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Functional implications of a cancer associated $p16^{INK4A}$ mutation insilico.

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Abstract

$P16^{INK4A}$ a tumour suppressor protein is found frequently mutated in a variety of human cancers. We have previously identified a novel 7 base pair deletion that associates with esophageal squamous cell carcinoma (ESCC). Here we describe its functional consequences through 3 D modeling, insilico structural analysis and ligand docking. Our data indicates that the structural perturbations, together with energy changes impose a serious constraint on the mutant protein to optimally interact with its ligands (CDK4 and CDK6).

Introduction

Tumour suppressor gene, $p16^{INK4A}$, located at chromosome 9p21, is a specific CDK 4/6 inhibitor. It is considered to be one of the most altered genes in a variety of human cancers. Its inactivation is known to be through a variety of processes including homozygous deletions, point mutations and hypermethylation in CpG islands (1-16) Homozygous deletions of p16INK4A gene and other loss of function mutations, reported in a variety- of tumours, substantiate its tumor suppressor role thereby pointing to the considerable role the protein P16 plays in the cell cycle progression at G1-S checkpoint (1,17). Loss of P16 function, rendering the protein unable to bind or inhibit CDKs, can result in uncontrolled cell proliferation leading to tumorogenesis (18-27). A variable spectrum of $p16^{INK4A}$ mutations has been observed in esophageal squamous cell carcinoma among different populations of the world (28).

P16 is comprised mainly of four ankyrin repeats, which are believed to mediate protein-protein interactions. There is a large contact surface between p16 and Cdk4, and many amino acids throughout the four ankyrin repeats are important for the interaction (29). Protein-protein interaction studies alongwith the recent developments in proteomics and computational prediction of protein-protein interactions have contributed to a surge in the amount of protein structural and functional information or structural protein interaction knowledge (30). Molecular dynamic simulation studies have been of lately used to study the probable impact of various gene mutations on the structure of $p16^{INK4A}$. These simulation studies have been helpful in gaining insight into the key interactions between cyclin-dependent kinases and the $p16^{INK4A}$. Thus designing small molecules/compounds which mimic the inhibition of $p16^{INK4A}$ appears to be a promising way to treat cancer (31).

Computer simulation techniques have become a major tool in the analysis of biomolecular properties and behavior. These techniques are used extensively in drug and protein design projects, because they can provide information that is complementary to experimental data.

Docking is a term used for computational schemes that attempt to find the "best" matching between two molecules: a receptor and a ligand. The molecular docking problem can be defined as predicting the "correct" bound association of these two molecules, when their atomic coordinates are given (32). Since molecular docking explores the binding modes of two interacting molecules, a phenomenon fundamental to the course of drug designing, this technique has now become increasingly popular for studying protein-ligand interactions and for drug design. (33). It has been shown in the literature that these computational techniques can strongly support and help the design of novel, more potent inhibitors by revealing the mechanism of drug-receptor interaction (34-35).

More ever drug discovery process has been greatly changed by the adoption of computational methods which help in designing of new drug candidates rapidly and at lower costs. Most importantly various anti-cancer drugs are being created thereby helping millions of cancer patients (36-37).

Therefore a better understanding of key interactions between tumour suppresor protein p16 and Cdk4/6 is necessary to gain information relevant for designing small molecules having similar inhibitory potential as that of p16. This can be considered as a first step in the development of new therapeutic agents targeting p16, to treat cancer (38).

The study was undertaken to predict the effect of 7 bp deletion on structural and functional attributes of p16, by predictive molecular modeling and molecular docking. Molecular docking studies like this help in making decisions regarding drug designing at various steps such as identification of molecular targets and their selection and modifications to obtain better affinities, including pharmacokinetic and pharmacodynamic properties (37).

Methodology

Sequence Submission for 3D Modeling

The amino acid sequence of the wildtype, mutant protein, and CDK6 in fasta format obtained from (NCBI) (www.ncbi.nlm.nih.gov) was submitted to an automated server (I-TASSER) (zhang.bioinformatics.ku.edu/I-TASSER) for 3D structure prediction (38-42). The server furnishes the predicted 3 D structure in a pdb format.

Viewing the PDB Files and Free Energy Calculations

Swiss PDB Viewer was used for viewing pdb files and computing the free energy of the predicted 3D structures as well as those of docked conformations obtained by DOT 1.0 (http://nrc.bu.edu/cluster/clusdoc.html) (43-45).

Quality assessment of the predicted 3 d structures.

Quality assessment of the model structures was performed by using, Ramachandran Plot, one of the several toolchains available on iMolTalk (http://i.moltalk.org). iMolTalk is an interactive web server for protein structure analysis. As an input, a protein structure in pdb file format is required to be submitted to the server. The server provides results as user friendly two-dimensional graphical representations and in textual format, ideal for further processing (46). The graph showing Ramachandran plot is divided into core, allowed, generous and disallowed regions.

Molecular Docking

Docking is a computational process used for finding the best matching between two molecules. Many application packages and servers performing docking, such as HEX, DOT, AUTODOCK, and ZDOCK are now available. DOT 1.0 Beta was used to generate the docked conformations of the two proteins viz, Cyclin dependent kinase 6 and P16. CDK 6 was docked with both wildtype as well as mutant p16 protein.

Results and Discussion

3d modeling and prediction of protein tertiary structure:

I TASSER server furnished five PDB files in each case, wildtype and mutant, representing the probable tertiary structures of the protein, with the C-Scores as -0.16, -0.46, -0.22, -2.32, -0.40 and +0.51, -4.13, -2.56, -4.67, -4.96 respectively. C-score is a confidence score for estimating the quality of predicted models by I-TASSER. C-score is typically in the range of [-5, 2], where a C-score of higher value signifies a model with a high confidence and vice-versa. CDK6 was also modeled by I-TASSER (Fig 1d) to obtain its PDB structure for docking purposes.

Incase of wildtype p16 protein, analysis of the four PDB files predicted by I-TASSER, using PDB Viewer confirms the first structure (Fig 1a) with C- score -0.16 as the most energetically favourable one (-5303.213 kJ/mol). While in the case of mutant p16 protein, analysis of the PDB structures confirms that the first structure (Fig 1b) with C-score of +0.51 as the most energetically favourable one (-1923.096 kJ/mol). The total energies of the rest of the structures calculated by the Swiss PDB Viewer are given in table 1.

Table 1: Table shows the total energy of the I-TASSER predicted p16 tertiary structures calculated by Swiss PDB Viewer. Model Wildtype 1(in bold) has the highest C-score of -0.16 among the five I-TASSER server furnished PDB files in case of Wildtype p16 protein and Mutant 1(in bold) with a highest C-score of +0.51 incase of the mutant p16 protein.

S.NO.	PROTEIN MODEL NAME	C-SCORE	ENERGY kJ/mol
1	Wildtype 1	-0.16	-5303.213
2	Wildtype 2	-0.46	- 4699.242
3	Wildtype 3	-0.22	-4584.513
4	Wildtype 4	-2.32	-5237.172
5	Wildtype 5	-0.40	-5395.397
6	Mutant 1	+0.51	-1923.096
7	Mutant 2	-4.13	-717.067
8	Mutant 3	-2.56	-1417.703
9	Mutant 4	-4.67	-1747.270
10	Mutant 5	-4.96	-1358.682

Quality assessment of predicted 3 d structures.

Ramachandran Plot, one of the several toolchains available on iMolTalk (http://i.moltalk.org) is a very powerful tool to identify errors in protein structures. The Ramachandran plot displays the dihedral angles phi (a-carbon to nitrogen) against psi a-carbon to carbonyl carbon) of all residues in a protein molecule.

For individual models obtained from I-TASSER, and for the docked conformations (Fig 2a) obtained by DOT docking program, Ramachandran plots were generated for quality assessment.

The Ramachandran plot of p16 wildtype protein (1A5E) (Fig 3a) shows 97.7 % and I-TASSER modeled p16 wildtype (Fig 3b) shows 96.1 % residues in core and allowed regions respectively, whileas 96.6 % residues of I-TASSER modeled p16 mutant protein (Fig 3c) fall in core and allowed regions. With 0.8 % (1) residue in disallowed region in case of the wildtype p16 protein (1a5e), 1.6% (2) residues in modeled wildtype P16 and 2.6 % (3) in disallowed regions of the mutant (modeled) protein.

Ramachandran Plot was generated for crystallized p16 protein (1A5E) and crystallized p16-CDK6 complex (1bi7) (Fig 2b), for comparison with that of the predicted P16 and P16-CDK6 docked structures.

The Ramachandran plots of the individual chains in the docked wildtype complex (4a&b) and in crystallized p16-CDK6 complex (1bi7) (4c&d), and in the mutant conformation (5a&b) were generated.

The plots of chain A (CDK6) and chain B (P16) of the wildtype (docked) conformation show 88.1 % (generous region included) and 97.2 % residues respectively, in core and allowed regions as compared to the crystallized complex (1bi7), where the plot shows 94.9 % residues in core and allowed regions in case of chain A (CDK6) and 96.2 % residues in case of chain B (P16).

Whileas in case of mutant (docked) complex the Ramachandran plots of chain A (CDK6) and chain B (P16) show 88.1 % and 43.7 % residues respectively, in core and allowed regions (Fig 5a&b). The figure 43.7 % as against 96.2 % residues in core and allowed regions indicates a significant structural distortion in the mutant protein.

Energy Computation

The energies of the individual protein chains and docked conformations (complexes) were calculated by *Energy Minimization* option in SWISS PDB Viewer (Method Section. (Table 2)

Table 2: The total energies of the individual proteins (crystallized p16, modeled & mutant) and docked conformations (complexes).

S.NO	PROTEIN MODEL	ENERGY kJ/mol
1.	Crystallized p16 (1a5e)	-4782.008
2.	Modeled p16	-5303.213
3.	Modeled p16 mutant	-1923.096
4.	Docked Wildtype Complex	-12632.216
5.	Docked Mutant Complex	-11788.572

The total energy of the crystallized wildtype p16 was calculated to be -4782.008 kJ/mol, whileas that of the modeled p16 structure was found to be in close range, -5303.213 kJ/mol.

The total energy of the modeled p16 mutant structure was calculated as -1923.096 kJ/mol.

The total energy of the docked complexes was found to be -12632.216 kJ/mol and -11788.572 kJ/mol for the wildtype and mutant respectively. This was reported by us recently (47).

The assessment of the protein structures by Ramachandran plot shows there is a significant distortion in the mutant p16 structure, where the mutant individual chain has more number of residues in disallowed regions, thereby decreasing the likelihood of its appropriate folding. In addition the plot shows that mutant (docked) complex has only 43.7 % residues in core and allowed regions, with 33 % residues in the disallowed region.

This data is supported by the energy values computed for the individual chains as well as the docked complexes, where the total energy of the modeled p16 mutant structure was found to be higher than that of the wildtype p16 chain. In case of the docked conformations, the mutant p16-CDK6 complex has higher total energy as compared to the wildtype one. This proposed structural instability in the mutant protein with 7 bp deletion can be predicted to be the cause of its functional inefficiency.

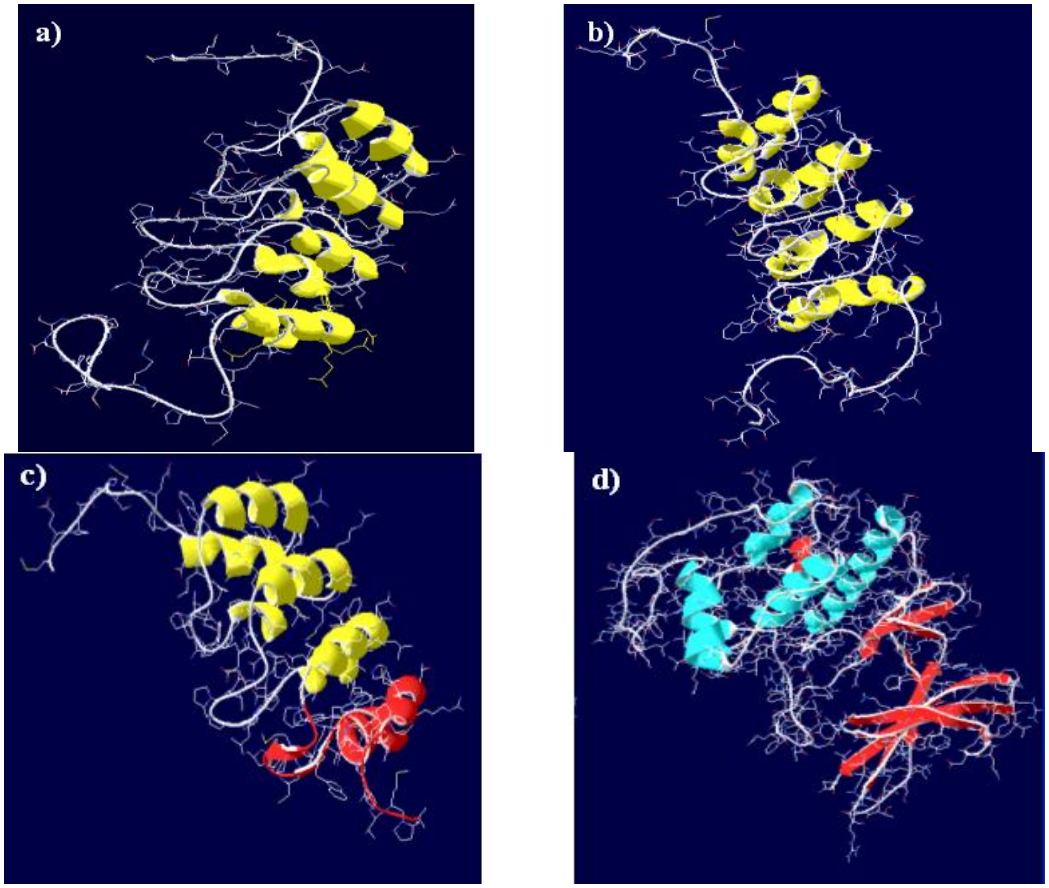


Figure 1: PDB structures viewed by using SWISS PDB Viewer. a) PDB structure of p16 available at Protein Data Bank (entry 1a5e); PDB file furnished by I-TASSER server representing the probable tertiary structure; b) the wildtype p16 protein very similar to 1a5e and (c) mutant p16 protein, mutation observed at the beginning of loop 3 joining ankyrins III and IV of P16, causes frameshift. The altered sequence is shown in red colour; (d) PDB structure of CDK6.

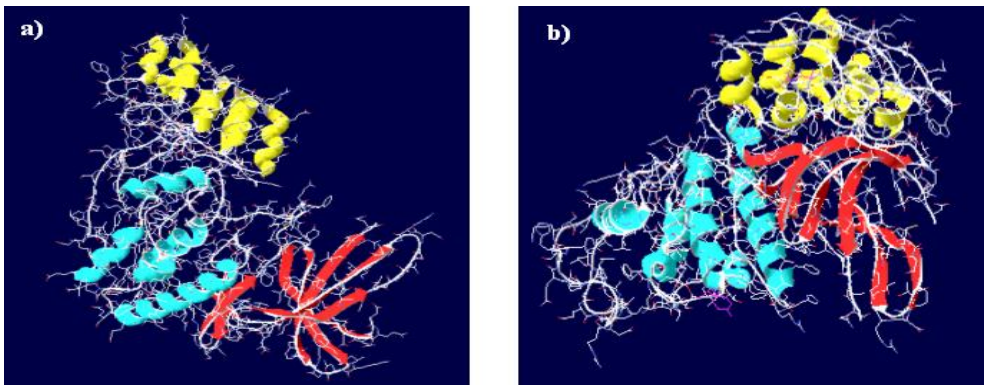
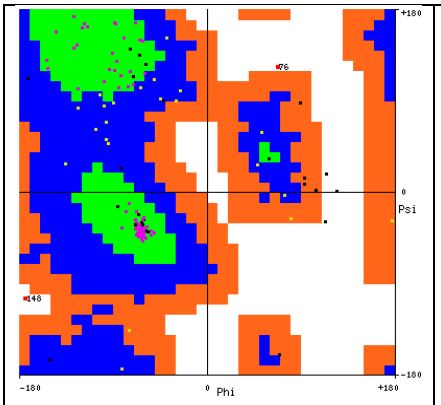
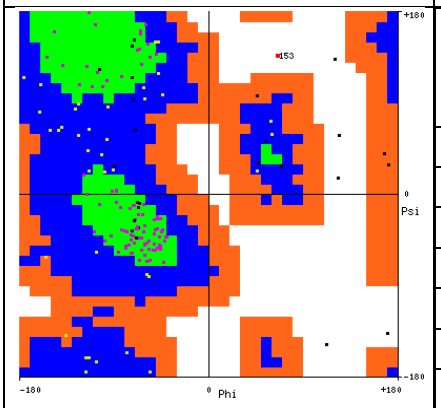


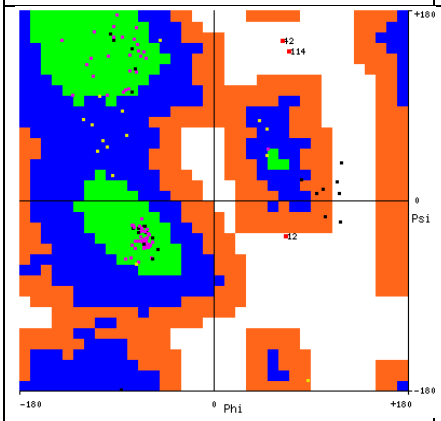
Figure 2: PDB structures viewed by using SWISS PDB Viewer. a) PDB structure of p16-CDK6 docked structure obtained using DOT 1.0; b) crystallized p16-CDK6 complex (1bi7).



The following numbers include residues with Phi/Psi angles calculated, but not GLY and PRO.	
residues in CORE	81.4% (105)
residues in ALLOWED	14.7% (19)
residues in CORE+ALLOWED	96.1 %
residues in GENEROUS	2.3 % (3)
residues in DISALLOWED	1.6 % (2)
number of GLY	14 (9.1%)
number of PRO	11 (7.1%)

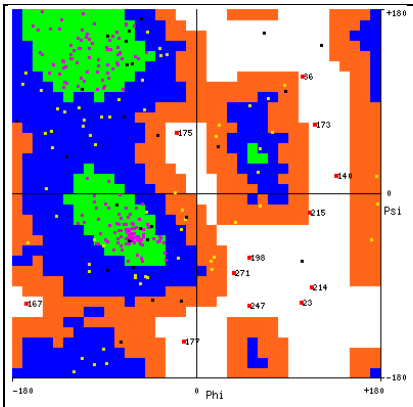


The following numbers include residues with Phi/Psi angles calculated, but not GLY and PRO.	
residues in CORE	70.5 % (91)
residues in ALLOWED	27.1 % (35)
residues in CORE+ALLOWED	97.7 %
residues in GENEROUS	1.6 % (2)
residues in DISALLOWED	0.8 % (1)
number of GLY	14 (9.1%)
number of PRO	11 (7.1%)



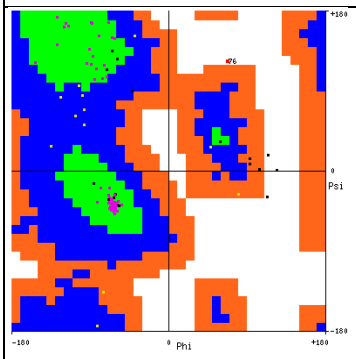
The following numbers include residues with Phi/Psi angles calculated, but not GLY and PRO.	
residues in CORE	84.5 % (98)
residues in ALLOWED	12.1 % (14)
residues in CORE+ALLOWED	96.6 %
residues in GENEROUS	0.9 % (1)
residues in DISALLOWED	2.6 % (3)
number of GLY	11 (7.9 %)
number of PRO	13 (9.3 %)

Figure 3: Ramachandran plot showing the phi-psi torsion angles for all residues in most stable predicted 3 D conformation of P16. The colouring/shading on the plot represents the different regions: the darkest areas (here shown in green) correspond to the "core" regions representing the most favourable combinations of phi-psi values. The different regions on the Ramachandran plot are, as described by Morris *et al.* (49). (a) Crystallized P16 Protein (1a5e); (b) Modeled P16 Protein; c) Modeled P16 Mutant Protein.



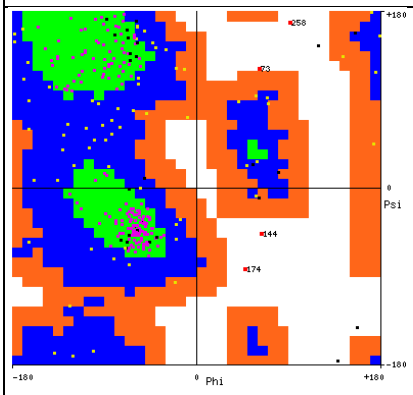
a

The following numbers include residues with Phi/Psi angles calculated, but not GLY and PRO.	
residues in CORE	69.1 % (163)
residues in ALLOWED	19.1 %(45)
residues in CORE+ALLOWED	88.1 %
residues in GENEROUS	6.8 % (16)
residues in DISALLOWED	5.1 % (12)
number of GLY	15 (5.7 %)
number of PRO	14 (5.3 %)



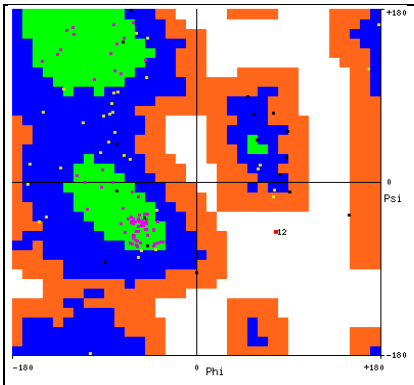
b

The following numbers include residues with Phi/Psi angles calculated, but not GLY and PRO.	
residues in CORE	87.7 % (93)
residues in ALLOWED	12.1 % (14)
residues in CORE+ALLOWED	97.2 %
residues in GENEROUS	0.9 % (1)
residues in DISALLOWED	2.6 % (3)
number of GLY	9 (7.3 %)
number of PRO	8 (6.5 %)



c

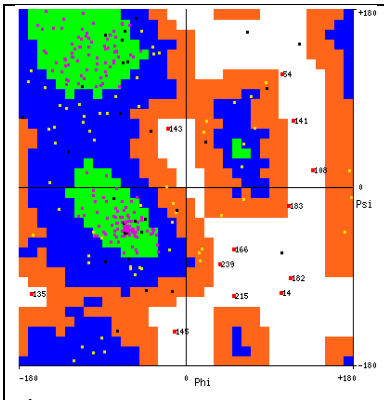
The following numbers include residues with Phi/Psi angles calculated, but not GLY and PRO.	
residues in CORE	70.3 % (166)
residues in ALLOWED	24.6 % (58)
residues in CORE+ALLOWED	94.9 %
residues in GENEROUS	3.4 % (8)
residues in DISALLOWED	1.7 % (4)
number of GLY	15 (5.7 %)
number of PRO	14 (5.3 %)



d

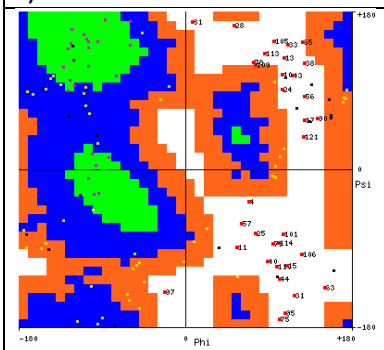
The following numbers include residues with Phi/Psi angles calculated, but not GLY and PRO.	
residues in CORE	67.9 % (72)
residues in ALLOWED	28.3 % (30)
residues in CORE+ALLOWED	96.2 %
residues in GENEROUS	2.8 % (3)
residues in DISALLOWED	0.9 % (1)
number of GLY	9 (7.3 %)
number of PRO	8 (6.5 %)

Figure 4: Ramachandran plot showing the phi-psi torsion angles for all residues in most stable predicted 3 D conformation of P16. The colouring/shading on the plot represents the different regions: the darkest areas (here shown in green) correspond to the "core" regions representing the most favourable combinations of phi-psi values. The different regions on the Ramachandran plot are, as described by Morris et al., (49), (a) Chain A (CDK6) in wildtype docked Complex; (b) Chain B (p16) in wildtype docked Complex; (c) CDK6 in crystallized structure (1bi7); (d) p16 in crystallized structure (1bi7).



a)

The following numbers include residues with Phi/Psi angles calculated, but not GLY and PRO.	
residues in CORE	69.1 % (163)
residues in ALLOWED	19.1 % (45)
residues in CORE+ALLOWED	88.1 %
residues in GENEROUS	6.8 % (16)
residues in DISALLOWED	5.1 % (12)
number of GLY	15 (5.7 %)
number of PRO	14 (5.3 %)



b)

The following numbers include residues with Phi/Psi angles calculated, but not GLY and PRO.	
residues in CORE	22.3 % (23)
residues in ALLOWED	21.4 % (22)
residues in CORE+ALLOWED	43.7 %
residues in GENEROUS	23.3 % (24)
residues in DISALLOWED	33.0 % (34)
number of GLY	10 (8.1 %)
number of PRO	10 (8.1 %)

Figure 5: Ramachandran plot showing the phi-psi torsion angles for all residues in most stable predicted 3 D conformation of P16. The colouring/shading on the plot represents the different regions: the darkest areas (here shown in green) correspond to the "core" regions representing the most favourable combinations of phi-psi values. The different regions on the Ramachandran plot are, as described by Morris *et al.* (49), (a) Chain A (CDK6) in mutant docked Complex; (b) Chain B (p16) in mutant docked Complex.

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