibitor complexes are analyzed as shown in Fig. 8. It is easy to note from Table 2 that the van der Waals interactions (ΔE_{vdw} , -51.18 kcal/mol) between CDK5 and A1 are significantly larger than that (-32.73 kcal/mol) between CDK5 and A4, leading to stronger bioactivity of A1 compared to A4. As shown in Fig. 8, the number of key residues for CDK5/A1 is considerably larger than that of CDK5/A4, resulting in stronger van der Waals interactions (ΔE_{vdw}) of CDK5/A1 than those of CDK5/A4, which is consistent with the result from Table 2. A1 and A4 possess different R^1 group and the same R^2 group as mentioned above. The above results imply larger van der Waal interactions of A1 are ascribed to larger R^2 group ((R)-1-hydroxy-but-2-

ylamino). As shown in Fig. 8, the van der Waals interactions between A1 and key residues of I10, V18, Y33, F80, F82, C83, Q85 and L133 of CDK5, especially the key contacts with I10 and L133 have larger contributions to the binding free energy. Although the van der Waals interactions of A2 with key residues of V18 (A3 with V18; A4 with F82) are relatively stronger, clearly, the key residues I10 and L133 play important roles in distinguishing the variant bioactivity of four inhibitors. The sum values of van der Waals interactions (CDK5/I10 and CDK5/L133) for CDK5/A1, A2, A3, and A4 are -5.36, -4.92, -4.31, and -2.72 kcal/mol, respectively, making the bioactivity ordering of A1>A2>A3>A4.

To further confirm above deduction about biological activity of the four inhibitors, the total interactions between the major residues and four inhibitors are deeply examined as shown in Fig. 9. Note from Fig. 9A that for CDK5/A1, CDK5/A2, CDK5/A3, the major residues include I10, Y33, F80, E81, C83, Q85, and L133. In fact, A1 has considerably stronger interactions with Y33, E81, C83, Q85, and L133 than A2 (or A3), making bioactivity ordering A1>A2 (or A3). Although the interactions between A2 and I10/F80, A3 and N144 are stronger, totally, the total interaction ordering is CDK5/A1>CDK5/A2>CDK5/A3, confirming bioactivity order A1>A2>A3. As shown in Fig. 9B, CDK5/A1 and CDK5/A4 have the major residues of I10, V18, Y33, F80, E81, C83, Q85, and L133. It is easy to see from Fig. 9B that A1 has significantly stronger interactions with the major residues except I10 and C83, which is in agreement with the bioactivity ordering of A1>A4. However, for the binding of cyclobutyl-substituted imidazole inhibitors with CDK5, Lys33 and Lys89 are the key residues to distinguish the activity of two inhibitors (cis-N-acetyl and cis-OH)[54]. In the work of Qiong Wu, et al.[25], for important inhibitors R-roscovitine, aloisine-A and indirubin-3'-oxime, the

key residues ranking the top five are “Cys83, Leu133, Ile10, Phe82 and Glu81” in energy analyses. Different inhibitors bound with CDK5 have slight differences in the binding mechanism as expected.

In order to better understand that the different parts (or fragments) of each inhibitor molecule play roles in the binding affinity of each inhibitor with CDK5, we performed energetic analyses of the interactions of individual fragments of each inhibitor with CDK5 (Table 1 and Table 4) using the ANAL module of Amber [57]. The contribution of fragment A is the most favorable for each inhibitor binding (-27.31, -28.02, -21.643, -28.88 kcal/mol for CDK5/A1, CDK5/A2 CDK5/A3 CDK5/A4, respectively), which confirm the structural reasonability of scaffold. Clearly, the energy contribution of isopropyl group (fragment D) for each CDK5/inhibitor is relatively small, which provide a new clue for further improving the bioactivity of A1, that is, it is possible to further enhance the bioactivity of A1 through modifying the fragment D of A1.

Based on the above analyses and discussion, the implications for inhibitor design can be described as follows. First, the above analyses about binding mechanism (or bioactivity) of four inhibitors with CDK5 suggest that the van der Waal and net electrostatic interactions play critical roles in binding affinity of potent inhibitor A1 with CDK5. In fact, the vdW energy term of CDK5/A1 has already larger contribution to the binding free energy (or bioactivity), but the electrostatic interaction contribution to binding is relatively small. Increasing the electrostatic contributions can help improve activity. Second, the analysis of the interactions of individual fragments of inhibitor A1 in CDK5/A1 demonstrates that the modifying of the isopropyl group (fragment D) in A1 could help to further improve bioactivity of A1. Therefore, to increase the electrostatic contribution of A1 to binding of CDK5/A1, we substitute the isopropyl group of A1 using polar 2-hydroxyethyl group and

n-propanol group, respectively, leading to two new designed inhibitors A1-a and A1-b.

To better verify our discovery, then the docking simulations, 20 ns MD simulations, and binding free energy calculations on CDK5/A1-a and CDK5/A1-b are carried out, respectively. The relevant results are shown in Fig. 10. The binding free energies of CDK5/A1-a and CDK5/A1-b are -36.33 kcal/mol and -38.85 kcal/mol, respectively, which are lower than that of CDK5/A1. CDK5/A1-a and CDK5/A1-b are lower in electrostatic energy than CDK5/A1 by 11.88 and 14.1 kcal/mol, respectively, which are consistent with the original intention of our design. It can be ascribed to stronger hydrogen bond interactions. The stable hydrogen-bonds include the pairs of N10(A1-a)-O(Cys83), N9(A1-a)-N(Cys83), and O20(A1-a)-ND2(Asn144) in CDK5/A1-a complex with the occupancies of 95.58%, 93.50% and 31.66%, respectively, and pairs of O45(A1-b)-OE2(Glu51), O45(A1-b)-ND2(Asn144), N10(A1-b)-O(Cys83), and N9(A1-a)-N(Cys83) in CDK5/A1-b complex with the occupancies of 99.64%, 96.88%, 91.58% and 69.98%, respectively. Moreover, we analyze the contribution of per-residue to the binding for the new designed inhibitors. Fig. S7 reveals that CDK5/A1-a and CDK5/A1-b include extra key residues that is, Phe82 (replacing Phe80 for CDK5/A1) and Asn144 in CDK5/A1-a, and Glu51, Ala143 and Asn144 in CDK5/A1-b compared to CDK5/A1 complex, resulting in more negative binding free energies (or stronger bioactivity). The binding of new designed inhibitors (A1-a and A1-b) with CDK5 is enhanced over synthesized compounds in the reference [5], ($\Delta\Delta G = -3.35$ kcal/mol, and -5.87 kcal/mol, respectively, where $\Delta\Delta G$ represents the relative binding free energy of the new inhibitor with R-roscovitine (A2) combining with CDK5) as shown in Table 2 and Fig. 10. The above thermodynamic analysis suggests that our designed A1-a and A1-b have stronger

inhibitory potency to CDK5 than A1, which remains to be confirmed by future experiment.

Totally, we can conclude from current work that the change of the group in the backbone of roscovitine affects the binding activity strongly. In current work, two new CDK5 inhibitors are designed based on same backbone. As for different kinds of CDK5 inhibitors (like staurosporine ($K_i=39\text{nM}$ [58]), flavonoidol ($IC_{50}=170\text{nM}$ [1]), indole, indirubin, thiazole and paullone), there are slightly different molecular contacts between residues of CDK5 and groups of inhibitors. In future, we will extend this work to the other inhibitors for CDK5 to explore a more universal method to reveal the binding mechanism and design new inhibitors.

4. Conclusions

In summary, the 20 ns MD simulations, free energy calculations, and energy decomposition analysis are carried out on four representative inhibitors (roscovitine derivatives) of CDK5 to examine and reveal the binding mechanism of four roscovitine derivatives with CDK5 and distinguish the different bioactivity of four CDK5 inhibitors. The computed binding free energies of CDK5/inhibitor complexes are in qualitatively consistent with experimental bioactivity (IC_{50} data) ranking of four inhibitors. Results demonstrate that the stable hydrogen bonds with Cys83 and Lys33 are favorable to the binding affinity of four CDK5/inhibitor complexes. The van der Waals interactions, especially the key contacts with Ile10 and Leu133, govern in the binding free energy, and play an important role in distinguishing the binding affinity of the four inhibitors. Based on the binding mechanism of the four inhibitors with CDK5 and energy contribution of fragments of each inhibitor, the

implications for inhibitor design are revealed. The thermodynamic analysis demonstrates that the two newly designed inhibitors have stronger inhibitory potency to CDK5 than A1, which provide valuable clues to the future design of CDK5 inhibitors.

Acknowledgements

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Figure captions

Fig. 1. Most favorable docking conformations of inhibitors in CDK5/p25 (p25 is colored in cyan). Carbon atoms of A1-A4 are colored in magenta, orange, green and blue, respectively.

Fig. 2. The detailed binding modes of the four CDK5/inhibitor complexes with lowest-energy conformations. Green dotted lines represent polar interactions or hydrogen bonds. A: CDK5/A1; B: CDK5/A2; C: CDK5/A3; D: CDK5/A4. This figure is prepared by Pymol.

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Graphical abstract

The molecular dynamics simulation was utilized to reveal the binding mechanism of the four Roscovitine derivative inhibitors with CDK5. Based on the above results and thermodynamic analysis, and new inhibitors are designed and predicted to have relatively higher bioactivity.

Table 1
Structures and in vitro activity of roscovitine derivative inhibitors of CDK5.

Inhibitors ^a	R ¹	R ²	IC50/(μM) [5]
A1	-4-pyridine	(R)-1-Hydroxy-but-2-ylamino	0.017
A2	-benzyl	(R)-1-Hydroxy-but-2-ylamino	0.2
A3	-2-pyridine	(R)-1-Hydroxy-but-2-ylamino	2.3
A4	-4-pyridine	Cl	7.4

^a Each CDK5 inhibitor is divided into four fragments, which are displayed by different colors, that is, black represents fragment A (9*H*-Purin-6-amine group); blue, fragment B(R¹ group); red, fragment C (R² group); and green, fragment D(isopropyl group)

Table 2

Binding free energy components for the protein–inhibitor complexes by using the MM-PBSA method.^a

Component^b	CDK5/A1 Mean^c	CDK5/A2 Mean^c	CDK5/A3 Mean^c	CDK5/A4 Mean^c
ΔE_{ele}	-17.67 (2.61)	-17.05 (3.45)	-16.78 (2.49)	-19.91 (4.56)
ΔE_{vdW}	-51.18 (2.12)	-52.48 (2.56)	-51.96 (2.45)	-32.73 (1.92)
ΔE_{gas}	-68.85 (2.91)	-69.54 (3.91)	-68.74 (3.18)	-52.64 (4.67)
ΔG_{pol}	40.68 (2.98)	43.08 (3.47)	44.84 (2.23)	32.67 (5.08)
$\Delta G_{\text{nonpolar}}$	-5.88 (0.08)	-6.52 (0.12)	-6.24 (0.09)	-4.47 (0.17)
ΔG_{sol}	34.80 (2.97)	36.56 (3.48)	38.6 (2.21)	28.2 (4.98)
ΔG_{bind}	-34.06	-32.98	-30.14	-24.44
$\text{IC}_{50}^{\text{d}}$	0.017	0.2	2.3	7.4

^aAll values are given in kcal/mol.

^bComponents: ΔE_{ele} : electrostatic energy in the gas phase; ΔE_{vdW} : van der Waals energy; ΔE_{gas} : total gas phase energy, which is the sum of ΔE_{ele} and ΔE_{vdW} ; ΔG_{pol} : polar solvation free energy; $\Delta G_{\text{nonpolar}}$: nonpolar solvation free energy; ΔG_{sol} : sum of nonpolar and polar contributions to solvation; ΔG_{bind} : total binding free energy

^cStandard error of mean values.

^dUnits of Inhibitory activity are given in μM .

Table 3
Hydrogen bonds formed between inhibitors and CDK5 during MD simulations

System	Donor	Acceptor	Occupancy (%)	Distance (Å)	Angle(°)
CDK5/A1	:83@O	:A1@H9-:A1@N13	89.96	3.081(0.18)	24.76(10.06)
	:A1@N7	:83@H-:83@N	85.16	3.182(0.14)	39.76(11.21)
	:A1@O37	:33@HZ-:33@NZ	70.46	3.023(0.16)	44.38(10.58)
CDK5/A2	:A2@N9	:83@H-:83@N	97.62	3.099(0.14)	20.36(11.47)
	:83@O	:A2@H1-:A2@N10	82.78	3.016(0.20)	38.68(8.91)
	:10@O	:A2@H13-:A2@O21	45.22	2.760(0.16)	17.91(9.91)
CDK5/A3	:A3@O37	:144@HD21-:144@ND2	31.79	3.085(0.18)	40.92(13.97)
CDK5/A4	:83@O	:A4@H9-:A4@N13	97.04	2.930(0.16)	26.34(11.19)
	:A4@N7	:83@H-:83@N	92.80	3.131(0.15)	28.09(12.73)

Table 4

The interaction energies (kcal/mol) of the different fragments of each inhibitor with CDK5.

Fragment ^a	CDK5/A1	CDK5/A2	CDK5/A3	CDK5/A4
A	-27.31	-28.02	-21.64	-28.88
B	-22.75	-15.96	-14.75	-9.03
C	-22.41	-23.38	-18.60	-3.48
D	-10.81	-10.67	-13.13	-8.56

^a see Table 1 for fragments A~D

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