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Molecular Docking of Known Carcinogen 4-(Methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) with Cyclin Dependent Kinases towards Its Potential Role in Cell Cycle Perturbation

Mohd Haneef[1], Mohtashim Lohani[1]*, Anupam Dhasmana[2], Qazi M.S. Jamal[1], S.M.A. Shahid[1] & Sumbul Firdaus[3]

[1]Department of Biosciences, Integral University, Lucknow-226026, UP, India; [2]Department of Bioengineering, Integral University, Lucknow-226026, UP, India; 3Department of Physics, Integral University, Lucknow-226026, UP, India; Mohtashim Lohani - Email: mlohani@rediffmail.com; Phone: 0522-2890812, 2890730, 3296117, 6451039; *Corresponding author

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Abstract:
Cell cycle is maintained almost all the times and is controlled by various regulatory proteins and their complexes (Cdk+Cyclin) in different phases of interphase (G_1, S and G_2) and mitosis of cell cycle. A number of mechanisms have been proposed for the initiation and progression of carcinogenesis by abruption in cell cycle process. One of the important features of cancer/carcinogenesis is functional loss of these cell cycle regulatory proteins particularly in CDKs and cyclins. We hypothesize that there is a direct involvement of these cell cycle regulatory proteins not only at the genetic level but also proteins level, during the initiation of carcinogenesis. Therefore, it becomes significant to determine inconsistency in the functioning of regulatory proteins due to interaction with carcinogen 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Hence, we investigated the interaction efficiency of NNK, against cell cycle regulatory proteins. We found a different value of ΔG (free energy of binding) among the studied proteins ranging between *-3.29 to -7.25 kcal/mol* was observed. To validate the results, we considered Human Oxy-Hemoglobin at 1.25 Å Resolution, [PDB_ID:1HHO] as a *+ve control*, (binding *energy -6.06 kcal/mol*). Finally, the *CDK8* (PDB_ID:3RGF) and *CDK2* (PDB_ID:3DDP) regulatory proteins showing significantly strong molecular interaction with NNK *-7.25 kcal/mol, -6.19 kcal/mol* respectively were analyzed in details. In this study we predicted that CDK8 protein fails to form functional complex with its complementary partner cyclin C in presence of NNK. Consequently, inconsistency of functioning in regulatory proteins might lead to the abruption in cell cycle progression; contribute to the loss of cell cycle control and subsequently increasing the possibility of carcinogenesis.

Keywords: Cell cycle, Carcinogen NNK, Check points, CDKs, Oncoinformatics and Z-Dock.

Background:
Cell cycle is controlled by various regulatory proteins (check points) for proper cell division. Infact, cell division of cell is divided into two stages: mitosis (M) and interphase (I) including G1, S and G2 transition phases **[1].** The transition from one phase to another phase occurs in an orderly fashion and is regulated by different type of cellular proteins, particularly cyclin-dependent kinases (CDKs-family of serine/threonine protein kinases) and cyclins are activated/deactivated at specific points. Therefore, CDKs

induce downstream processes by phosphorylating the regulatory proteins in cell cycle **[2].** However, different types of cyclins D (cyclin D1, cyclin D2 and cyclin D3) are essential for the regulation of cell cycle. Further, cyclins bind to CDKs and form CDK-cyclin complex. This complex/s is/are very important for phase progression in cell cycle **[3].** The functional activity of that CDK-cyclin complex is induced conformational changes due to phosphorylation in conserved threonine and tyrosine residues of CDKs part. Thus the complex (Cdk+Cyclin) enhances or suppresses the binding efficiency of their specific cyclin partner **[4].** In case of cancer, mutations have been observed in genes encoding CDKs, Cyclins (D1, D2, D3, A, H, T & C), CDK-activating enzymes (CAK), CKI and CDK-substrates **[5].** Consequently, changes in functioning of CDKs activity to formed Cdk+Cyclin complex, an important part of many cancers, as well as other disease states, generally through elevated and/or inappropriate activation **[6].** One of the probable ways of loss of cell cycle control could be disruption of these regulatory proteins CDKs (check points) by a direct interaction with chemical/carcinogen. In the present study, we investigated the possible molecular interactions between CDKs, cyclins with potent cigarette smoke carcinogen 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-butanone (NNK).

NNK is a yellow crystalline compound with a molecular formula $C_{10}H_{13}N_3O_2$, (Mol Weight = 207.2316) derived from tobacco alkaloids (nitrosamine) as a potent carcinogen **[7].** The concentrations of NNK in tobacco substances such as 1-20 µg/g, 20-310 ng/cigarette and ≤26 ng/m^3 in snuff, cigarette mainstream smoke, and in indoor air respectively **[8].** The existences of substantial amount of NNK in tobacco products play a very significant role as a main cause of cancer in population **[9].** Ultimately, NNK is considered as a major contributor as well as risk factor to lung carcinogenesis **[10-11].** NNK and its metabolite NNAL, has already been reported to molecular interaction with DNA repair proteins primarily by our group **[12].**

We analyzed the molecular interaction, based on binding efficiency of potent carcinogen NNK against CDKs (check points) and cyclins involved in the cell cycle process. In case of cancer, cells develop an autonomous set of instructions against normal rules, leading to uncontrolled, undifferentiated growth and proliferation called an abnormal condition of a cell known as carcinogenicity**.** The carcinogen NNK and its metabolite NNAL directly bind with DNA repair proteins to make DNA adduct. Therefore, possibility exists that NNK may directly interact with these regulatory protein (CDKs, cyclin) and affect the functional activity of CDK-cyclin complex in cell cycle regulation. The carcinogen NNK induced cell cycle abruption may in turn result in hastened DNA replication, with a compromised proofreading by DNA pol/RNA pol. This process in a whole may give rise to daughter cell with loads of mutations in their DNA. Therefore we designed this study to investigate and determine whether the carcinogen NNK, apart from directly causing damage to regulatory proteins, is also capable of affecting their functionalities in term of binding efficiency of CDKs to their specific cyclins partner (A, C, D, D_1, D_2, D_3, E, and H) for proper signal to execute cell cycle phases normally.

Methodology:

Preparation of receptor- protein structures

The 3D structures of check point proteins (CDKs & Cyclins) involved in cell cycle regulation were obtained from PDB (Protein Data Bank) and some other proteins retrieved from MODBASE server **Table 1 (see supplementary material)**. MODBASE is a queryable database of annotated protein structures models, theoretically calculated models, which may contain significant errors, not experimentally determined structures **[13].** Published protein structures were edited, to remove HETATM by using Discovery Studio Visualizer (Version 2.5.5). And Chimera was used for energy minimization, removal of steric collision (forces) with the steepest descent steps 1000, steepest descent size 0.02 Å, conjugated gradient steps 1000 and gradient step size 0.02 Å for the conjugate gradient minimization **[14-15].** Protein structure visualization and image generation were performed using PyMOL software (DeLano Scientific, Palo Alto, CA).

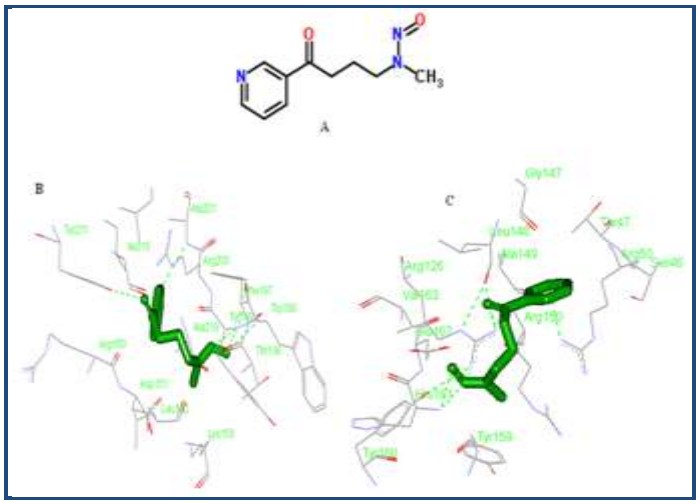

Figure 1: (A) 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanonePubChem Compound ID- 47289,ChemSpider ID-43038; **(B)** 3RGF:CDK8 interact with carcinogen NNK; **(C)** 3DDP:CDK2 interact with carcinogen NNK

Preparation of ligand structure

Potent cigarette smoke carcinogen NNK (4-(Methylnitrosamino)-1-(3-pyridyl)-1- butanone) ligand file was retrieved in (dot).mol format **Table 2 (see supplementary material) (Figure 1)** from latest version of Chem Spider Chemical Database. This file format could not directly use by Autodock (4.0) tool **[16].** Thus that file was finally converted it into.(dot)pdb file format using DS Visualizer (version 2.5.5), so it easier to comprehend chemoinformatics and molecular mechanics of ligand and different receptor proteins interacting molecules. Further, ligand was submitted for minimization by using Chimera (version 1.5.3) with Genetic Algorithm Steps 2000 and 0.5 grid units Optimized **[17]**.

In silico Studies

In silico studies were performed by Autodock version 4.0 suit with Cygwin interface tool **[18-19]**. We selected molecular docking methods for CDKs and NNK interaction, followed by retrieving the best conformations of check point (CDKs) as regulatory proteins and carcinogen (NNK), on the basis of binding energy value (kcal/mol). First of all, we marked all

water molecules (H_2O) in proteins then removed from targeted protein structure, before apply docking performance. Then only hydrogen atoms were added to all target proteins. After that Kollman united charges and salvation parameters were applied to selected regulatory proteins (CDKs and cyclins). Gasteiger charge also was charged to ligand (NNK). Then defined Grid box was set to cover the maximum part (including target site) of selected protein for ligand interaction. The value was set to standard 60×60×60 Å in X, Y and Z coordinate of grid point with default value of grid points spacing 0.375 Å. Lamarckian Genetic Algorithm (LGA) was applied for receptor protein and ligand for flexible docking calculations **[20]**. The LGA parameters like population size (ga_pop_size), energy evaluations (ga_num_generation), mutation rate, crossover rate and step size were set to 150, 2500000, 27000, 0.02, 0.8 and 0.2 Å, respectively. The LGA runs were set to as standard 50 runs. We observed all 50 conformations of selected proteins with ligand complex were analyzed for the interaction orientations including binding energy of the docked structure using Discovery Studio Visualizer version 2.5.5.

Protein-Protein Interaction analysis

The interacting regulatory proteins (CDKs) and cyclins were found using STRING 9.0 database that predict, interacting proteins against your query. We found interface residues in CDKs & Cyclins using PDBe PISA, an interactive tool for the exploration of macromolecular (protein, DNA/RNA and ligand) interfaces **[21]**. The Discovery studio 2.5 was used for Zdock (Dock Proteins) and Zdock score obtained from Protein-Protein interactions (Cdk+Cyclin) as well as from Protein-Complex (NNK+CDK & Cyclin) interaction.

Z dock calculations

Zdock is one of the successful suites that have shown great prediction abilities in Critical Assessment of Predicted Interactions (CAPRI) **[22].** Zdock uses a fast Fourier transform to search all possible binding modes for the proteins, evaluating based on shape, desolvation energy, and electrostatics. The top 2000 predictions from Zdock where they are minimized by CHARMM with create fixed atom constraint in backbone of protein and again create Harmonic restraint in selected protein for improving the energies and eliminate clashes. ZDOCK is an initial stage rigid body molecular docking algorithm that uses a fast Fourier transform (FFT) algorithm to improve performance for searching in translational **[23]**. All of the available structures from NMR were used to calculate docking poses and the structures obtained were subjected to energy minimization using, smart minimize algorithm (Max steps 200, RMS gradient 0.01) in the program D.S. 2.5. The resulting highest values of score were used as appropriate conformational pose with Zdock score value **Table 3 (see supplementary material).**

Results:

The ligand structure NNK (4-(Methylnitrosamino)-1-(3-Pyridyl)-1-butanone) as a potent cigarette smoke carcinogen described in this study was retrieved from latest version of Chem Spider Chemical Database in (dot).mol format **(Table 2 & Figure 1A)** with PDB-ID: 1B17. The structures of the cell cycle regulatory proteins CDKs (CDK8:3RGF, CDK2:3DDP, CDK7:1UA2, CDK6:1BLX, CDK9:3BLH & CDK4:3G33) obtained from PDB and other complementary partner protein cyclin (cyclin A: 1JSU, cyclin H: 1KXU, cyclin D3: 3G33, cyclin D: 2W9F, cyclin C: 1ZP2 and cyclin T: 3BLR) were retrieved from MODBASE server. **Table 1 & Table 4 (see supplementary material)** show the docked score (binding energy) of ligand NNK against cyclin dependents kinases (CDKs), complementary partner proteins cyclins. While **Figure 1 B & C** were indicated the docking images with best molecular interaction orientation. In this *in silico* study, the docking scores (binding energy) of *CDK8* and *CDK2* regulatory proteins against the carcinogen NNK, ***-7.25 kcal/mol & -6.19 Kcal/mol*** respectively were more than that of *positive control Human oxy-hemoglobin* binding energy ***-6.06 kcal/mol***. The residues *ARG* 150, *ASP* 151, *LEU* 152, *LYS* 153, THR 196, PHE 197, TRP 198, *TYR* 199, ARG 200, *ALA* 201, LEU 204, TYR 211, ILE 215 and ALA 219 of CDK8 and ILE 10, *VAL* 18, *ALA* 31, *LYS* 33, *VAL* 64, PHE 80, GLU 81, PHE 82, *LEU* 83, HIS 84, GLN 85, *ASP* 86, *LEU* 134 and *ALA* 144 of CDK2 actively participate in molecular interaction with NNK as shown in **Table 5 (see supplementary material)**. The residues ARG, ASP, LEU, LYS, TYR, VAL and ALA from all regulatory proteins (CDKs) commonly interacted with carcinogen NNK through hydrogen bonds. The functional integrity with interaction efficiency of CDKs once bound to NNK was also evaluated by using Zdock method. We obtained Zdock-scores of CDK8 with its corresponding partner cyclin C was *30.12*, and of CDK2 with its corresponding partner cyclin A was *21.62*. Whereas, the complex *CDK8+NNK* when docked with its partner cyclin C showed Zdock score *19.12*, and *CDK2+NNK* when docked with its partner cyclin A showed Zdock score *21.34*. Reduction in the Zdock score of CDK8 after it forms complex with NNK may represent a loss in its capacity to bind with partner cyclin C, once bound with NNK. Whereas, regulatory protein *CDK2* doesn't show significant change in Zdock value, after binding with NNK as shown in **(Table 3).**

Discussion:

An *insilico* approaches were applied to evaluate protein-ligand and protein-protein interaction (PPI) for identifying possible targets of carcinogen (NNK) amongst cyclin dependent-kinases (CDKs) as well as with their respective partner cyclins. In order to gather in-depth knowledge on, an important issue that how the cigarette smoke carcinogen NNK interfere the mechanism of signals through CDK complex (Cdks+Cyclins) in proliferation, cell division and in abruption of cell cycle. In this study, we characterized and identified the molecular interaction of NNK with all regulatory proteins (CDK2, CDK4, CDK6, CDK7, CDK8, and CDK9) and with their respective partner cyclins (Cyclin A, Cyclin E, Cyclin D, D1, D2, D3, Cyclin C, Cyclin H, and Cyclin T) by using Autodock and Zdock (protein-protein interaction) methods. To validate our *in silico* study, we considered Human oxy-hemoglobin at 1.25 Å resolution, [PDB ID-1HHO] as a *+ve control*, which showed binding energy of *-6.06 kcal/mol,* as the binding of Human oxy-hemoglobin with NNK has previously been quantified in tobacco users and is considered as a biochemical marker for uptake of tobacco specific nitrosamines **[24].** The docking outputs indicated that potent cigarette smoke carcinogen NNK shows the binding efficiency (ΔG) against cell cycle regulatory enzymes/proteins (CDKs) ranging**, *-3.95 to -7.25 kcal /mol*** **(Table 1).** Simulations depicted that *two* regulatory proteins *CDK8 (3RGF) & CDK2 (3DDP)* showed better potential to bind

carcinogen NNK as *compared to +ve control*, *i.e.* ***-7.25 & -6.19 Kcal/Mol*** (**Figure 1 B & C**) respectively. During the protein-ligand interaction, the amino acid residues of CDK8 involved in interaction with ligand (NNK) were identified as ARG150, ASP151, LEU152, LYS153, THR196, PHE197, TRP198, TYR199, ARG200, ALA201, LEU204, TYR211, ILE215, and ALA219. While, in case of protein CDK2 the amino acid residues, namely ILE10, VAL18, ALA31, LYS33, VAL64, PHE80, GLU81, PHE82, LEU83, HIS84, GLN85, ASP86, LEU134, and ALA144 were found to be involved efficiently in the interaction with ligand NNK. In fact, amino acid residues, particularly ARG, ASP, LEU, LYS, TYR, VAL and ALA of both CDK8 and CDK2 were found to be essential for the interaction of carcinogen NNK **(Table 5).** This molecular interaction between CDKs and NNK is validated on the basis of their binding energy (ΔG) obtained from best docked conformations. It has been reported that *Cyclin Dependent Kinase-8* is actively involved in the regulation of mRNA transcription and considered as a potent oncogene in colon carcinogenesis. In addition, mutated or amplified CDK8 with increased expression is a common observation during a variety of human cancers **[25]**. However, we further explored the interaction impact of NNK on *CDK8 (3RGF)* in term of binding efficiency towards its respective partner cyclin C. For this purpose, we applied an *in silico* Zdock method for calculating Zdock score of protein-protein interaction (PPI) between CDK8 Vs Cyclin C complex and further compared it with protein complex interaction (CDK8+NNK Vs Cyclin C) by using Discovery studio 2.5. Results clearly depicted that significant loss of binding energy of CDK8 Vs Cyclin C from *30.12 (PPI) to 19.12* for NNK bound with CDK 8 complex Vs Cyclin C (PCI) at coordinates X -17.585, Y 11.939 & Z 17.689. These results predicted that binding of NNK at the active site of CDK8 strongly interferes with the natural binding of cyclin C to the active site of CDK8, rendering it unable to form functional complex (CDK8+cyclin C). It has been reported that CDK8 positively regulates transcription, by directly phosphorylating p53 and histone H3, or by facilitating assembly of Pol II elongation complex [**26**]. The CDK8-cyclin C complex is the part of RNA Polymerase II, which regulate the transcription of general transcription initiation factor IIH (TFIIH), controlling the basal transcription machinery. Failing the formation of CDK8-cyclin C complex may eventually will result in failure of the transcriptional regulation of a member of RNA-Pol-II dependent genes **[27].** CDK8 plays significant role in regulating cell cycle progression [**28**] And CDK 8-cyclin C complex abnormality has many times reported to result in tumorogenesis **[29].** When we performed same Zdock study with other protein CDK2, it was observed that NNK binding with the CDK2 didn't significantly interfere natural binding of its partner cyclin A **Table 3.** Previously QMS Jamal, et al., 2012 presented Zdock based analysis to determine the loss/incapability in formation of functional complex of regulatory proteins in DNA repair pathways after its binding with a concerned chemical/carcinogen **[12].** The binding of NNK with CDK8 therefore, may be an important event in carcinogenesis caused by cigarette smoke carcinogen and should be studied in depth.

Conclusion:

In silico study explores the interaction of CDK8 (cyclin dependent kinase-8) with NNK, a widely inhaled potent cigarette smoke carcinogen among the young generation of population. CDK8, cyclin C and its complex (CDK8+cyclin C) are the key mediator of cell cycle progression which play a vital role in cell cycle perturbation due to potential interaction of cigarette smoke carcinogen NNK. The hydrogen bonds and certain amino acid residues ARG, ASP, LEU, LYS, TYR, VAL and ALA play a key role in the correct positioning of CDKs within the active site of NNK to permit docking interaction. The effect of molecular interaction of NNK on the binding with CDKs is elucidated. Study indicated the loss of functional complex of these enzymes/regulatory proteins (Cdk+Cyclin), which probably could be a reason for perturbation in cell cycle process resulting in occupied active site of CDKs by NNK. Furthermore, our study suggests that carcinogens (NNK) positively alter the mechanisms of cell cycle progression pathways and enzymes functioning could be affected by carcinogens. Computer based structural analysis of bio macromolecules and their molecular interactions (ligand and protein) could play an important role in assessment of risk to a number of diseases including cancer**.** At last but not least, a deep analysis is needed to elucidate the perturbation of cell cycle mechanisms with best suitable techniques and tools. *In vivo* and *In vitro* validation is needed to authenticate *insilico* results obtained from this study.

Conflict of Interest:

We have no conflict of interest with anybody working in the area and among the authors in the manuscript.

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Supplementary material:

Table 1: Cyclin Dependent Kinases (Cdks) docked with carcinogen NNK, results obtained by Autodock 4.0

S.N.	PDB_ Id	Name of Proteins	Binding Energies (Kcal/Mol)	Estimated Inhibition Constant (μm)	Reference r.m.s.d.*
1	**3RGF**	**CDK8**	**-7.25**	**4.84**	**24.50**
2	**3DDP**	**CDK2**	**-6.19**	**28.79**	**34.47**
3	1UA2	CDK7	-6.02	38.41	34.41
4	1BLX	CDK6	-5.68	68.61	73.14
5	3BLH	CDK9	-4.29	711.60	56.20
6	3G33	CDK4	-3.95	128.00	71.85

***rmsd: root mean square deviation**

Table 2: Fact of Potent Carcinogen NNK

Carcinogen	IUPAC Name	Mol. Formula	Mol. Weight	Smiles
NNK	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone	$C_{10}H_{13}N_3O_2$	207.22912 g/mol	CN(CCCC(=O)C1=CN=CC=C1)N=O

Table 3: Protein–Protein Interaction of Cyclin Dependent-Kinases (CDKs) and their Respective Regulatory Proteins (Cyclins)

S. N.	PDB _Id	Name of Proteins	Name of Regulatory Proteins	Coordinates of Docked Pose (X, Y, And Z Respectively)	Zdock Score	Protein-Complex * Zdock Score
1	3RGF	CDK8	Cyclin C	-17.585, 11.939 & 17.689	**30.12**	**19.12**
2	3DDP	CDK2	Cyclin A	13.731, 29.461 & 8.810	21. 62	21.34

***Complex contain NNK+CDKs Vs Cyclins**

Table 3 4: Regulatory Proteins (Cyclins) Docked with Carcinogen NNK, Results Obtain By Autodock 4.0

S.N.	Pdb_Id	Name of Proteins	Binding Energies (Kcal/Mol)	Estimated Inhibition Constant (μm)	Reference r.m.s.d.
1	1JSU	Cyclin A	**-6.00**	40.24	55.67
2	1KXU	Cyclin H	-5.68	68.93	93.16
3	3G33	Cyclin D3	-5.35	119.87	67.33
4	2W9F	Cyclin D	-4.98	222.64	88.45
5	1ZP2	Cyclin C	-4.85	285.63	98.37
6	3BLR	Cyclin T	-3.29	3880.0	39.84

Table 5: Interacting Amino Acid Residues Involved in Complexes formation with NNK in Cell Cycle Regulatory Proteins

S.N	Pdb _Id	Name of Proteins	Number of Hydrogen Bonds	Residues Involved In Hydrogen Bonding	Amino Acid Residues Involved In Interaction With NNK Carcinogen	Common Interacting A. A. Residues In Regulatory Proteins
1	3RGF	**CDK8**	6	TYR 199: N- - -PHE 197: O TRP 198: N- - -O 15: NNK TYR 199: N- - -N 14: NNK TYR 199: N- - -O 15: NNK ALA 201: N- - -N 10: NNK TYR 211: OH- - -O 7: NNK	**ARG 150, ASP 151, LEU 152, LYS 153**, THR 196, PHE 197, TRP 198, **TYR 199,** ARG 200, ALA 201, LEU 204, TYR 211, ILE 215, **ALA 219.**	
2	1BLX	**CDK6**	7	ARG 144: HN- - OD1:ASP201 LEU 146: HN - -O15: NNK ARG 186: HH11—O7: NNK ARG	TYR 185, ARG 186, ALA 187, VAL 190, TYR 196, VAL 200, ASP 201, SER 204.	**ARG, ASP, LEU, LYS, TYR, VAL, ALA,**

				186: HH12—OD1:ASP 145 SER 204: HN—O : VAL 200 SER 204: HG— N 14: NNK SER 204: HN—-O 15: NNK	
3	1UA2	**CDK7**	2	ARG 179: N- - -O7: NNK ALA 180: N- - -O15: NNK	ARG 136, ASP 136, LEU 138, LYS 139, THR 175, TYR 178, ARG 179, ALA 180, LEU 183, VAL 194, ALA 198.
4	3BLH	**CDK9**	5	SER115: N- - -O: ALA 111 SER115: N- - -O: GLY 112 ALA152: N - - - O: LYS 151 ALA152: N - - - O 15: NNK TYR 194: OH- - - OE1: GLU 221	ALA 111, LEU 114, SER 115, LYS 151, ALA 152, ALA 153, TYR 194, GLU 221, ARG 225, SER 226, PRO 227.
5	3G33	**CDK4**	9	LYS 35: HZ1- - - ASP158:OD1 LYS 35: HZ1- - - ASP158:OD2 LYS 35: HZ1- - -N 14: NNK LYS 35: HZ1- - -O 15: NNK LYS 35: HZ3- - - ASP158:OD1 LYS 35: HZ1- - - ASP158:OD2 ASN 140: HD21- - - O:ASP140 LYS 142: HZ3- - - -N10: NNK ALA 197: HN - - -O: ASN 145	GLY 57, ALA 16, TYR 17, LYS 35, ASP 140, LYS 142, GLU 144, ASN 145, LEU 147, ALA 157, ASP 158.
6	3DDP	**CDK2**	6	LYS 33: NZ- - -OE1: GLU 51 LYS 33: NZ- - -N 14: NNK LYS 33: NZ- - -O 15: NNK LEU 83: N- - - O 7: NNK GLN 85: N- - -O: ILE 135 ILE 135: N- - -O: 85 GLN	ILE 10, VAL 18, ALA 31, LYS 33, VAL 64, PHE 80, GLU 81, PHE 82, LEU 83, HIS 84, GLN 85, ASP 86, LEU 134, ALA 144.