Significance of Water Molecules in the Inhibition of Cylin-Dependent Kinase 2 and 5 Complexes

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Interest in CDK2 and CDK5 has stemmed mainly from their association with cancer and neuronal migration or differentiation related diseases and the need to design selective inhibitors for these kinases. In the present paper, eight Molecular Dynamics (MD) simulations are carried out to examine the importance of structure and dynamics of water in the active site of both CDK2 and CDK5 complexes with roscovitine and indirubin analogues. Together with previous results, the current work shows a highly conserved water-involved hydrogen bonding (HB) network in both CDK2− and CDK5−indirubin combinations to complete information from the X-ray crystallography. The simulations suggest the importance of such a network for combining the inhibitor to the host protein as well as the significance of using an activated CDK as a template when designing new inhibitors. Different binding patterns of roscovitine in CDK2 and CDK5 are detected during the simulations because of the different binding conformations of the group on the C2 side chain, which might offer a clue toward finding highly selective inhibitors with regards to CDK2 and CDK5.

INTRODUCTION

Water is a highly versatile and mobile component in biomolecular complexes.[1] The structural and biological functions of biosystems are controlled by their solvent environment. Most water molecules, including those that appear to be fixed in a crystal structure, undergo rapid motion in solution. Water molecules in interior cavities are generally conserved in different homologues structures and are seen both in crystals and in solution; these buried water molecules exchange much more slowly (10 ns to 0.01 s).[2] Biomolecules are dynamic systems, and X-ray diffraction studies can give snapshots of the dynamic process to provide detailed structural information on the positions of protein atoms. However, even if the crystal structures are made under the same experimental conditions at the same resolution, the snapshots might reflect a different dynamic phase of the biosystems. Generally, high resolution (<2.0 Å) crystal structures are preferred for a reliable analysis of structural aspects of water associated with a host protein. These bound water molecules in the binding pockets of proteins play an important role in the noncovalent association of proteins and small drug compounds. The water molecules are often highly structured and localized and may form water bridges between the drug molecules and the binding sites and optimize the interactions of the drug molecules. Unfortunately, even high-resolution crystal structures might sometimes overlook such water molecules since the large mean square displacement fluctuations of water make their contribution to high-resolution X-ray diffraction spectra negligible.[3] For example, two to four water molecules which were not observed in high-resolution crystal structures and actually residing within human interleukin-1β for times longer than 1 ns were first reported by Ernst et al.[4] In a subsequent analysis of low-resolution diffraction data, Yu et al.[3] showed that the disordered water molecules reported by Ernst et al. indeed exist.

There is an increasing number of examples[5−7] in drug design literature on how binding affinity may be improved when a tightly bound water molecule, which may form water bridges between the drug molecules and the binding sites, is displaced by a ligand. For instance, it has been shown that the displacement of a localized water molecule in the binding pocket of the HIV-1/KNI-272[6,7] complex by an inhibitor similar to KNI-272 causes the new inhibitor to bind more strongly than KNI-272 although it is not always the case.[8] Other studies have shown that both natural substrates and designed inhibitors can make use of existing tightly bound water molecules to bridge their interactions with the protein.[9,10]

Both Cyclin-Dependent Kinase 2 (CDK2) and Cyclin-Dependent Kinase 5 (CDK5) belong to a large family of heterodimeric serine/threoine protein kinases comprising a catalytic CDK subunit and an activating subunit.[11−15] The subunits of these CDKs have very similar three-dimensional structures as reflected in their sequence identity of 60%. Both the kinases are folded into a typical bilobal conformation. They have an N-terminal domain of approximately 85 residues in a mainly β-sheet structure, a predominantly α-helix C-terminal domain of about 170 amino acids, and a deep ATP-binding cleft between the two lobes. These two kinases have generated a marked interest among cell biologists, neuroscientists, and biochemists. CDK2 is targeted in cancer therapy due to its role in the cell division cycle,[11,16,17] while CDK5 presents an attractive pharmacological target because its deregulation is implicated in various neurodegenerative diseases such as Alzheimer's disease, Parkinson's

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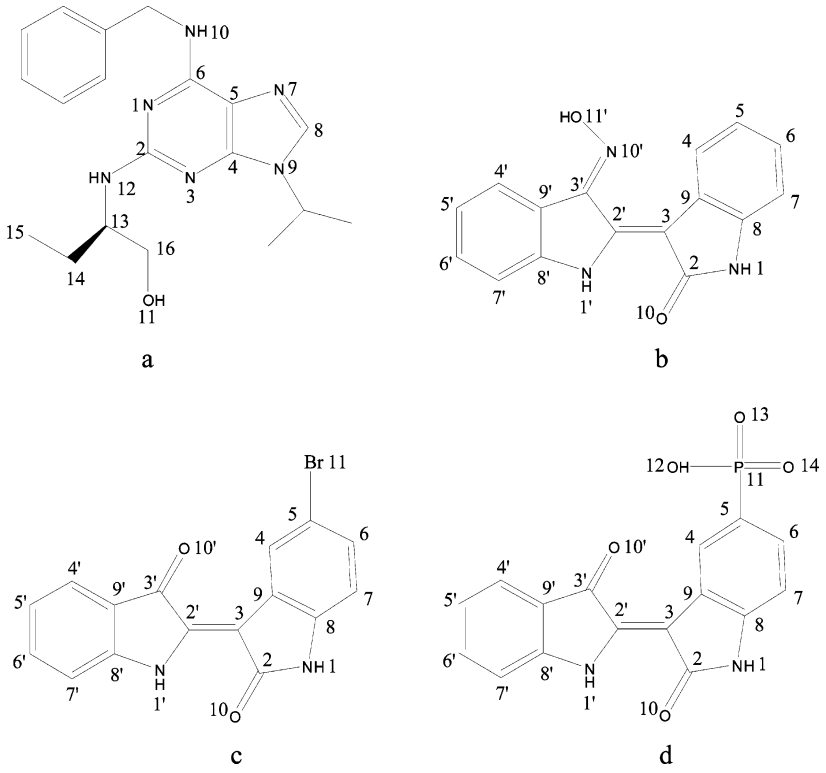
Scheme 1. Schematic Representation of the *R*-Roscovitine (a), the Indirubin-3′-oxime (b), the 5-Bromoindirubin (c), and the 5-Sulfonateindirubin (d)



disease, etc.[18−22] Many ATP-competitive inhibitors of cell cycle control related CDKs have been suggested,[23−28] and several of them have progressed to clinical trials targeting diverse types of cancer. Within the CDK group of kinases, CDK inhibitors fall into three classes—those that are not selective for any specific CDK, those that inhibit CDK1, -2, and -5, and those that are selective for CDK4 and -6. Although specific inhibition of a protein kinase is highly desirable, it remains a challenging goal in drug design.[23,29−31] No inhibitor that is selective for a single CDK has been discovered probably due to the conservation of the amino acids lining the CDK ATP-binding pocket.[32−33] The 20 important amino acids in the active sites of CDK1 and CDK2 are all the same, and 18 of these 20 amino acids are the same as in CDK5′ implying that most of the inhibitors share similar binding patterns in these CDKs. (*R*)-Roscovitine [2-(*R*)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine] (Scheme 1a) containing an asymmetric carbon is a purine analog that has been shown to potently inhibit CDKs with inhibitory concentration (IC_{50}) values ranging from 0.16 μM (for CDK5) and 0.70 μM (for CDK2) to over 100 μM (for CDK4 and CDK6) while maintaining good selectivity with respect to a number of other kinases.[34] Indirubin is the active ingredient of Danggui Longhui Wan,[24,35] a mixture of plants that is used in traditional Chinese medicine to treat chronic diseases. A series of indirubin analogues which have been suggested as potent inhibitors of CDKs,[36] unfortunately, show less specific inhibition among CDK1, CDK2, and CDK5. The structures of indirubin-3′-oxime[37] and 5-bromindirubin and 5-sulfonateindirubin[35] are also given in parts b−d, respectively, of Scheme 1.

Dynamical studies on binding modes are necessary to understand key structural features and interactions to provide valuable information for the design of efficient inhibitors. Several theoretical studies on CDKs based on both molecular dynamic simulations and quantum mechanical calculations[38−49] have been carried out to supplement the abundance of experimental works. Our previous simulation works[38,39] showed (1) the importance of the electrostatic contributions to the binding of a water molecule in the CDK5/indirubin-3′-oxime system, and the displacement of the water molecule in the active site of the water-included CDK5/indirubin-3′-oxime system results in a more conserved binding pattern than the water-excluded structure, and (2) the importance of activator included models of the kinases. This paper presents the analyses of bound water molecules determined from MD studies performed on the following eight CDK complexes to supplement information from X-ray crystallography: Apo CDK2/indirubin-3′-oxime (referred to as CDK2/indirbuin hereafter), CDK2/cyclinA/indirubin-3′-oxime (CDK2/cyclinA/indirubin-W) with a water originally placed among Asp86, Gln131, and the $-O^{11'}H$ of the inhibitor (imitating the water molecule in the active site of the CDK5/p25/indirubin-3′-oxime crystal structure), CDK2/cyclinA/indirubin-3′-oxime (CDK2/cyclinA/indirubin-WO) without any water molecule in the active site, CDK2/cyclinA/5-bromoindirubin (CDK2/cyclinA/indirubin-Br), CDK2/cyclinA/5-sulfonateindirubin (CDK2/cyclinA/indirubin-Sul), CDK5/p25/indirubin-3′-oxime (CDK5/p25/indirubin), CDK5/p25/roscovitine, and CDK2/cyclinA/roscovitine. In the following, this paper shows the crucial role of the bound water molecules in maintaining proper binding patterns. Together with detailed comparisons and analyses of the reported CDK2/inhibitor crystal structures in the Protein Database Bank, our simulations suggest the importance of detailed analyses of the involvement of bound water in the binding pattern for an individual ligand although some water molecules are well conserved in some homologue proteins or in some analogue inhibitors—same receptors complexes.

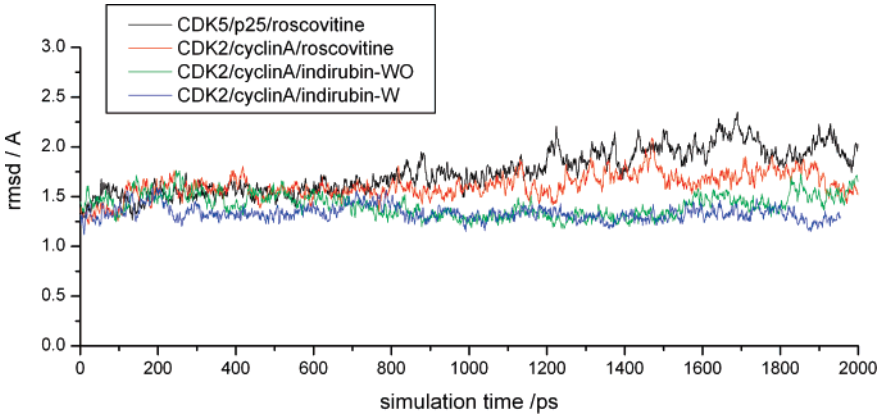


Figure 1. Time-dependent Cα rmsd of all the complexes with respect to the initial structures for CDK5/p25/roscovitine, CDK2/cyclinA/roscovitine, CDK2/cyclinA/indirubin-WO, and CDK2/cyclinA/indirubin-W.

Finally, different binding patterns of roscovitine in CDK2 and CDK5 are found, and modification of the skeleton structure to improve the inhibition selectivity is suggested.

MATERIALS AND METHODS

Molecular Dynamics Simulations. Molecular dynamics simulations were carried out using the SANDER module of AMBER 8.0 with the Cornell et al. all-atom force field.[50] Prior to the MD simulations, the values for some of the force field parameters for the ligands had to be developed because of the lack of reported data. Optimization of the five ligands was first achieved with the Gaussian98 package at the HF/6-31G* theoretical level. Electrostatic potentials (ESP) were then generated with Merz−Singh−Kollman van der Waals parameters.[51] Fitting of the charges to the ESP was performed with the RESP program[52] of the AMBER package. GAFF[53] force field parameters and RESP partial charges were assigned using the ANTECHAMBER module.

The starting geometries for the simulations of CDK2/cyclinA/indirubin-Sul, CDK5/p25/roscovitine, and CDK5/p25/indirubin were generated from X-ray structures obtained from the Protein Data Bank (PDB ID code: 1E9H, 1UNL, and 1UNH, respectively). The initial structure of CDK2/indirubin was derived from a manual modification of the crystal structure of CDK2/indirubin-Br, and the starting structures of CDK2/cyclinA/indirubin-W, CDK2/cyclinA/indirubin-WO, and CDK2/cyclinA/indirubin-Br were all generated from the crystal structure of 1E9H. The beginning structure of the CDK2/cyclinA/roscovitine was derived from an autodock simulation. All simulations are at neutral PH. Lys and Arg residues are positively charged, and Asp and Glu residues are negatively charged. The default His protonation state in AMBER8 is adopted. Counterions were added to maintain the eletroneutrality of all the systems. Each system was immersed in a 10 Å layer truncated octahedron periodic water box. The layer of water molecule in all cases contained around 11 000 TIP3P[54] water molecules in each of the complexes. A 2 fs time step was used in all the simulations, and long-range electrostatic interactions were treated with the particle mesh Ewald (PME) procedure[55] using a cubic B-spline interpolation and a 10^{-5} tolerance for the direct-space and with a 12 Å nonbonded cutoff. Bond lengths involving hydrogen atoms were constrained using the SHAKE algorithm.[56] All systems were minimized prior to the production run. The minimization, performed with the SANDER module under constant volume condition, consists of 7 steps. All heavy atoms in both proteins and ligands were restrained with degressive forces of 500, 200, 100, 50, 5, and 1 kcal/mol, respectively. In the first 3 steps, minimization of the solvent molecules and hydrogen atoms of the systems involved 250 cycles of steepest descent followed by 250 cycles of conjugate gradient minimization. In the next 3 steps, 100 cycles of steepest descent minimization were performed followed by 500 cycles of conjugate gradient minimization. All systems were then relaxed by 500 cycles of steepest descent and 1000 cycles of conjugate gradient minimization. After the relaxation, the systems were heated to 300 K.

Automated Molecular Docking. To effect the docking of roscovitine onto the CDK2/cyclinA, a Thr160-phosphorylated/CDK2/cyclinA/4-(6-cyclohexylmethoxy-9H-purin-2-ylamino)benzamide complex was first aligned with the CDK2/roscovitine complex. The crystal structure of CDK2/R-roscovitine was kindly provided by Prof. Laurent Meijer (CNRS, Station Biologique, France) and Dr. Sung-Hou Kim (University of California, Berkeley, U.S.A.). The CDK2 in the latter complex and the inhibitor in the former system were then excluded, and the position of roscovitine in the CDK2/cyclinA was used as the initial position for docking. In this docking simulation, we used the AutoDock 3.0 program.[57] The Lamarckian genetic algorithm (LGA)[58] was employed to treat the inhibitor−protein interactions. The number of generations, energy evaluations, and docking runs were set to 50 000, 1 500 000, and 20, respectively. Of all the conformations obtained from the docking runs, only those with positional root-mean-square deviation less than 1.5 Å were accepted. The conformation of the docked complex with the lowest energy was used for the analysis reported in this paper.

RESULTS AND DISCUSSION

NPT MD simulations were performed on complexes of CDKs with inhibitors. The root-mean-squared deviations (rmsd) fluctuations of backbone atoms from the X-ray structures were obtained. The curves in Figure 1 indicate that the solvated systems attained equilibrium after some initial fluctuations. Only rmsd values of the CDK2/cyclinA/indirubin-W, CDK2/cyclinA/indirubin-WO, CDK2/cyclinA/roscovitine, and CDK5/p25/roscovitine are presented for clarity. A comparison of the calculated temperature B-factors of the CDK2/indirubin and the CDK2/cyclinA/indirubin is

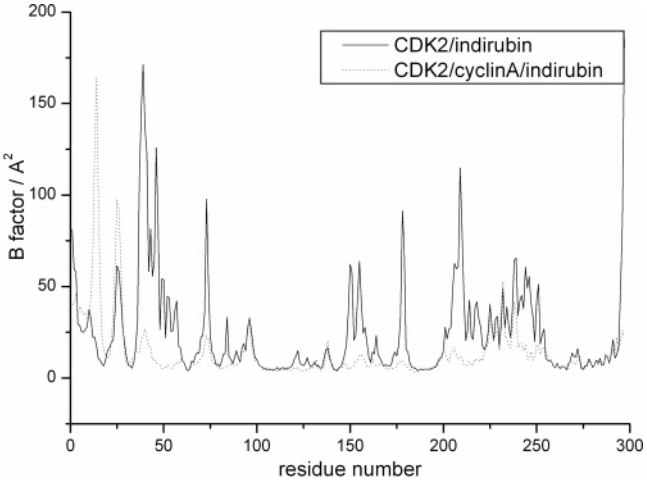


Figure 2. B-factors of CA atoms of CDK2 in CDK2/indirubin and CDK2/cyclinA/indirubin.

presented in Figure 2 to show that the cyclinA binding increases the stability of the PSTAIRE helix (residues 46−56) and the activation loop (residue 153−164) significantly and decreases thermal movements in these regions by direct interactions. This stabilization, which has been reported in several crystallographic studies,[59] serves as one measure of verification of the molecular dynamics simulations.

Water Bridged Hydrogen Bonding (HB) Networks in the Indirubin Complexes. Three regular HBs which the indirubin skeleton directly makes with the backbone of the kinase are all detected in the CDK5/p25/indirubin, just as in CDK2/cylinA/indirubin and CDK5/indirubin-3′-oxime[39,60]—the lactam amide nitrogen N^1H of the inhibitor donates a HB to the peptide oxygen of Glu81; the NH group of Leu83 of CDK2 (Cys83 in CDK5) donates a HB to the lactam amide oxygen O^{10}; and the cyclin nitrogen $N^{1'}H$ acts as a HB bond donor to the backbone oxygen of Leu83 (Cys83) (Table 1). Our previous simulations on CDK5/indirubin-3′-oxime reported a displacement of a water molecule toward the Gln130 from the Asp86, and, thus, a water bridged HB network between the hydroxyl group $O^{11'}H$ of indirubin and the side chain of Gln130 was formed. An energy calculation also supports the involvement of the water molecule in the active site. Hence, it is not surprising that a similar water bridge is revealed in the CDK5/p25/indirubin complex, considering the previous simulation was also based on an activated kinase. As presented in Figure 3, the distances between the water and the inhibitor and between the water and the residues show that the network is established from the beginning of the equilibrium and is maintained throughout the whole 2 ns process. According to Angel E. García et al.,[61] the time scale of a strongly bounded water molecule in proteins can reach 0.1−1 ns, and water molecules tend to form clusters or linear chains of two or more water molecules. Although no water cluster or linear chain has been detected in the CDK5/p25/indirubin system, the water molecule in the active site is strongly bound as indicated by its long residence time of 2 ns. Interestingly, a double-water-molecule chain is found in another CDK complex; this will be discussed later.

Observations of similar water bridged HB networks in indirubin analogues complexes with CDK2/cyclinA in the following confirm the important role of water molecules in the combination of inhibitor and protein and validate our previous discussion. Moreover, the water molecules make the binding pattern of indirubins more conserved.

Table 1. Hydrogen Bonds between the Inhibitors and the Active Sites as Obtained from Simulations

complex	hydrogen bond[a]	duration[b]	mean distance (Å)	mean angle (deg)
CDK2/indirubin	$N^{10'}H\cdots O$(Glu81)	99.90	2.91	19.49
	$O^{10}H\cdots N$(Leu83)	94.59	2.99	20.66
	$O^{11}H\cdots$OD2(Asp145)	96.20	2.64	12.86
CDK2/cyclinA/ indirubin-W	$N^1H\cdots O$(Glu81)	99.50	2.95	16.60
	$O^{10}\cdots$HN(Leu83)	99.80	2.82	22.90
	$N^{1'}H\cdots O$(Leu83)	88.35	3.13	37.69
CDK2/cyclinA/ indirubin-WO	$N^1H\cdots O$(Glu81)	98.95	2.99	18.00
	$O^{10}\cdots$HN(Leu83)	99.95	2.81	19.90
	$N^{1'}H\cdots O$(Leu83)	93.15	3.09	40.63
CDK2/cyclinA/ indirubin-Br	$N^1H\cdots O$(Glu81)	100.0	2.90	15.93
	$O^{10}\cdots$HN(Leu83)	99.80	2.81	22.74
	$N^{1'}H\cdots O$(Leu83)	89.92	3.13	38.78
CDK2/cyclinA/ indirubin-Sul	$N^1H\cdots O$(Glu81)	100.0	2.94	15.31
	$O^{10}\cdots$HN(Leu83)	99.52	2.83	24.36
	$N^{1'}H\cdots O$(Leu83)	96.81	3.02	40.25
	$O^{13}\cdots$HN(Asp145)	60.79	3.09	46.43
	$O^{13}\cdots$HZ3NZ(Lys33)	43.95	2.93	21.72
	$O^{13}\cdots$HZ1NZ(Lys33)	26.43	2.93	19.52
	$O^{13}\cdots$HZ2NZ(Lys33)	25.94	2.97	20.80
CDK5/p25/indirubin	$N^1H\cdots O$(Glu81)	98.75	3.01	21.91
	$O^{10}\cdots$HN(Cys83)	99.40	2.82	22.85
	$N^{1'}H\cdots O$(Cys83)	48.10	3.13	46.64
CDK5/p25/roscovitine	$N^{10}H\cdots O$(Leu83)	97.96	2.97	40.70
	$N^7\cdots$HN(Leu83)	72.34	3.27	28.96
	$O^{11}H\cdots O$(Gln130)	97.60	2.73	15.35
CDK2/cyclinA/ roscovitine	$N^{10}H\cdots O$(Leu83)	96.25	2.99	39.05
	$N^7\cdots$HN(Leu83)	75.45	3.53	22.43
	$O^{11}\cdots$HNE2(Gln131)	12.00	3.04	28.22
	$O^{11}H\cdots O$(Glu12)	43.10	2.75	20.78
	$O^{11}H\cdots O$(Ile10)	20.00	2.75	17.21

[a] Hydrogen bond obtained by measuring distances between heavy atoms. [b] % of equilibration simulation time.

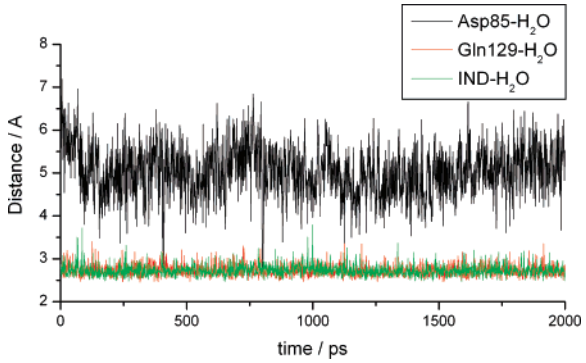


Figure 3. Intermolecular distances of Asp85-H^2O, Gln129-H^2O, and inhibitor-H^2O in the CDK5/p25/indirubin complex.

The calculations still suggest that all the inhibitors bind predominantly in the region of the pocket occupied by the ATP adenine ring in all the complexes and form hydrogen bonds with the backbone of the CDKs at the hinge region (Table 1). However, without the activator and the water molecule in the active site, the indirubin-3′-oxime in our simulations does not locate in the host protein at the position seen in the crystal structures.[37] The crucial amino acids around the active sites in CDK2/indirubin, CDK2/cyclinA/indirubin-W, and CDK2/cyclinA/indirubin-WO and the inhibitors are superimposed in Figure 4 to show the differences of the binding patterns of these complexes. In the latter two complexes, the inhibitors locate in the position in the

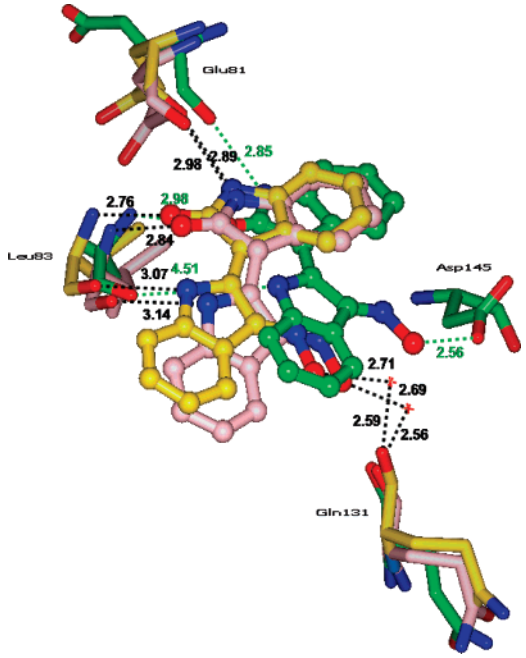


Figure 4. HB networks around the active sites of CDK2/cyclinA/indirubin-WO (yellow), CDK2/cyclin/indirubin-W (pink), and CDK2/indirubin (green). In this figure, oxygen atoms are in red and nitrogen in blue, and the oxygens of water molecules are displayed with stars.

active site obtained from the crystal structures. However, in the CDK2/indirubin system without a water bridged HB network between the inhibitor and the Gln131, the indirubin-3′-oxime moved away from the original location. The very conserved HB between $-N^{1'}H$ of the inhibitor and the −NH-(Leu83) is broken, and the $-O^{11'}H$ group, therefore, approached the Asp145 to form a very stable and strong HB with the atom OD2, as given in both Table 1 and Figure 4. The duration of the HB is 96.20% and the bond length is only 2.64 Å. After cyclinA and a water molecule were added, the three classical HBs, $N^{1}H-O$ (Glu81), $O^{10}-HN$(Leu83), and $N^{1'}H-O$(Leu83), in the averaged structures are detected, indicating a binding pattern consistent with that in the crystal structure.[37] Additionally, HBs between the water molecule and the inhibitor are detected, as presented in Table 2. However, a closer look at the HB networks in this complex shows that the original water molecule placed at the active site has displaced to form a water bridge between the $-O^{11'}H$ and the Gln131 for a short time (Figure 5). After the displacement, a second water molecule moved in and formed a similar bridge between the inhibitor and the protein. Some time later, a third water molecule replaced the second one to form a similar bridge at the same position. Within the 2 ns simulation, four water molecules entered and left to maintain the presence of the water-bridge between the $-O^{11'}H$ and the Gln131 and, hence, maintain the correct binding pattern of the inhibitor.

This phenomenon led us to question if the complex is able to grasp water molecules from the solvent and form water bridges to keep the inhibitor in the right position in the absence of a water molecule in the active site, or if the inhibitor would simply displace, like what was observed for the CDK2/indirubin complex. The water molecule in the active site, therefore, was removed from the active site in the CDK2/cyclinA/indirubin-WO simulations for comparison

Table 2. Water Molecules in the Active Site To Build Bridged HB Network

complex	hydrogen bond[a]	duration[b]	mean distance (Å)	mean angle (deg)
CDK2/cyclinA/ indirubin -W	$O(WAT1)\cdots O^{11'}H$	10.02	2.75	18.80
	$OH^{1}(WAT1)\cdots O(Gln131)$	3.95	2.82	22.97
	$OH^{2}(WAT1)\cdots O(Gln131)$	5.25	2.83	22.19
	$O(WAT2)\cdots O^{11'}H$	23.10	2.72	16.32
	$OH^{1}(WAT2)\cdots O(Gln131)$	10.05	2.74	21.25
	$OH^{2}(WAT2)\cdots O(Gln131)$	16.90	2.73	20.52
	$O(WAT3)\cdots O^{11'}H$	14.95	2.74	18.82
	$OH^{1}(WAT3)\cdots O(Gln131)$	5.55	2.75	21.84
	$OH^{2}(WAT3)\cdots O(Gln131)$	9.00	2.74	19.41
	$O(WAT4)\cdots O^{11'}H$	7.10	2.73	16.28
	$OH^{1}(WAT3)\cdots O(Gln131)$	2.65	2.75	21.59
	$OH^{2}(WAT3)\cdots O(Gln131)$	2.65	2.79	23.16
CDK2/cyclinA/ indirubin -WO	$O(WAT1)\cdots O^{11'}H$	19.80	2.78	23.43
	$OH^{1}(WAT1)\cdots O(Gln131)$	7.95	2.82	22.58
	$OH^{2}(WAT1)\cdots O(Gln131)$	7.35	2.86	23.72
	$O(WAT14684)\cdots O^{11'}H$	18.50	2.76	23.15
	$OH^{1}(WAT2)\cdots O(Gln131)$	7.15	2.81	20.15
	$OH^{2}(WAT2)\cdots O(Gln131)$	5.75	2.86	19.83
	$O(WAT9517)\cdots O^{11'}H$	15.65	2.74	21.27
	$OH^{1}(WAT3)\cdots O(Gln131)$	6.85	2.80	19.83
	$OH^{2}(WAT3)\cdots O(Gln131)$	6.85	2.82	21.52
CDK2/cyclinA/ indirubin -Sul	$O(WAT)\cdots HN(Tyr15)$	74.73	3.12	20.33
	$O(WAT)\cdots HN(Gly16)$	31.17	3.17	28.38
	$O(WATI)\cdots O^{12}H$	95.55	2.73	17.43
	$OH^{1}(WAT1)\cdots O(Gln131)$	12.68	2.79	21.00
	$OH^{2}(WAT1)\cdots O(Gln131)$	10.16	2.77	20.64
	$OH^{1}(WAT2)\cdots O(Gln131)$	21.97	2.79	20.97
	$OH^{2}(WAT2)\cdots O(Gln131)$	7.94	2.80	20.80
	$OH^{1}(WAT3)\cdots O(Gln131)$	10.26	2.82	21.80
	$OH^{2}(WAT3)\cdots O(Gln131)$	3.68	2.81	23.92
CDK5/p25/ indirubin	$O(WAT)\cdots HO^{11'}$	99.70	2.73	16.66
	$OH^{1}(WAT)\cdots O(Gln129)$	43.05	2.72	22.18
	$OH^{2}(WAT)\cdots O(Gln129)$	55.40	2.73	22.42
CDK5/p25/ roscovitine	$O(WAT)\cdots HN^{12}$	26.94	3.32	41.39
	$OH^{1}(WAT)\cdots ND2(Asn144)$	10.05	2.93	33.03
	$OH^{2}(WAT)\cdots ND2(Asn144)$	7.35	2.87	40.08

[a] Hydrogen bond obtained by measuring distances between heavy atoms. [b] % of equilibration simulation time.

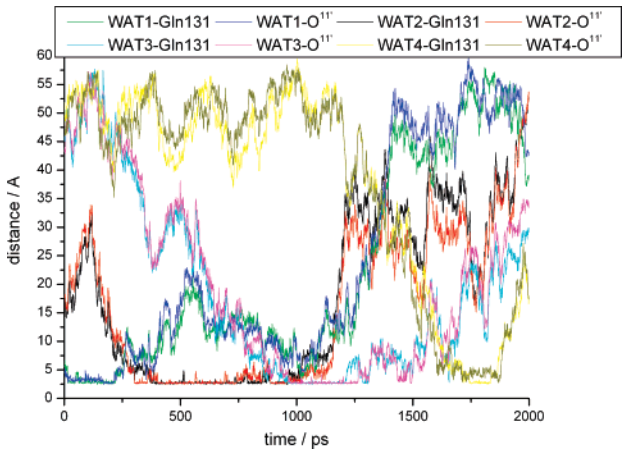


Figure 5. Distances of water molecules between the Gln131 and the $O^{11'}$ atom of the inhibitor in the CDK2/cyclinA/indirubin complex.

studies. The results show that the system can seize water molecules from bulk solvent to establish bridges between the inhibitor and the kinase (Table 2), and, as a result, the binding mode in this complex also can replicate the crystal structure.[37] Thus, it brings to question why the CDK2/indirubin complex is not able to grab water molecules from the environment to form such HB networks to prevent the

displacement of the inhibitor. To answer this, the different structures of apo-CDK2 and activated CDK2 have to be taken into account. The activation loop (known as T-loop), in apo CDK2, blocks the entrance of the catalytic cleft, and the activation of the kinase introduces large conformational and positional changes of the T-loop[59] in the CDK2/cyclinA complex. Consequently, the T-loop is directed away from the entrance of the cleft and, thus, significantly relieves the blockade of the cleft observed in monomeric CDK2. So it seems that the conformation of the T-loop influences the solvent environment of inhibitors in the ATP-binding cleft and in free CDK2 due to the obstruction of the T-loop. It is hard for water molecules outside of the active site to penetrate to build bridged HB networks. Accordingly, our conclusion that simulations using monomeric CDKs as templates may not yield correct results is consistent with our previous suggestion.[38]

From the above discussion, it is clear that the bound water molecule is crucial to maintain the correct binding pattern, and the binding modes of the inhibitor in both CDK2 and CDK5 systems are very conserved because of the involvement of the water molecule, which supports the findings of Michael Levitt et al. that the bound water molecules are generally conserved in different homologous systems.[2] In addition, our comparisons and analyses of the abundant CDK2/inhibitor structural data in the Protein Data Bank show that when a series of inhibitor analogues is enclosed in same receptor proteins, the locations of water molecules in the crystal structures are usually very close to each other in different complexes. Of course, this is partly because the structures are crystallized under the same experimental conditions and the resolutions are very similar. For example, 1H00, 1H01, 1H07, and 1H08 are the high-resolution crystal structures of the CDK2 complex with four 4,6-bisanilinopyrimidine inhibitors (resolution, 1.60 Å, 1.79 Å, 1.85 Å, and 1.80 Å, respectively). By superimposing all the structures, it is found that most of the water molecules are in similar locations. An inhibitor-Asp86-water-Gln131 chain is detected in all the complexes, indicating the conservation of the bound water molecule. The same phenomena also appeared in the 1OIQ, 1OIR, 1OIT, 1OIU, and 1KE5-1KE9 et al. CDK2/analogue inhibitors complexes, where similar water bridged HB networks can be detected. However, in the five high-resolution crystals 2C6I, 2C6K, 2C6M, 2C68, and 2C69, five triazolo[1,5-α]pyrimidines are cocrystallized with CDK2. Within two out of the three SO_2NH_2 involved complexes (2C6I and 2C6M), a water molecule is caught together by Ile10 and the inhibitor SO_2NH_2 group, respectively. When the SO_2NH_2 is replaced by a pyridine group in the 2C68 and 2C69 systems, respectively, the water molecule cannot be detected anymore. From these observations, it seems the binding of the analogues could be improved comparably by replacing the conserved water molecules in all the analogue/receptor complexes, whereas, if the structure of individual ligand in the group can be modified to replace some nonconserved water molecules in a single complex, then the ranking of the binding affinity of the compound might be raised, and ideas of which subgroup of the structure to modify to give stronger binding would be generated. Thus, the analyses suggest all the analogous systems deserve to be analyzed case by case, in spite of the similarities of the ligand structures, before one can deduce whether or how many water molecules can be retained in the active site and participate in the binding when analogues bind to the same receptors. Based on these findings, two more simulations on indirubin analogue complexes with CDK2/cyclinA were carried out to investigate the bound water molecules involved in the binding patterns, although no water molecules are originally detected in the crystal structures. It was found that the binding modes of CDK2/cyclinA/indirubin-Br and CDK2/cyclinA/indirubin-Sul are both consistent with those in the crystal structures.[37,62] Due to the big sulfonate group of the 5-sulfonateindirubin molecule, the HB network in the CDK2/cyclinA/indirubin-Sul is more complicated than that in the indirbuin-3′-oxime and the 5-bromo-indirubin complexes.[62] Our simulations, nevertheless, not only reproduced exactly the binding patterns reported by Davies et al.[62] but also produced information which cannot be seen in the crystal structure. As shown in Tables 1 and 2, the $-O^{12}H$ on the sulfonate group forms a strong and stable HB with a water molecule (WATI) during the whole process. An analysis on this water molecule reveals an interesting double-water-molecule bridged HB network between the inhibitor and the Gln131. As seen in Figure 6 and Table 2, the second water molecule acts in the same way as that in the HB networks of CDK2/cyclinA/indirubin, i.e., water molecules from the solvent keep moving in and out of the active site to interact with the stable water molecule caught by both the carbonyl oxygen and the hydroxyl group of the inhibitor and the side chain of the Gln131 to form the double-water-molecule chain until a similar and conserved binding pattern as that in the CDK2/cyclinA/indirubin and the CDK5/p25/indirubin appears. Figure 7 shows that the first water molecule in the CDK2/cyclinA/indirubin-Sul is at a position similar to that of the $O^{11'}$ atom of indirubin-3′-oxime in the CDK2/cyclinA/indirubin and the CDK5/p25/indirubin, and the second water molecule plays the same role as the water in the two complexes. Within this network, the sulfonate O^{12} plays an indispensable role to seize the first water molecule because a similar network is not found in the CDK2/cyclinA/indirubin-Br simulations. The only difference between 5-bromoindirubin and 5-sulfonateindirubin is the 5-position. Without any help from a group at this position, the $O^{11'}$ by itself cannot grab a water molecule from the outside to build

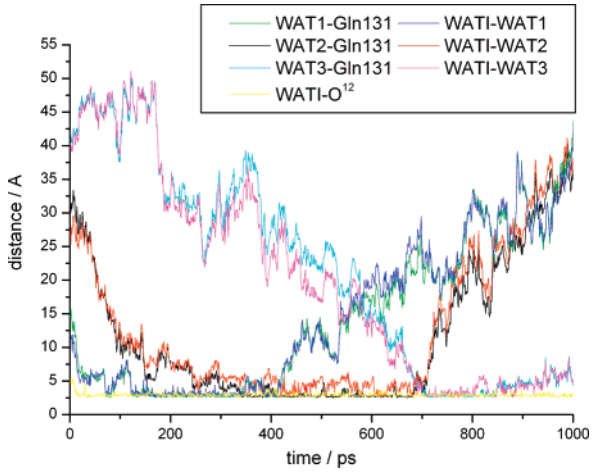


Figure 6. Distances between the second water molecules (WAT1, 2 and 3) and the first molecule (WATI), the second water molecules and the Gln131, and the WATI and the O^{12} of the inhibitor in the CDK2/cyclinA/5-sulfonateindirubin complex.

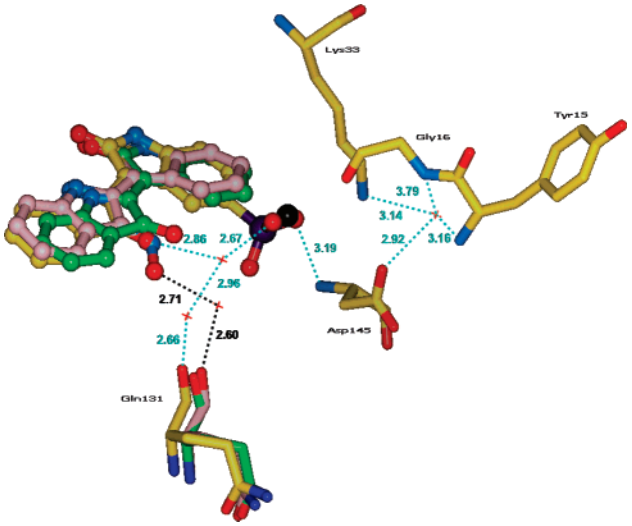


Figure 7. HB networks around the active sites of the CDK2/cyclinA/indirubin-Br (green) the CDK2/cyclinA/indirubin-Sul (yellow), and the CDK2/cyclinA/indirubin (pink). In this figure, oxygen, nitrogen, phosphorus, and bromine are in red, blue, dark purple, and black, respectively, and the oxygens of water molecules are displayed as crosses. HB networks of the second system are in light blue.

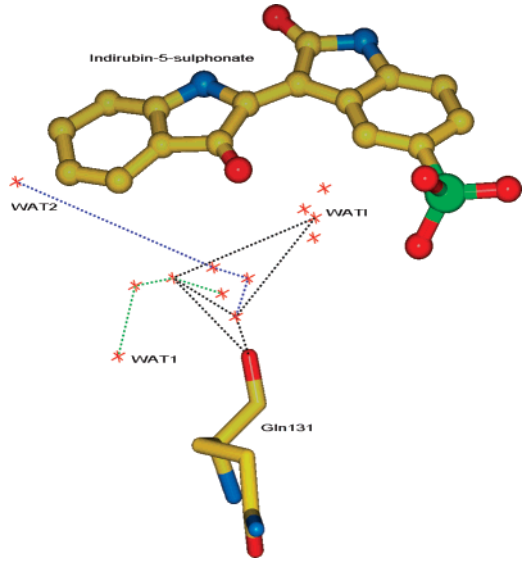


Figure 8. Water molecules exchange in the CDK2/cyclinA/indirubin-Sul system. Routes of water molecules going into and out of the active site are colored with green and blue, respectively. The transition state formed by the three water molecules is in black.

a bridge with the Gln131. Although a water molecule was placed at the active site to replicate the position in other systems before the simulations were started, it escaped at the beginning of the simulation. Thus groups with reasonable volume and that might establish HBs with water molecules together with the $O^{11'}$ should be preferred in the 5-position.

Figure 8 shows the dynamics of the water molecules exchange in the CDK2/cyclinA/indirubin-Sul system. It can be seen from Figures 8 and 6 that a water molecule (WAT2) from the bulk solvent approaches the inhibitor first and then forms a triangular transition state with the WATI and the water molecule which bridges the WATI and the Gln131 (WAT1). Under this situation, both the water molecules interact with the WATI and the Gln131, and then the WAT1

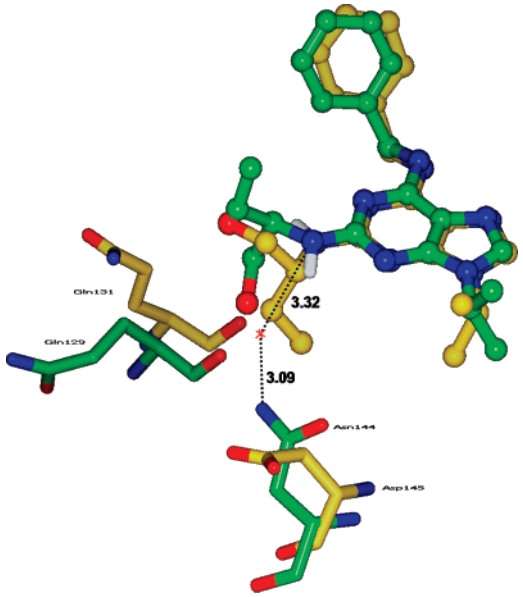


Figure 9. HB networks around the active sites of CDK5/p25/rosocitine (green) and CDK2/cyclinA/roscovitine (yellow). In this figure, oxygen, nitrogen, and hydrogen atoms are in red, blue, and white, respectively, and the oxygens of water molecules are displayed with stars.

is replaced rapidly by the new water molecule and escapes from the active site to the bulk solvent.

Finally, as seen in Table 2, both hydrogen atoms of the water molecules are involved during their combination with the residues, indicating these atoms interact with the receptors in turn. This is consistent with the reported description on the characteristics of water molecules in proteins, which pointed out that water molecules keep translating and/or rotating during biological processes.[63] Additionally, comparing Figure 3 with Figures 5 and 6, it seems the water included HB network in the CDK5 is more stable than those in the CDK2 systems because the duration of the network is much longer.

Water Bridged Hydrogen-Bonding Networks in Roscovitine Systems. Similarities and differences in the binding patterns of inhibitors between CDK2/cyclinA/roscovitine and CDK5/p25/roscovitine have been described in detail.[38] We focus on water molecules involved in the active site of these two complexes. As displayed in Figure 9 and Table 1, no water molecule is observed in the active site of the CDK2 system in our simulations. However, in the CDK5 complex, a relatively stable water bridged HB network is established between $N^{12}H$ of roscovitine and the side chain of the Asn144. Our results show that the difference is caused by the different binding conformation of the C^2 side chain of the inhibitor in the receptor kinases.

It can be seen in Figure 10 that the duration of the water bridge in the CDK5 system is considerably long, implying a stable and strong connection. By retrieving the different averaged conformations of roscovitine and of the Gln131 side chain in these kinases,[38] it is easy to explain the absence of water between the inhibitor and the Asp145 in CDK2. Due to the torsional rotation of $N^1-C^2-N^{12}-H^{N12}$ (numbered according to Scheme 1), $N^{12}-H$ of roscovitine points in opposite directions in both the complexes (Figure 9). The hydrogen on the N^{12}, the oxygen of the water molecule, and the Asn144 in CDK5 are all at the same side of the C^2 side

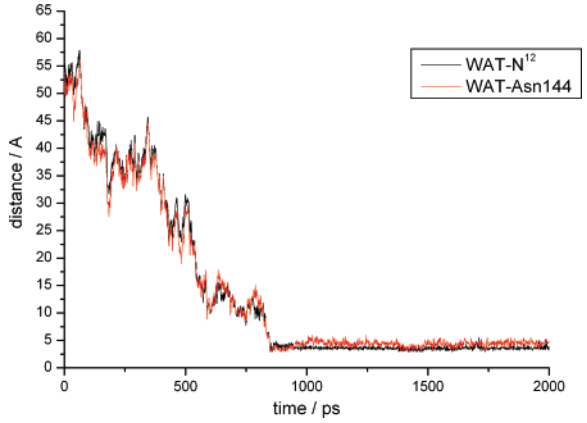


Figure 10. Distances between the water molecule and N^{12} of the inhibitor and the Asn144 in the CDK5/p25/roscovitine complex.

chain of the inhibitor, so the N^{12}−H ties up a water molecule together with the Asn144. Nevertheless, the N^{12}−H diverts to the other side of the C^2 chain of roscovitine in the CDK2 complex, and the system, accordingly, loses the chance to establish bridges with the Asp145. On the other hand, the tilted conformation of the side chain of the Gln131 shown in the previous simulations acts as a barrier to the ATP-binding cleft, preventing water molecules outside from entering the active site. As a result, no water molecule is detected in the CDK2 active site.

Because roscovitine is an ATP competing inhibitor and all the competing inhibitors try to imitate one or more binding interactions between ATP and the kinases, the CDK5/p25/roscovitine complex is superimposed onto both CDK2/cyclinA/ADP[62] and CDK2/cyclinA/ATP systems[65] to see whether the water molecule in CDK5 helps to conserve some of the interactions. It turns out that the position of the water molecule is similar to that of O5* atom on the phosphate chain in both the CDK2 complexes. Although there is no indication that the phosphate O5* is involved in any HB interactions in both the CDK2 systems, our simulations indicate the displacement of the oxygen in CDK5, however, can extend the interactions between roscovitine and CDK5 to a new area. This differs from the interactions between roscovitine and CDK2 and thus might be studied further for the design of lead compounds with more specificity between CDK2 and CDK5.

The interaction of inhibitor with Asp145 directly or via a water bridged HB network is found in many other CDK2/inhibitor crystal structures[66] but not in the CDK2/roscovitine crystal structure or our simulations. In the limited CDK5 involved crystal structures, it is the first time such a water molecule is detected and leads to a different binding pattern with respect to that in the CDK2 complex. Considering the similarity of the 3D structures of the two kinases, and the very similar binding patterns of roscovitine in both the CDKs, one can expect a longer but rigid hydrophilic group on the N^{10} position to replace the water molecule and to extend the interaction to the Asn144 area only in the CDK5 system to increase the inhibition selectivity of the roscovitine-like molecules between CDK2 and CDK5.

CONCLUSION

Detailed analyses of eight MD simulations were conducted to elucidate the roles of water molecules in CDKs, which are complementary to the X-ray crystallography.

The simulations first present the conservation of bound water molecules in homologous proteins complex with the same inhibitors. A conserved water molecule is detected in both the CDK5/p25/indirubin and CDK2/cyclinA/indirubin simulations to keep the conservative binding patterns of the ligand. Our simulations show the importance of the involvement of the bound water molecule in maintaining the correct binding pattern of indirubin-3′-oxime in the activated CDK2 by comparing it with the incorrect binding pattern in monomeric CDK2/indirubin-3′-oxime system. To investigate the common properties of the bound water in the indirubin analogues, two more simulations were carried out. A double-water-molecule chain in the CDK2/cyclinA/indirubin/Sul was found to conserve the HB network in the CDK5/p25/indirubin and the CDK2/cyclinA/indirubin complexes but not in the CDK2/cyclinA/indirubin-Br combination. The maintenance of the HB network by the double-water-molecule chain implies the importance of a suitable sized substituent on the 5-position, which can help to grab water molecules from bulk solvent.

Because of the different binding conformations of roscovitine in CDK2 and CDK5, different binding patterns of the C^2 side chain have been observed in the complexes. Due to the rotation of the C^2 side chain of roscovitine and the blockage of the tilted conformation of the Gln131 in the CDK2/cyclinA/roscovitine system, no part of the inhibitor could interact with the Asp145. In CDK5, however, the side chain can establish interactions with the residue via a water bridge. The binding modes of roscovitine between CDK2 and CDK5, therefore, can differ even more than previously reported. Thus, longer but rigid hydrophilic groups on the N^{10} position to replace the water molecule and to extend the interaction to the Asn144 may be exploited to find or design more selective inhibitors with respect to CDK2 and CDK5.

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