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Original Research Paper

DOCKING AND MOLECULAR DYNAMICS STUDIES ON CHROMONE BASED CYCLIN DEPENDENT KINASE-2 INHIBITORS

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ABSTRACT

Many chromone analogues have been reported as cyclin dependent kinase-2 inhibitors. Falvoperidol, a flavones alkaloid is currently under clinical investigations. In order to study the binding mode, structural requirement of such inhibitors, docking with Autodock 4.2 and molecular dynamics studies with gromacs 4.6.3 was undertaken. The ligands 2-(3,4-dihydroxyphenyl)-8-(1,1-dioxidoisothiazolidin-2-yl)-3-hydroxy-6-methyl-4H-chromen-4-one, baicalin, fisetin, flavoperidol, genestein, P-276-00, quercetin and rohitukine were used in the studies. Hydrogen bond acceptors or donors were found essential at 2 and 3rd position of chromone ring. Favourable coulombic interactions are also crucial in deciding the potency of such chromone based CDK2 inhibitors.

Keywords: CDK-2 inhibitors, Chromone, Flavones, Isoflavone, Docking, Autodock 4.2, Molecular Dynamics, Gromacs 4.6.3.

INTRODUCTION

Chromones are naturally occurring compounds, not common but found widely in many plant species. Benzopyran-4-one moiety is present in their structure. 2-Phenylchromones and 3-phenylchromones commonly referred as flavones and isoflavones[1] constitutes important group of naturally occurring compounds called flavonoids. Substituted flavones and isoflavones possess diverse biological activities like cytotoxic[2], anti-HIV[3], antimicrobial[4], antifungal[5], antiviral[6], antioxidant[7], nuroprotective[8], cardioprotective[9] and antihistaminic[10]. Many synthetic and natural flavonoids possess cytotoxic activity by inhibiting important targets like aromatase[11-12], topoisomerase[13-14], protein kinase C[15-16], cyclin-dependent kinases (CDKs) [17-20] and tyrosine kinases.[21-23] Among many regulators (CDKs) have been recognized as key regulators of cell cycle progression. Hyper activation of CDKs through mutations, resulting in alteration and deregulation of CDK activity, leads to cell cycle related disease like cancer. Therefore, CDK inhibitors are of great interest in intervening cancer.[24] Among 20 CDKs identified so far[25] CDK1, 2, 3, 4 and 6 have been investigated as potential therapeutic target as these are involved directly in cell cycle progression while other CDKs work indirectly as activators or regulators. CDKs require a regulatory protein called cyclin. The CDK-cyclin complex is activated kinase and phosphorylates serine/threonine residues on their substrates. ATP-competitive CDK inhibitors are divided into two classes' viz. unspecific CDK inhibitors and selective CDK inhibitors. The later class block all CDKs in an equipotent manner and some selective CDK inhibitors show preference for specific CDK. Many natural products have been investigated as CDK inhibitors, but flavonoids, chromones, flavones and isoflavones remain choice among them. Flavopiridol[19], a

flavones analogue has been identified as a potent selective CDK inhibitor and is currently under clinical investigation. In CDK family CDK2 has been attributed an important role in the occurrence and progression of melanoma and its inhibition significantly reduces the growth of melanoma cells.[26] Numerous studies have been reported on binding mode analysis of CDK2 inhibitors.[27-29]

Molecular dynamics (MD) studies highlights key structural features and interactions at active site which is essential to design new efficient inhibitors. In this pursuit, the CDK2 complex with its flavones inhibitor (PDB: 2DUV) from protein data bank has been used in current studies. The prominent flavones and isoflavone analogues quercetin, baicalin, fisetin, rohitukine, flavoperidol, P-276-00 and isoflavone analogue genestein were docked in to the active site of CDK2 and the resulting complexes were used for 1ns MD simulation. The results of docking studies and MD studies are reported in this paper. The paper also describes how the binding modes of various flavones and isoflavone based inhibitors can be useful for drug design.

MATERIALS AND METHODS

Computer Hardware and Software

Computational work was carried out on Ubuntu Linux 12.0 and windows XP operating system. Various software used include Autodock 4.2 with mgltools 1.5.6[30], Marvin Sketch (a structure drawing program), UCSF Chimera1.8rc[31], ArgusLab 4.0.1 (from Thomson and Planaria Software LLC), Discovery Studio 3.5 (Accelrys Inc.), Pymol version1.3 (from Schrodinger, LLC), Modeller 9.13 (a program for protein structure modelling from Sali lab)[32], VMD (a structure visualization program)[33] and Gromacs 4.6.3 (a package to perform molecular dynamics).[34-35]

Preparation of Protein Target Structure

In the present study, the X-ray crystal structure of CDK2 (PDB ID: 2DUV) in complex with compound **(1)**, 2-(3,4-dihydroxyphenyl)-8-(1,1-dioxidoisothiazolidin-2-yl)-3-hydroxy-6-methyl-4H-chromen-4-one, was obtained from Protein Data Bank.[36] The resolution of protein structure with 298 amino acid residues was 2.20 A^0. The amino acid residues 36-48 and 151-163 were not included in this X-ray crystal structure. These missing residues were modelled and included in the structure by using Modeller 9.13 program. Missing residues make the loop region of the protein (figure 1). The modelled protein was further processed by removing water and other residues including co-crystallized ligand. The resulted clean protein was further refined by energy minimization in UCSF Chimera with Amber ff12SB force field. Combination of 10,000 steepest descent and conjugate gradient steps with 0.02 A^0 step size were used during energy minimization. The energy minimized protein structure was used for docking procedure.

Ligand Preparation

2D structures of **(1)** and flavones and isoflavone analogues baicalin **(2)**, fisetin **(3)**, flavoperidol **(4)**, genestein **(5)**, P-276-00 **(6)**, quercetin **(7)** and rohitukine **(8)** (figure 2) were drawn and converted to 3D structures using Marvin Sketch. Geometry optimization was carried out in ArgusLab 4.0.1 on semi empirical quantum mechanical basis with parameterized model number 3 (PM3) hamiltonian, until restricted closed shell hartree-fock self consistent field formalism converses to 10^{-10} kcal/mol and steepest descent geometry search criteria until gradient converses to 10^{-6} kcal/mol. Gasteiger partial atomic charges of optimized molecules were computed in UCSF chimera.

Molecular Docking Simulation

Docking simulation was carried out by using Autodock 4.2 with mgltools 1.5.6. Clean protein was pre-processed by adding polar hydrogen and gasteiger charges and subsequently converted to pdbqt format. Pre-optimized ligands **1-8**, were also pre-processed similarly and converted to pdbqt format. All the torsion angles in the small-molecules were set free so as to perform flexible docking. Grid box of size 26.792 x 29.082 x 27.468 with 0.375 A^0 spacing was defined along x, y and z axis. The defined grid was large enough to cover active site of protein. Lamarckian genetic algorithm (LGA) was used for docking with the following settings: a

maximum number of 25,000,000 energy evaluations, an initial population of 150 randomly placed individuals, a maximum number of 27,000 generations, a mutation rate of 0.2, a crossover rate of 0.80, an elitism value (number of top individuals that automatically survive) of 1 and 10 docking runs. Results were clustered according to the root-mean square deviation (RMSD) values. The best docked conformations of ligands were selected as initial active/binding conformations to build the complexes for MD studies.

Molecular Dynamics Simulation

Molecular dynamics (MD) simulations were performed using the GROMACS 4.6.3 package with GROMOS96 43a1field.[37] The CDK2-ligand complexes obtained in docking simulation were used for performing MD simulations. Topology file of ligands were generated using the PRODRG program.[38] The charges generated by PRODRG are manually replaced with gasteiger charges. CDK2 solvated in cubic box using periodic boundary conditions and the SPC water model (figure 3). During MD simulation all the systems of individual complexes were neutralized by adding Cl^- counter ions by replacing water molecules. Energy minimization of complexes was carried out using the steepest descent algorithm. Energy minimized complexes were subjected to 100 ps position restraining simulation to relieve close contacts. This included NVT and NPT equilibration phases. During these equilibration phases leap-frog integrator was used for 100 ps simulation. Coordinates, energies and velocities were updated every 0.2 ps with LINCS algorithm to constrain bond lengths. Electrostatic interactions were calculated with Particle Mesh Ewald (PME) method with long-range electrostatics, a 14A˚ cut off for van der Walls interactions and 9A˚ cut off for Coulomb interactions. Modified Berendsen thermostat was used for temperature coupling and Parrinello-Rahman method for pressure coupling. Protein, ligand, water and ions were separately coupled with constants of tau 0.1 and 0.1 ps. The reference temperature and pressure was kept 300 K and 1 bar respectively. Finally, 1000 ps production phase MD was performed at the NPT canonical ensemble. MD analysis was carried out with respect to root mean square deviation (RMSD), root mean square fluctuations (RMSF), pressure, temperature, volume, total energy, leenard jones and coulomb energies, gyrate, number of hydrogen bonds, minimum average distance of hydrogen bond, number of contacts of ligands with active site residues and RMSD of residues at active site.

RESULTS AND DISCUSSIONS

Molecular Docking

Currently there are over 408 crystal structures of CDK2 in protein data bank. Many reports[39-42] are available for docking of CDK2 inhibitors in the active site of CDK2. We have chosen the PDB ID: 2DUV[39] as it has flavones based inhibitor as its co-crystallized ligand. Starting 2DUV structure aligns well with most of the crystal structures available in protein data bank (figure 4). The original interactions between CDK2 active site and co-crystallized ligand were hydrogen bonds with Leu83, Glu81 and Asp 145. The residues Ile10, Lys33, Ala31, Phe80, Leu134, Ala144 and Asp 145 show important hydrophobic interactions with co-crystallized ligand. After docking, the analysis of Autodock 4.2 built complexes revealed that the same interactions were reproduced with docking (figure 5). The RMSD between docked ligand and co-crystallized ligand was found 1.2 A^0 which suggests reliable docking protocol (figure 6). The binding free energy and other details from docking studies are presented in table 1. All the compounds under study found to interact with Leu83, Asp145 and Glu81 except compound 5 which interact with only Leu83 and Asp145. The residues Ala144, Gln131 and Asp86 also interact with compounds **2**, **3** and **7**. These interactions with additional hydrophopic interactions are shown in figure 7.

Molecular Dynamics Simulation

After subjecting the docked complexes to MD in GROMACS 4.6.3, various kind of analysis was undertaken. The MD analysis included stabilization of complex to pressure, temperature thermostat, stabilization of system with respect to

total energy, kinetic energy, potential energy, volume, deviation in the system from original structure in the form of RMSD, RMSF, stabilization of system with respect to gyrate, number of hydrogen bonds formed between ligand and selected residues, hydrogen bond distances. In terms of reported IC50 values in nM the order of inhibitory activity against CDK2 is **1** > **6** > **3** > **2** > **7** > **4** > **5** > **8**; contrary to this in terms of inhibitory constant, ki in nM this order was found **1** > **6** > **4** > **3** > **7** > **2** > **8** > **5**. Various energy terms calculated in MD are presented in table 2. It was observed that there was significant deflection in total energy, potential energy, coulombic and leenard jones interactions for **1** & **2** which accounts for the interactions produced during the simulation (figure 8). Coulomb –SR interactions between ligand and active site residues were fond crucial for activity. The order of activity as per lowest coulomb-SR interaction energy was found **1** > **6** > **3** > **7** > **4** > **5** > **8** > **2**. This order is similar to experimental IC50 values except compound **2**. The dynamic behaviour of sugar substituted compound **2** could not be revealed in this simulation and longer simulation can be devised to interpret its binding modes. Long range LJ-SR interactions were found moderately contributing in deciding the binding of ligand at its active site. Thought the MD simulation it was observed that the RMSD between energy minimized protein and MD simulated protein remains in the range of 0.25 A^0 (figure 9). Thus it is evident that CDK2 protein is quite stable during the entire simulation period of 100 ps. The analysis of root mean square fluctuations (RMSF) in CDK2 protein backbone was found stable over entire simulation for all the complexes with values of 0.05 to 0.6 A^0. The snapshot of backbone residues of complex **1** aligned over original CDK2 protein is shown in (figure 10). RMSF in ligand atoms was also analysed and was found between 0.004 to 0.1 A^0 for all the ligands. The analysis of radius of gyration of ligand center of gyration to center of gyration of protein was found deviating to considerable extent for ligand **2**, **5** and **6** (figure 11). The number of hydrogen bonds formed between ligand atoms and protein active site residues was analysed (figure 12). It was observed that maximum 9 hydrogen bonds were formed with ligands **1**, **4** and **7**; where as other ligands could form maximum 6 hydrogen bonds. Ligand **2** was found not forming hydrogen bonds during entire simulation. This can be due to the absence of hyroxyphenyl ring present on 2 or 3rd position of chromone ring in **2**. The analysis of last trajectory generated after simulation revealed that for all the ligands except **2** and **4** the conformer generated is very close to docked conformer as shown for **1** in figure 13.

CONCLUSION

CDK2 is a unique target in the treatment of cancer. Compounds **1**, **3**, **6** are most active CDK2 inhibitors. Flavoperidol, **4** is under clinical investigations as CDK inhibitor, but its specificity for CDK2 is less than compounds **1**, **3**, and **6.** We attempted to investigate the modes how various CDK2 inhibitors bind at the active site through docking and MD simulation. The docking scores and estimated inhibitory concentration in nm are comparable to experimental IC50 values. The docked conformers of ligands also showed interactions with residues Leu83, Glu81 and Asp145. Useful information about the dynamic changes when the ligands bound with CDK2 protein was obtained through MD simulation. From the results of RMSD, RMSF, total energy, Gyrate, number of hydrogen bonds formed, we concluded that the all the compounds under study could bind with CDK2 and induce its conformational change. The most active compound **1** was found to differ from other compounds in RMSD, total energy, RMSF and number of hydrogen bonds formed. Compound **2** on the contrary unable to form any hydrogen bonds with residues at active site and the energy terms, gyrate are poorer for this compound. Coulomb-SR interactions were also found very promising in MD simulation studies and lower such interactions higher the activity observed. It is evident from this finding that the substitution on chromone at 2 or 3rd position with hydroxyphenyl or aromatic ring with hydrogen

bond acceptor or donor decides the CDK2 activity.

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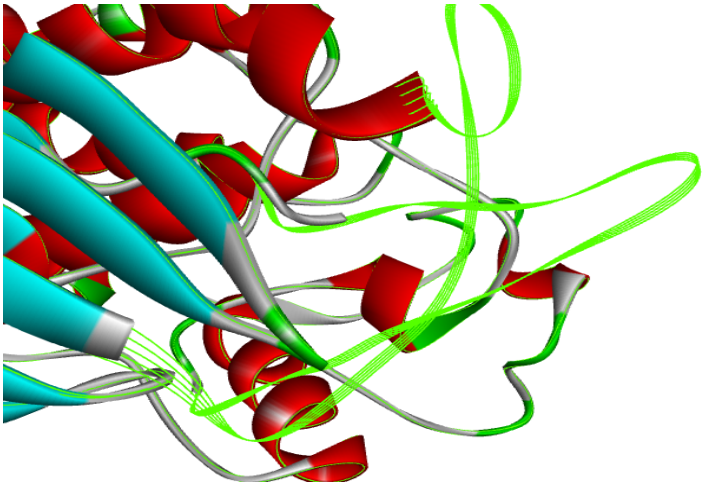

Figure 1: Modelled CDK2 protein (modelled missing residues shown green)

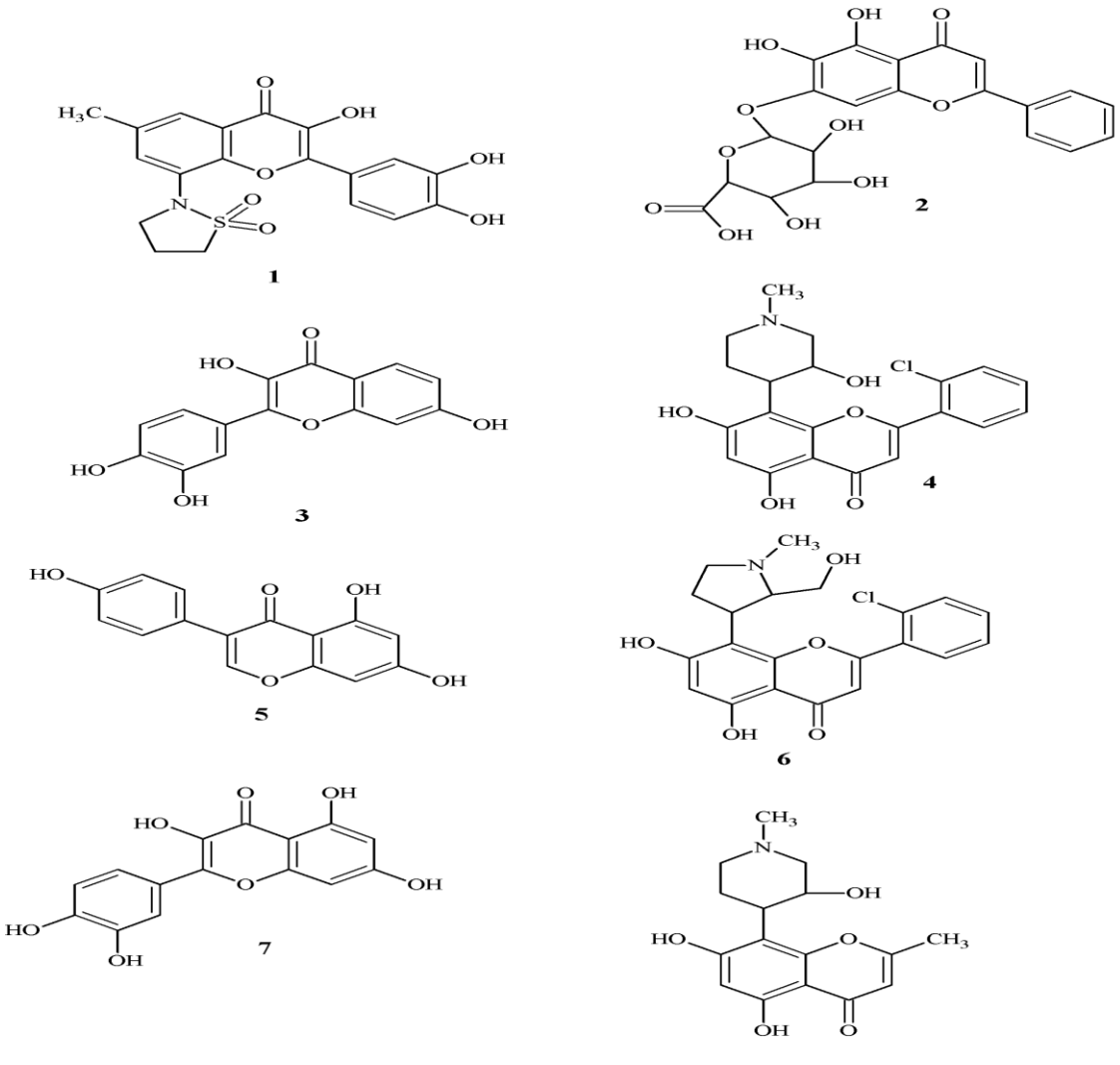

Figure 2: Structures of flavones and isoflavone analogues

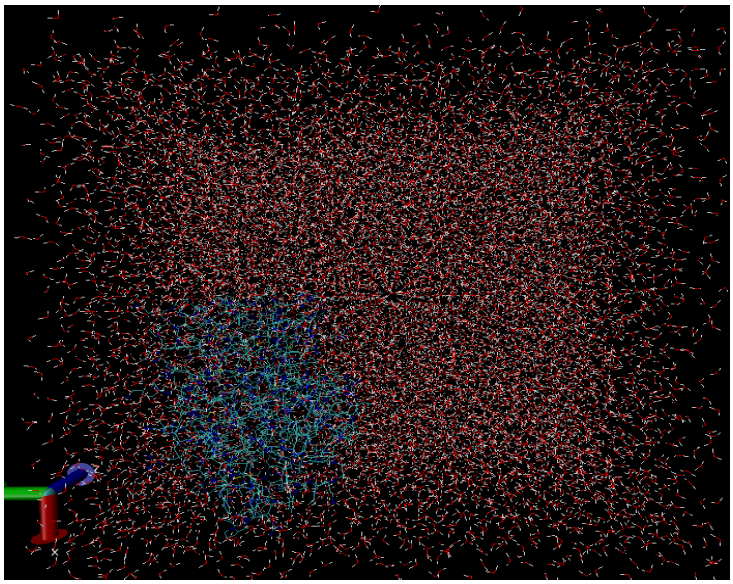

Figure 3: CDK2 embedded in cubic box of water

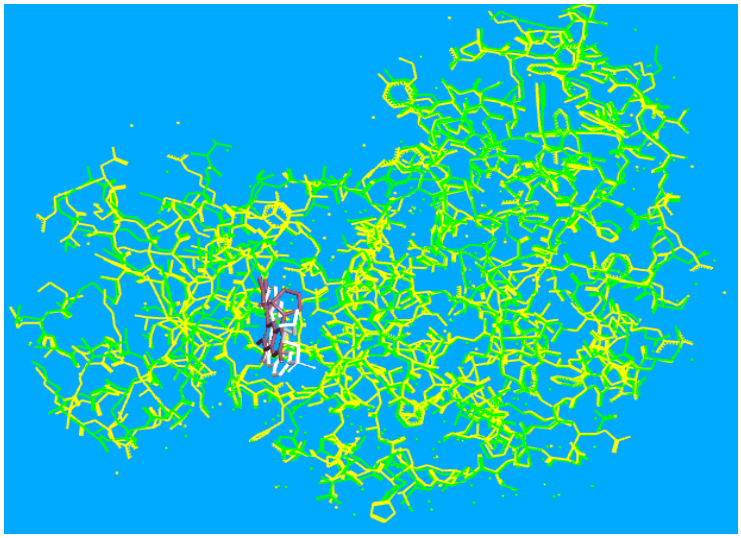

Figure 4: Aligned structures of 2DUV and 1URW (2DUV residues shown in yellow, 1URW shown in green, 2DUV ligand shown in brown and 1URW ligand shown in white color)

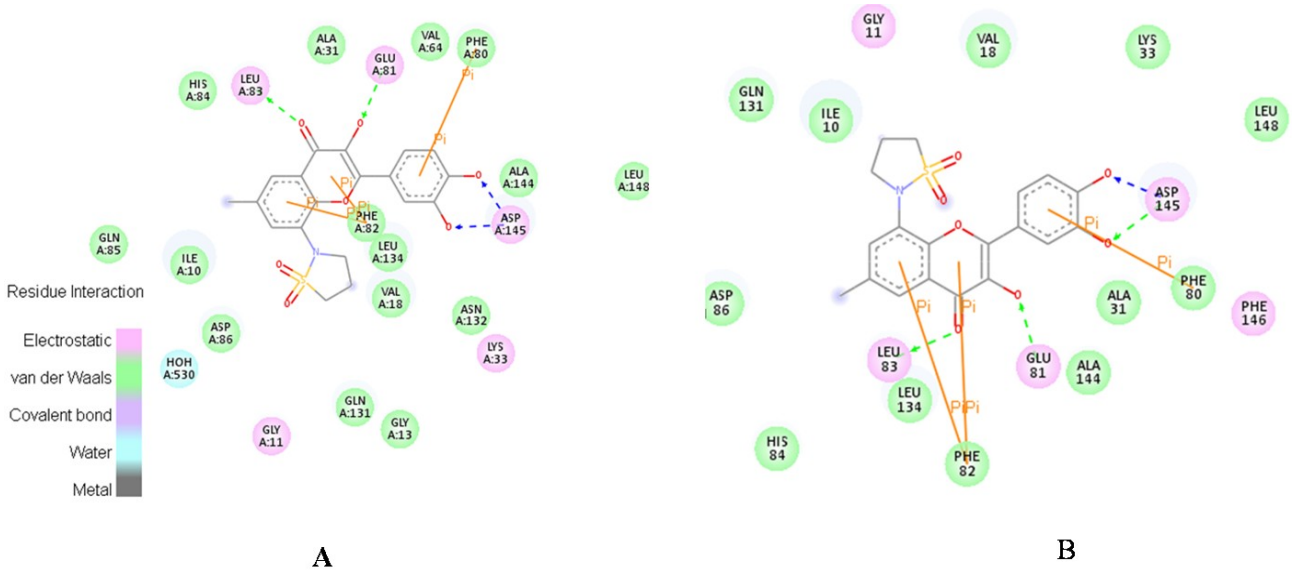

Figure 5: Interactions between ligand and important residues (A) co-crystallized ligand, (B) Docked ligand

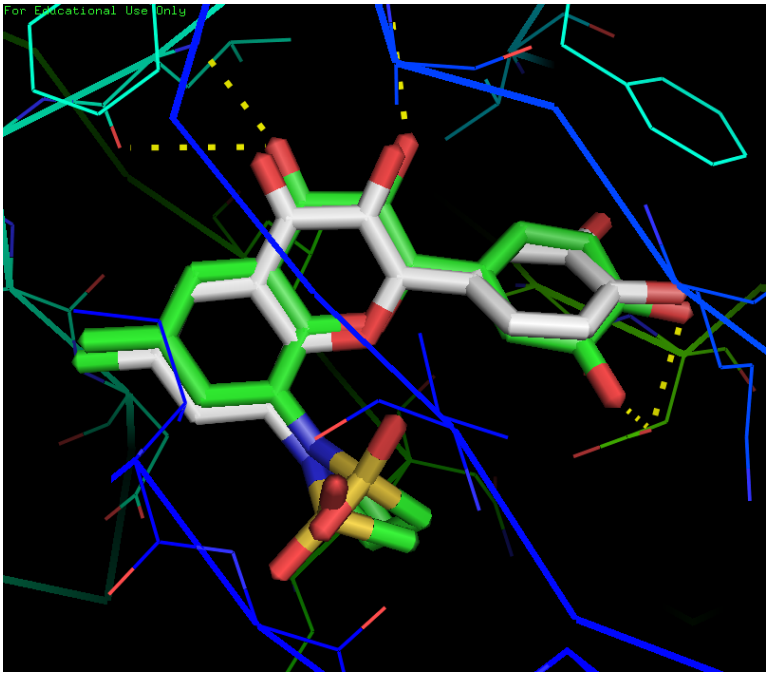

Figure 6: Docked and co-crystallized ligand (Except hetero atoms docked ligand shown green and cocrystallized ligand shown white)

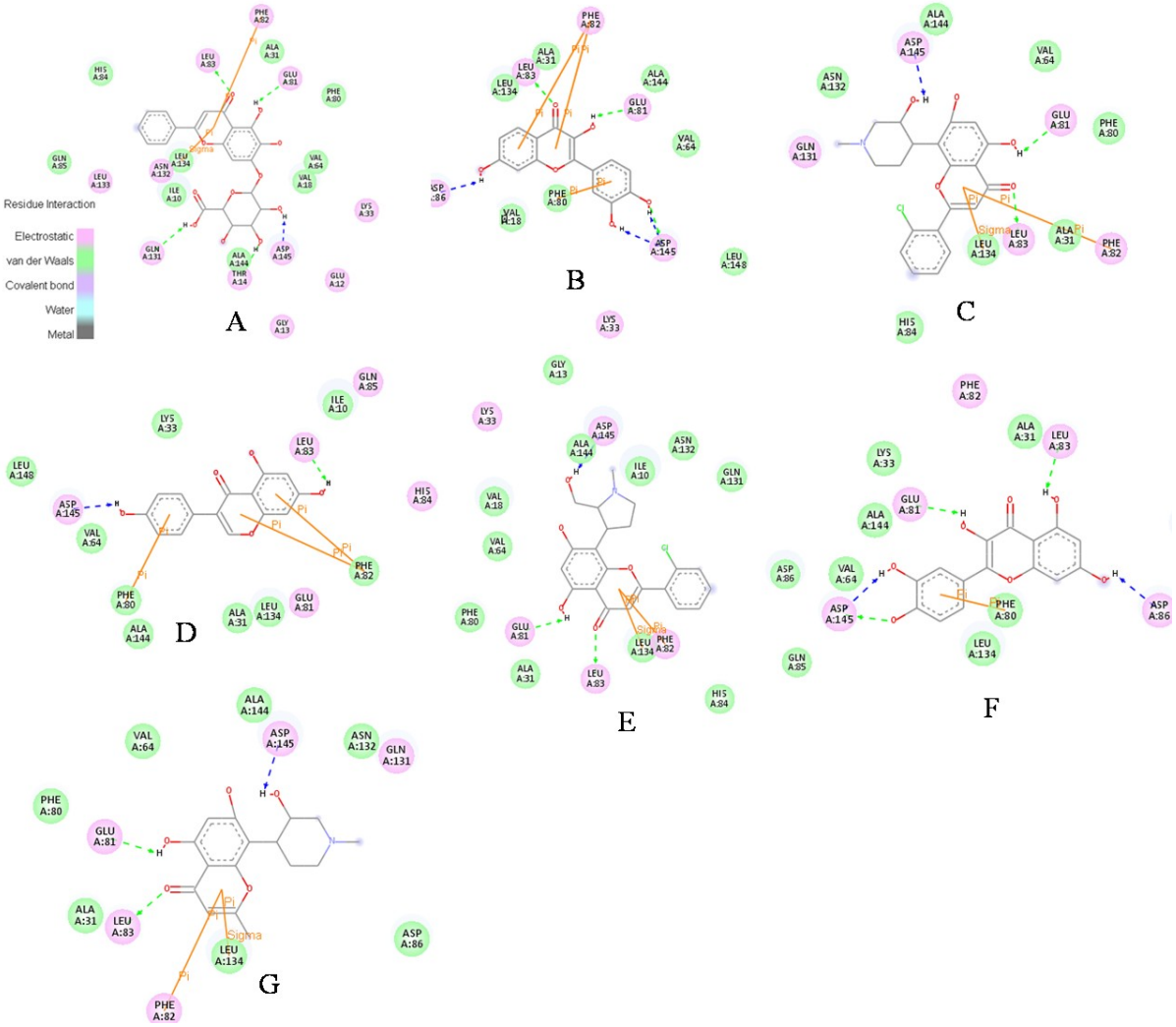

Figure 7: Interactions of important residues with ligands; interactions of (A) compound **2**, (B) compound **3**, (C) compound **4**, (D) compound **5**, (E) compound **6**, (F) compound **7** and (G) compound **8**

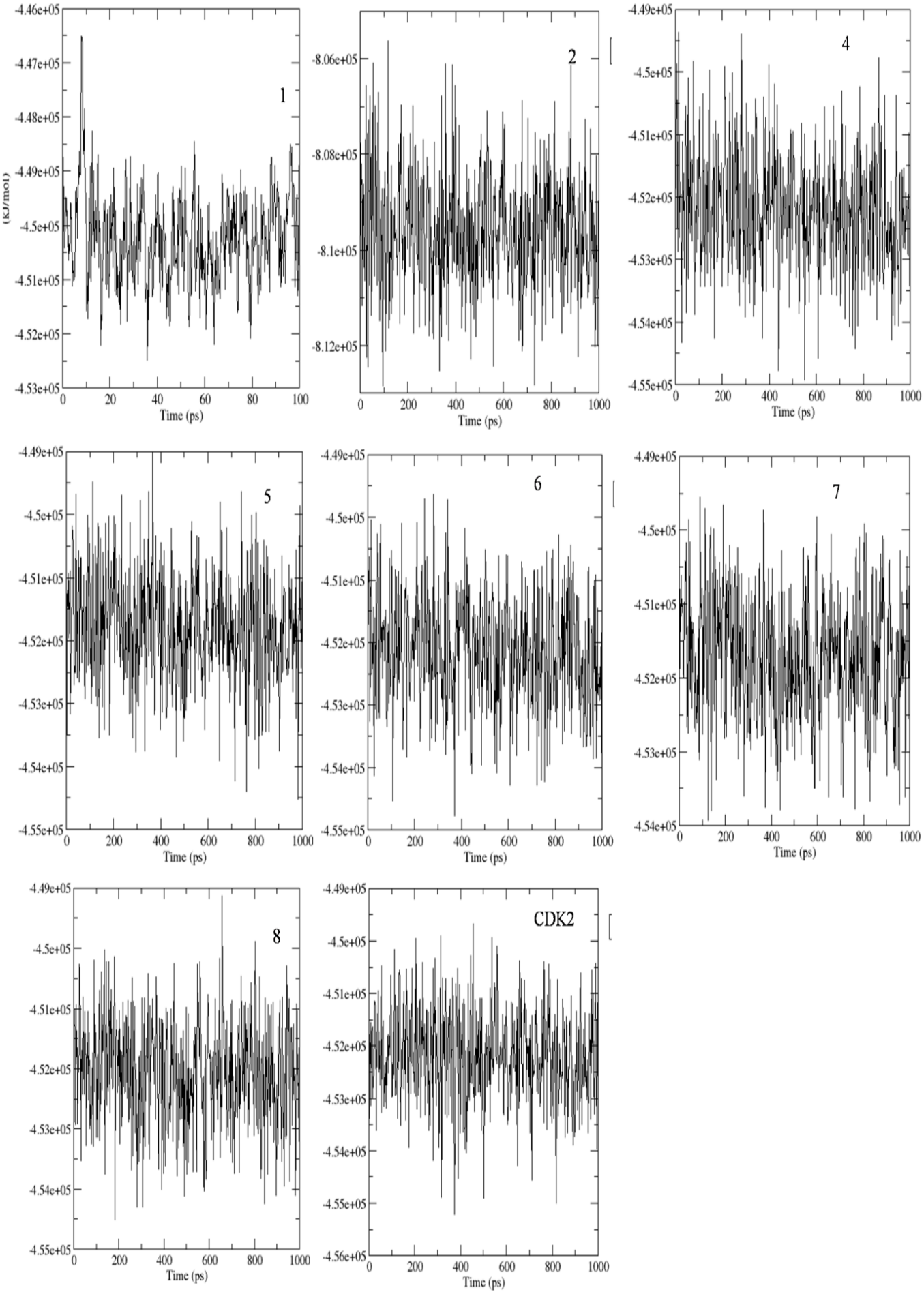

Figure 8: Total energy for complexes and CDK2 bare protein

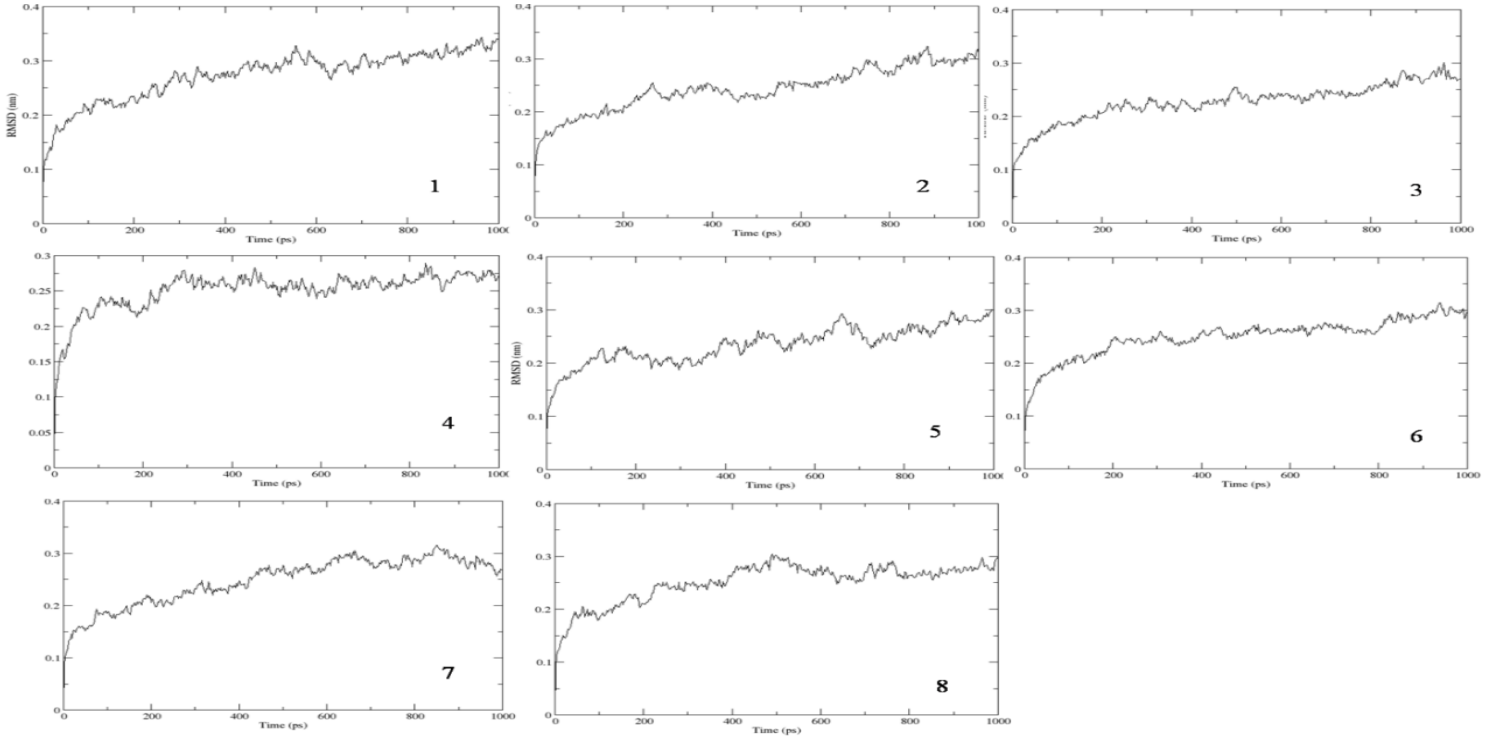

Figure 9: RMSD between energy minimized protein and protein subjected to MD simulation

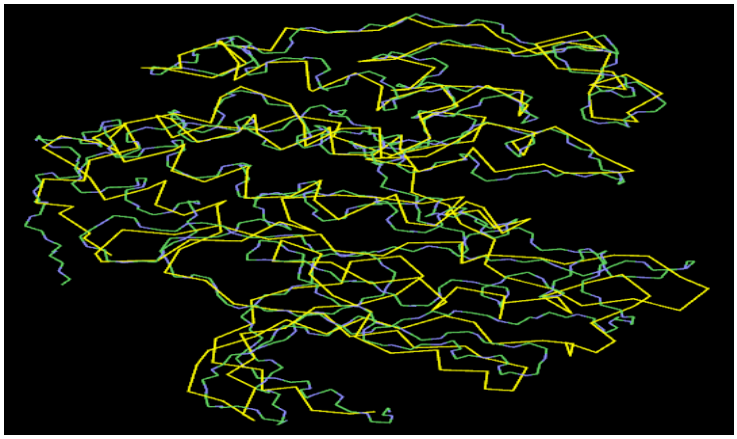

Figure 10: Backbone residues of complex **1** and original CDK2 (shown in yellow)

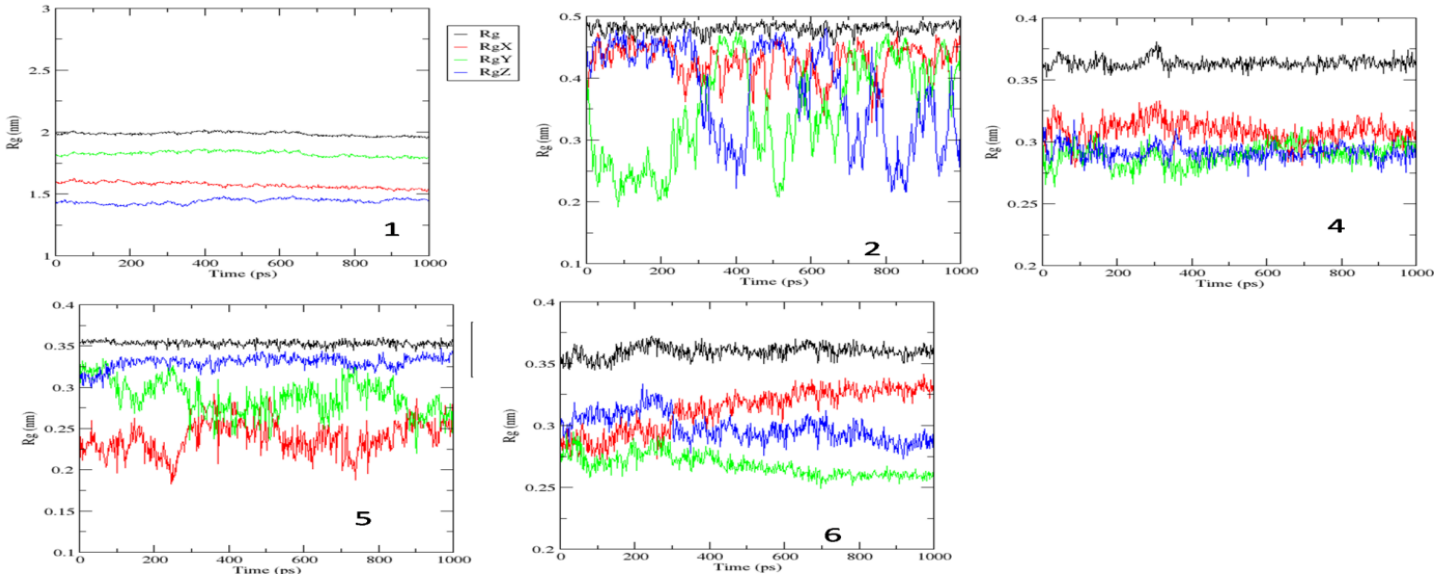

Figure 11: Radius of gyration of ligand center of gyration around the center of protein gyration for complex **1**, **2**, **4**, **5**, and **6**.

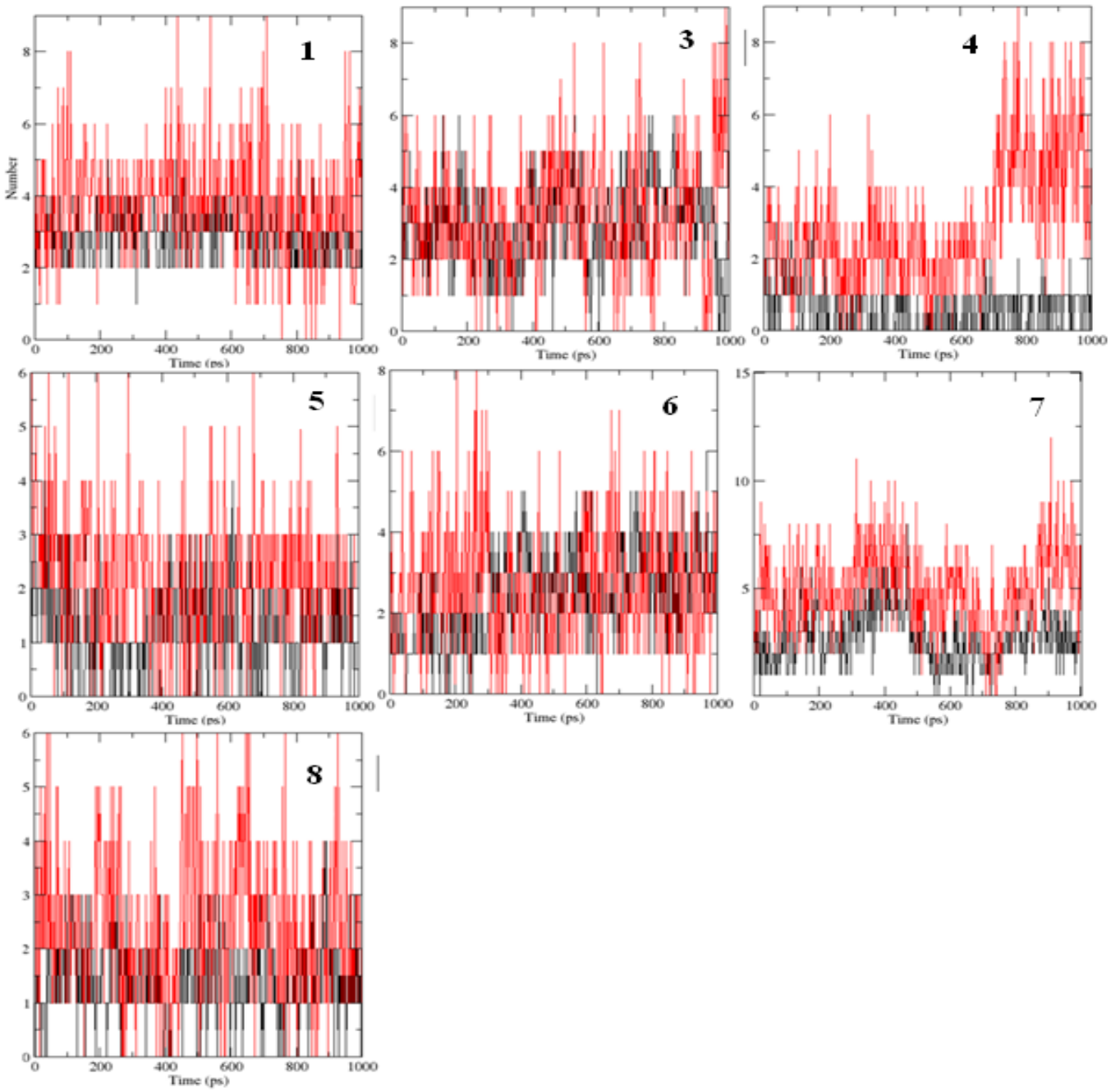

Figure 12: Number of hydrogen bonds formed during simulation between ligand and CDK 2 residues.

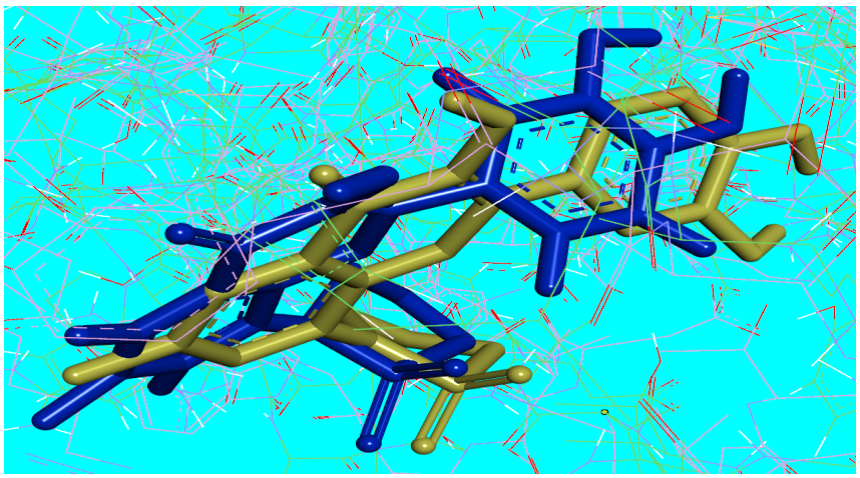

Figure 13: Representation of docked conformer shown in blue and conformer generated in last trajectory shown in yellow.

Table 1: Autodock 4.2 docking results

Comp.	Reported IC50 (nM)	Free Energy of Binding (kcal/mol)	Inhibition Constant, Ki (nM)	HB	Interacting residues
1	87	-10.24	31.29	4	Leu83, Glu81, Asp145(2)*
2	14360	-7.11	6140	5	Leu83, Glu81, Asp145, Ala144, Gln131
3	5000	-8.43	662.27	5	Leu83, Glu81, Asp145(2)*, Asp86
4	100000	-8.48	608.03	3	Leu83, Glu81, Asp145
5	370000	-6.88	9060	2	Leu83, Asp145
6	224	-8.79	361.03	3	Leu83, Asp145, Glu81
7	40000	-7.8	1920	5	Leu83, Glu81, Asp145(2)*, Asp86
8	NA	-6.91	8590	3	Leu83, Asp145, Glu81

HB: Number of hydrogen bonds formed, * Number of hydrogen bonds formed with residue

Table 2: Calculated energy terms for complexes

Energy type	CX- 1	CX- 2	CX- 3	CX -4	CX- 5	CX- 6	CX- 7	CX- 8	CX
Total (kJ/mol)	-4.5×10^5	-8.0×10^4	-5.9×10^5	-4.5×10^5	-4.5×10^5	-4.5×10^5	-4.5×10^5	-4.5×10^5	-4.5×10^5
Potential (kJ/mol)	-5.5×10^5	-9.8×10^5	-10.8×10^5	-5.5×10^5	-5.5×10^5	-5.5×10^5	-5.5×10^5	-5.5×10^5	-5.5×10^5
Temperature (K)	299.99	299.99	299.99	299.99	299.99	299.99	299.99	299.99	299.99
Pressure (bar)	3.75	-1.12	-0.63	-1.35	-0.85	-0.15	-1.01	-0.16	-1.51
Coulomb-SR (kJ/mol)*	-186.24	5.85×10^{-6}	-41.77	-35.42	-23.64	-67.19	-38.15	-17.85	NA
LJ-SR (kJ/mol)*	-173.71	-4.42×10^{-5}	-157.14	-189.41	-141.171	-188.902	-165.662	-132.531	NA
Volume (nm^3)	421	420.22	420.25	420.28	420.3	420.35	420.12	420.44	420.33
Density (kg/m^3)	1077.22	1016.50	1016.47	1016.92	1016.5	1016.75	1016.83	1016.65	1016
Enthalpy (kJ/mol)	-4.5×10^5	-4.5×10^5	-4.5×10^5	-4.5×10^5	-4.5×10^5	-4.5×10^5	-4.5×10^5	-4.5×10^5	-4.5×10^5

CX: Complex of CDK2 with referred ligand or ligand stripped bare CDK2 protein

* Determined for selected residues at binding site with ligand

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