Design of novel peptides to assess the inhibitory effect of Hsp90 alpha: An in silico approach

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UDAY KUMAR GUPTA

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Under The Supervision of

Dr. SUBHANKAR PAUL

Department of Biotechnology & Medical Engineering

National Institute of Technology

Rourkela-769008, Orissa, India

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National Institute of Technology, Rourkela

Certificate

This is to certify that the thesis entitled **"Design of novel peptides to assess the inhibitory effect of Hsp90 alpha: An in silico approach"** by **Uday Kumar Gupta (211bm2004)** submitted to the National Institute of Technology, Rourkela for the Degree of Master of Technology is a record of bonafide research work, carried out by him in the Department of Biotechnology and Medical Engineering under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/ Institute for the award of any Degree or Diploma.

Place: NIT Rourkela
Date:

Dr. Subhankar Paul
(Associate Professor)
Department of Biotechnology & Medical Engineering
National Institute of Technology
Rourkela-769008

Acknowledgement

If words are reflected as symbols of appreciation and token of acknowledgement, then words play the role of thanks to exhibit the deeply embedded feeling of gratitude. I am greatly indebted to, who either through guidance, discussion or providing facilities for the thesis work, have served as a beacon light or crowned my efforts with success. With an overwhelming sense of pride and genuine obligation I express my deep sense of heartfelt gratitude and regards to my guide **Dr. Subhankar Paul**, Department of Biotechnology and Medical Engineering for giving me an opportunity to do the project work in an independent arena.

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Date: Uday Kumar Gupta
Place: NIT Rourkela **Roll No.**-211BM2004

Abstract

Breast cancer is commonly found in women than in men and has been a serious issue for last two decades. High level of Hsp90 alpha expression has been documented in many breast cancer cells. Hsp90 alpha is dimeric protein with a molecular mass of 90 kDa, well conserved molecular chaperone and plays a key role in signal transduction pathways in cancer cells. Inhibition of Hsp90 alpha has been shown to be a common therapeutic approach by many reports. For proper chaperoning activity, Hsp90 alpha requires a host of other co-chaperones in proper functioning. Disrupting the interaction between Hsp90 alpha and co-chaperone will impair its chaperoning function and thus inhibit cancer.

In the present investigation, protein-protein interaction studies were done between Hsp90 alpha, its co-chaperones and client proteins. Results showed that complex formation is important for stabilization and maturation of client proteins. Hsp organizing protein also known as HOP brings together Hsp90 alpha and Hsp70 and therefore, inhibiting Hsp90 alpha and HOP interaction may lead to the disruption of Hsp90 alpha-Hsp70 complex formation and thus destabilize and degrade the client proteins including p53. In order to inhibit Hsp90 alpha and HOP interaction, ten numbers of peptides have been designed considering those residues involved in the interaction between Hsp90 alpha and HOP using computational methods. In-silico docking method Hex 6.1 was used to assess the binding energy between peptides and Hsp90 alpha. Based on the energy values we selected the peptide with highest negative energy that was the indicative of most stable interaction. As peptides commonly form aggregates, amyloidogenic properties of the selected peptide were predicted and the peptide was accordingly modified. When the modified peptide was interacted with Hsp90 alpha, docking results showed peptide INSAYKLKYARG binds with Hsp90 alpha with highest negative free energy of -861.81kcal/mole. The docking of the complex with HOP showed less affinity than Hsp90 alpha alone showing the conformational changes upon inhibition.

Keywords: Hsp90 alpha, Breast cancer, HOP, Docking, peptide designing

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Abbreviations

Hsps	Heat Shock Proteins
HOP	Hsps organizing protein
Asp	Aspartic acid
Thr	Threonine
Phe	Phenylalanine
Lys	Lysine
Gln	Glutamine
His	Histidine
Arg	Arginine
Asn	Asparagine
Ser	Serine
Leu	Leucine
Ile	Isoleucine
Cys	Cysteine
Pro	Proline
Met	Methionine
Val	Valine
Trp	Tryptophan
Glu	Glutamic acid
Tyr	Tyrosine
Gly	Glycine
Ala	Alanine

CHAPTER 1

INTRODUCTION

1.1 Introduction

Cancer is one of the most common diseases in the developed world and 1 in 4 deaths are due to cancer (Siegel et al., 2012). Lung cancer is most common in men and breast cancer is most common in women (Bray et al., 2013).There are over 100 different forms of cancer. Normal body cells grow, divide and die in a controlled and co-ordinated manner and these cells are in communication with each other involving delicate and complex signaling and communication pathways. Sometimes there is disturbance in the controlled and coordinated growth due to various external factors and genetic mutation and such loss of control can cause cancer. Before cancer formation, the cells undergo tumor formation. These tumors are classified into two types, benign and malignant. It is the malignant tumor that results in cancer and metastasis. Cancer may be caused by several reasons primarily due to mutations in DNA leading to disturbances in regulatory pathways and cell cycle. Although there are many types of cancer, they all are generates because of uncontrolled growth of abnormal cells.

1.1.1 Hsp90 alpha and Breast cancer

Hsps (Heat Shock Proteins) are highly conserved ubiquitous proteins among species which are involved in maintaining appropriate folding and conformation of other protein and thus known as molecular chaperones. Hsp90 alpha is one of the groups of the molecular chaperones responsible for protein folding and quality control in cellular environment. It comprises 1-2% of total proteins under non stress conditions. It regulates the stability and activation of more than 200 client proteins (Calderwood and Gong, 2012). Recent studies indicate that Hsp90 alpha is a major interaction node, regulating a diverse set of cellular functions.

Hsp90 alpha is involved in the maturation and stabilization of a wide range of oncogenic client proteins (Beliakoff and Whitesell, 2004)**.** Cancer cells are heavily dependent on proper Hsp90 alpha functioning. Many of the client proteins which turn oncogenic are either mutated e.g. p53 or over expressed or both (Ciocca et al., 1992). Many harsh environmental conditions are found in tumors such as hypoxia, low pH and bad nutritional status making client proteins heavily dependent on Hsp90 alpha. The expression of Hsp90 alpha is high compared to normal cells indicating that its inhibition would help check the proliferation of cancer cells, followed by proteasomal degradation offering a combinatorial impact. This approach also reduces the chances to develop resistance by cancer cells. As Hsp90 alpha is associated with a plethora of pathways viz, signal transduction, cell cycle, transcriptional regulation and many others it is a good target for many diseases especially cancer(Conroy and Latchman, 1996).

Cancer is the main cause of death found globally, around 7.6 million deaths (around 13% of all deaths) in 2008, with an estimated 13.1 million deaths in 2030. Out of which breast cancer covers 22.9% of all cancers in females. 13.7% of breast cancer deaths were recorded in 2008 in women all over the world (Soerjomataram et al., 2012) . The incidence of breast cancer in India is increasing day by day and is quickly becoming the number one cancer in females. The significance of the situation is obvious after running through present data from Indian Council of Medical Research (ICMR).

1.1.2 Computational approaches

Application of computational approaches to drug designing is becoming widespread since there is a plethora of public databases and free software. Main application of bioinformatics and chemoinformatics include the rapid and economical initial screening and lead identification of drug like compounds for further developments. Without these tools individual experimental screening would have been a costly affair. These have resulted in speeding of drug discovery process. Rational or knowledge based drug design facilitates and speeds up the drug designing processes that involves various method of identifying novel compounds. One advanced method is the docking of the drug molecule or ligand or inhibitor with the target also known as structure based virtual screening. Another form of virtual screening is the ligand based virtual screening where knowledge of known ligand is used for screening. In docking two molecules bind to each other in 3D space. There are three types of docking viz, rigid, flexible, and semi-flexible docking. In addition, regression based or knowledge based scoring functions can be useful to compute the free energy of ligand binding (Oprea and Matter, 2004). Various tools, software's and servers are available for docking calculations. Different databases are available where one can find the 3D structures of macromolecule and ligand which are extracted from NMR and X-RAY co-ordinates .Thus before doing the actual experimental work one can easily do a virtual study of his problem and after coming to a viable solution start the experiment which will reduce time and money.

1.2.1 Objective

1. To Design small peptides as the Hsp90 alpha inhibitor based on the residues involved in the interaction between Hsp90 alpha and HOP.
2. To estimate docking energies between designed peptides and Hsp90 alpha and the multi-chaperone complex.
3. To identify the best peptide based on the binding affinity.
4. Analysis of docking result with LIGPLOT^{+} to understand type of interaction.
5. To compare the binding affinity of the interaction between Hsp90, p53 and HOP before and after inhibition.

CHAPTER 2

REVIEW OF LITERATURE

LITERATURE REVIEW

2.1 Hsp90 alpha (molecular chaperones) and cancer

Heat-shock protein 90 (Hsp90 alpha) is a molecular chaperone (Csermely et al., 1998) that participates in the quality control of protein folding. Hsp90 alpha functions by recruiting many co-chaperones viz, Cdc37, Hop, p23, Hsp70 and Hsp40 (Li et al., 2012a). The complex formation is an energy requiring step and involves sequential ATPase cycles (Richter et al., 2004). Client proteins of Hsp90 alpha are several kinases viz, AKT, B-Raf mutant, MET and CDK4; Transcriptional factors HIF-1A, ERA-receptors p53 mutant regulating cell proliferation and survival and chimeric fusion proteins (Theodoraki and Caplan, 2012). Cancer cells are stressed cells and heavily depend on Hsp90 alpha chaperoning and thus show higher levels of expression of Hsp90 alpha, therefore Hsp90 alpha has emerged as a target for cancer therapy (Hong et al., 2013). Many drugs targeting ATP binding domain have been developed and are in clinical trials. Geldanamycin a prototype example of ansanamycins antibiotic showed exciting results disrupting multiple pathways but was toxic to normal cells and hence could not enter clinical trials (Fukuyo et al., 2010). Analogoues of geldanamycin 17-AAG and 17-DMAG were designed which was non toxic to human cells (Li et al., 2012b). Hsp90 alpha inhibitors found in literatures have mostly been discovered by structure based virtual screening, generating derivatives from already existing inhibitors or finding new scaffolds by HTS (Li et al., 2009). Recently Shepherdin, a novel anticancer agent was designed based on the interaction between

Hsp90 alpha and surviving (Plescia et al., 2005). Survivin is a mitotic regulator and antiapoptotic protein involved in many pathways (Duffy et al., 2007). The structure of shepherdin was based on modelled interface between Hsp90 alpha and survivin.

2.2 Structural organization of Hsp90 alpha

It contains 3 domains: an N-terminal approximately 25-KDa, a middle domain approximately 35KDa and C-terminal domain approximately 10KDa. ATP binding site is located in N-terminal domain of Hsp90 alpha (Beliakoff and Whitesell, 2004). Hsp90 alpha has two isomers alpha and beta encoded by two distinct genes, share approximately 81% sequence homology. While Hsp90 alpha beta is constitutively expressed at high abundance in most tissues, Hsp90 alpha is typically inducible in response to various cellular stress conditions (Cooper et al., 2011). The folding of the client protein mainly occurs in the middle domain. Hsp90 alpha function in a dimer form. It dimerizes through the C-terminal domain, besides this domain also interacts with the HOP (Csermely et al., 1998). HOP is an Hsp organizing protein .It helps in proper chaperoning of Hsp90 alpha. HOP through its tpr2a domain binds to the C-terminal of Hsp90 alpha and through its tpr1 domain to the N-terminal of Hsp70. Client protein comes to Hsp90 alpha via Hsp70 and both are joined by HOP(Onuoha et al., 2008; Pimienta et al., 2011; Southworth and Agard, 2011).

2.3 Breast cancer and over expression of Hsp90 alpha

In breast cancer, a number of client proteins interact with Hsp90 alpha. Some of the client proteins are estrogen receptors, p53 protein, hypoxia-induced transcription factor HIF-1alpha, protein kinase Akt, Raf-1 MAP kinase and a number of receptor tyrosine kinases, such as erbB2

(Beliakoff and Whitesell, 2004; Cho et al., 2006; Sreedhar et al., 2004). It has long been appreciated that tumor cells are “stressed” cells capable of coping with unfavorable environments via a generalized up-regulation of their stress response machinery(Kastan, 2007).

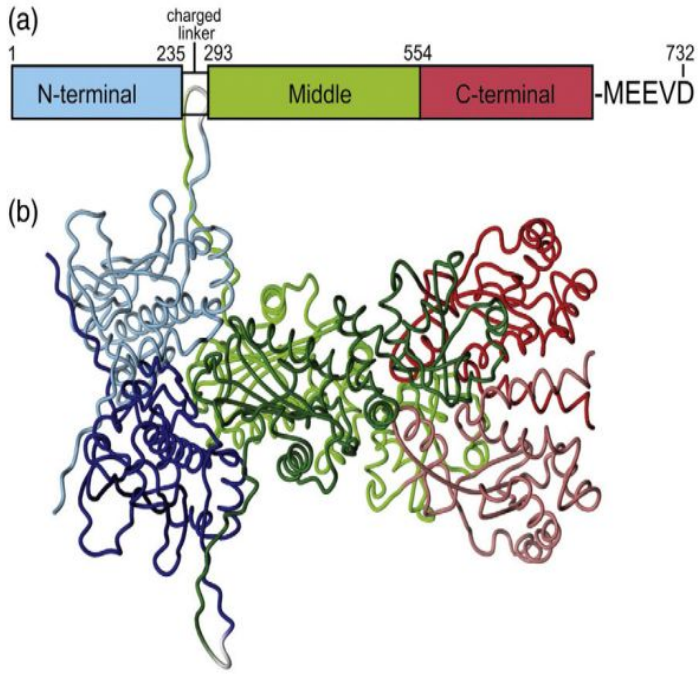


Figure 1: (a) Domain structure of human Hsp90 alpha showing domain boundaries (b) Ribbon diagram of the X-ray crystal structure of dimeric yeast Hsp82, from the complex with Sba1 (adapted from ref (Park et al., 2011b)).

This is largely centered on the expression and function of the molecular chaperone Hsp90 alpha, which is highly expressed in breast cancer and has provided an attractive target for therapeutic intervention in cancer (Beliakoff and Whitesell, 2004) . High Hsp90 alpha expression level has been detected in invasive breast carcinomas and is associated with reduced survival in breast cancer (Pick et al., 2007).

2.4 Hsp90 and its client p53

p53, also known as the “guardian of genome” is important client protein of Hsp90 and also the first tumor suppressor gene was discovered. Structurally it consists of four domains each with a different function and a total of 393 amino acid (Park et al., 2011b). The expression of p53 is low in normal cells and functions to inhibit and eliminate proliferation of normal cells (Lane and Hupp, 2003). Mouse double minute 2 (MDM2) is important regulatory protein of p53 and responsible for its degradation via proteosomal degradation pathway by binding to the C-terminus of p53 (Park et al., 2011b; Wang and Jiang, 2012). In cancer p53 is inactivated by multiple mechanisms. Mutant p53 has been found in number of breast cancer cases. Majority of mutation is located in the DNA-binding domain(Feki and Irminger-Finger, 2004). Experiments have shown the crucial role of Hsp90 in stabilizing mutant p53. Hsp90 blocks the E3 ubiquitin ligase activity of Mdm2 thus preventing the degradation of mutant p53 (Brooks and Gu, 2003; Michael and Oren, 2003).

Structural analysis of interaction between Hsp90 and p53 has been done by many researchers. NMR studies showed that the core domain of p53 is bound to Hsp90 in unstructured fashion. Various domains of Hsp90 interact with p53 with most of the association with middle domain. Besides middle domain it also binds loosely to N-terminal and C-terminal domain (Hagn et al., 2011). Molten globule like state of p53 has been reported to form upon interaction with middle domain of Hsp90 (Park et al., 2011a). In contrast, it has also been reported there is no structural changes and p53 binds natively to Hsp90 based on charge and shape (Hagn et al., 2011).

2.5 Hsp90 alpha inhibitors

Hsp90 alpha inhibitors are categorized in four classes based on their mechanism:

1) Blocking of ATP binding site
2) Disruption of co-chaperone/Hsp90 alpha interaction
3) Antagonism of client/Hsp90 alpha associations
4) Interference with post-translational modifications of Hsp90 alpha

2.5.1 Hsp90 alpha inhibitors targeting the ATP binding site

Benzoquinone ansamycins, represented by Geldanamycin (Figure 3) were the first class of natural Hsp90 alpha inhibitors to be discovered and substantially studied (Fukuyo et al., 2010). Geldanamycin, a natural occurring antibiotic, was originally isolated from Streptomyces hygroscopicusas early as 1970s. But GA exhibited high hepatotoxicity observed in animal models. Therefore GA derivatives like 17AAG,17 DMAG were synthesized and tested but still suffer from problems like low water-solubility, instability in solution, and low oral bioavailability may become the obstacle to further clinical application (Gartner et al., 2012). To avoid the problems of natural inhibitors synthetic small molecule and peptides have been designed with high specificity for Hsp90 alpha (Modi et al., 2011).It has been possible due to the crystal structure of Hsp90 alpha and powerful computational methods of screening called virtual screening. Compounds were designed based on purine and pyrazole scaffold mimicking the shape of natural nucleotide (Caldas-Lopes et al., 2009). Short peptide derivatives composed of three core amino acids Phe-D-Trp-Leu, were synthesized and reported to inhibit ATP (Orosz et

al., 2006) **.** Recently a novel peptide called shepherdin was designed to inhibit the interaction between Hsp90 alpha and survivin(Plescia et al., 2005).

2.5.2 Hsp90 alpha inhibitors targeting co-chaperone/Hsp90 alpha interactions

For the proper functioning of Hsp90 alpha, a series of co-chaperones are required. Binding and leaving of the co-chaperones at various stages provide regulatory control to the chaperoning process of Hsp90 alpha (Neckers, 2002).Therefore, blocking the chaperone cycle at these stages by targeting different co-chaperone/Hsp90 alpha interactions is likely to achieve similar results with the direct inhibition of Hsp90 alpha (Gray et al., 2008). Various co-chaperones targeted so far are cdc37/Hsp90 alpha, Hsp70/Hsp90 alpha, HOP/Hsp90 alpha and Aha1/Hsp90 alpha (Cortajarena et al., 2008; Holmes et al., 2008; Onuoha et al., 2008; Smith et al., 2009).

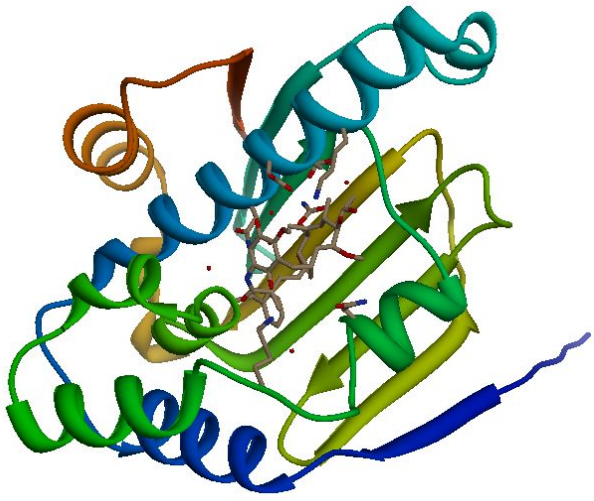


Figure 2: Crystallographic structure of Hsp90 alpha N-terminal domain bound to Geldanamycin (PDB ID 1YET).

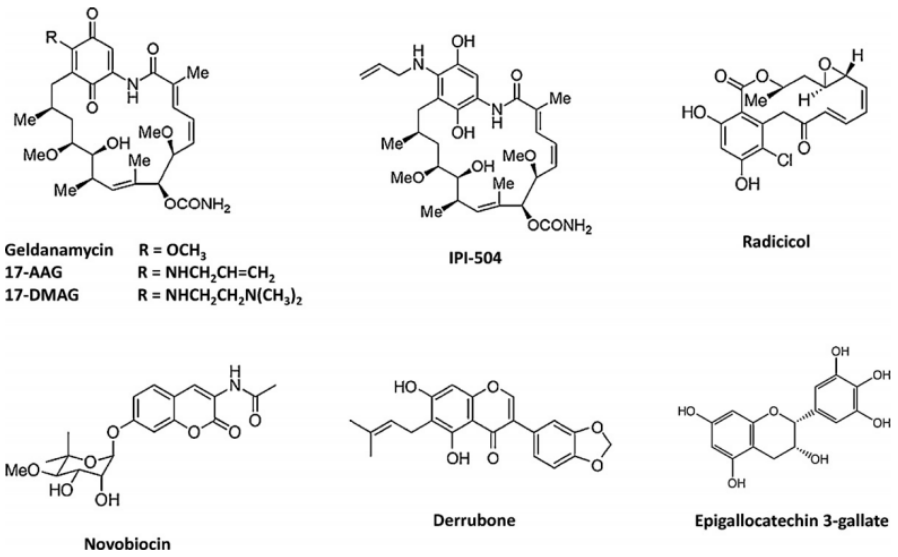


Figure 3: Chemical structure of the naturally occurring Hsp90 alpha inhibitors (adapted from ref (Li et al., 2009)).

2.5.3 Hsp90 alpha inhibitors targeting client/Hsp90 alpha associations

By targeting the client/Hsp90 alpha interactions selectivity might be possible , but not much is known for the structural basis of these interactions (Pearl et al., 2008). A peptidomimetic compound called as shepherdin was designed to disrupt the Hsp90 alpha/survivin interaction (Plescia et al., 2005) . However, shepherdin also interacted with the ATP pocket of Hsp90 alpha, and affects a range of Hsp90 alpha clients in addition to surviving (Pearl et al., 2008) .

2.5.4 Post-translational modifications of Hsp90 alpha

Out of the Post-translational modifications, studied so far S-nitrosylation, hyperphosphorylation and reversible hyperphosphorylation, have been implicated in regulating chaperone function of Hsp90 alpha (L, 2007). Indirect disturbance of the activity of Hsp90 alpha through post-translational modifications may open up new avenues for drug discovery (Wandinger et al.,

2006). Some of the post translational modifications studied so far are Hsp90 alpha hyperacetylation(HDAC'S), Hsp90 alpha thiol oxidation and Hsp90 alpha phosphorylation (Chen et al., 2008; Kovacs et al., 2005; Yang et al., 2008)

CHAPTER 3

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Database, Software's and Tools used

3.1.1 NCBI

NCBI (www.ncbi.nlm.nih.gov) stands for NATIONAL CENTRE FOR BIOTECHNOLOGY INFORMATION is part of the United States NATIONAL LIBRARY OF MEDICINE (NLM) a branch of the NATIONAL INSTITUTE OF HEALTH (NIH) and it is located in Bethesda, Maryland. NCBI has many databases cross linked to each other. Genome databases are in GenBank, biomedical research articles are in PubMed Central and PubMed both free access, and articles relevant to biotechnology. Entrez search engine provides searching through various databases.

3.1.2 PDB

RCSB PROTEIN DATA BANK (www.rcsb.org) is an information portal to biological macromolecule structure. It is a repository of 3D structural data of biological molecules such as nucleic acids and proteins. The datas are obtained experimentally from X-RAY crystallography studies and NMR studies by researchers and submitted from around the world. I t can easily be accessed from internet from its website. As of Tuesday November 27 2012 there are 86487 structures

3.1.3 PHYRE

PHYRE stands for **P**rotein **H**omology/analog**Y** **R**ecognition **E**ngine is a server for protein structure prediction using homology modeling techniques and is free for academic users. It is widely used with approximately 1000 citations and 4150 submissions per day is user friendly and have a simple interface to results (Kelley and Sternberg, 2009).

3.1.4 UCSF CHIMERA

Chimera is program for visualization and structural analysis of molecular structures and related data including including density maps, sequence alignments supramolecular assemblies, docking results, trajectories, energy minimization, conformational ensembles and many other tasks. Publication quality Images and movies can also be created in it. It has extensive documentation and is available free for academic users (Pettersen et al., 2004).

3.1.5 HEX

Hex is an interactive program for calculating and displaying feasible docking modes. It is an Interactive Molecular graphics program developed by Dave Ritchie for estimating docking calculations and displaying docking modes of pairs of protein and ligand molecules. HEX is used as the docking tool which calculates intermolecular “energies” by adding up all intermolecular interactions (e.g. van der Waals, electrostatic) that occur between a ligand and protein target. It can perform protein-protein docking taking receptor as rigid. Hex docking calculation each molecule is modeled using 3D expansions of real orthogonal spherical polar basis function to encode both surface shape and electrostatic charge and potential distributions. Typical docking

requires loading a receptor which is treated as rigid and ligand as movable. First it performs a spherical polar fourier transform based on 3D structure, then there is fast fourier transform steric scan and final search followed by a molecular mechanics refinements(Ritchie, 2008).

3.1.6 PEP-FOLD

PEP-FOLD is a program for de-novo structure prediction of peptides. The word de-novo means starting from the beginning .Rational peptide design and large scale prediction of peptide structure from sequence is a challenge .PEP-FOLD is a program for de-novo modeling of 3D conformation of peptides between 9 and 25 amino acids. It builds structure only from the sequence information based on the concepts of structural alphabets (SA) .It is a two step process prediction of a limited set of SA letters at each position from sequence and then assembling of the prototype fragments associated with each SA letters using a revised version of greedy algorithm and a generic protein coarse-grained force field (J. Maupetit et al., 2007; Maupetit et al., 2009; Maupetit et al., 2007).

3.1.7 PEPTIDE PROPERTY CALCULATOR

Various properties of the peptide can be calculated from INNOVAGEN'S (www.innovagen.com) peptide property calculator. The properties calculated are molecular weight, net charge, isoelectric point and solubility.

3.1.8 FOLDAMYLOID

Amyloidogenic regions in peptides are very important because such regions are responsible for amyloid formation and aggregation which might cause it to become non-functional and harmful. It has been seen that only small fragment are required for amyloidogenisis and the fragments are

able to form fibrils even without whole protein. Most of the fragments have an elevated content of hydrophobic amino acids residues called type1 and regions rich in ASN and GLN called type 2. FOLDAMYLOID predicts amyloidogenisis on the basis of sequence in fasta format. This program is based on hydrogen bond statistics and based on using expected characteristics scales either expected packing density or the probability of formation of hydrogen bonds(Garbuzynskiy et al., 2010).

3.1.9 LIGPLOT^{+}

LIGPLOT^{+} software is a program for plotting 2D protein-ligand (LIGPLOT) and protein-protein (DIMPLOT) interaction diagram and is free for academic users. It is an improved version of the earlier LIGPLOT. Hydrogen bonds and hydrophobic interactions are the two major interactions which are shown by dashed lines and arc with spokes respectively. It has several addition of new features viz, Superposition of related diagram, Improved DIMPLOT program and Links to PyMOL and RasMol (Laskowski 2011).

3.2 Protocol

3.2.1 Interaction between Hsp90 alpha, co-chaperones and client proteins

Retrieval of amino acid sequences of Hsp90 alpha protein from NCBI

3D structure modeling of Hsp90 alpha Protein using PHYRE server.

3-D structure of p53, Hsp70, Hsp40, HOP and client proteins retrieved from PDB (Protein Data bank).

Energy minimization of all 3D structure of proteins done by UCSF Chimera.

Docking of individual protein and protein complex respectively done by Hex 6.1.

Retrieval of the residues involve at the binding site by Dimplot analysis.

3.2.2 Design and assessing the binding efficiency of Hsp90 alpha peptide Inhibitors

Design of peptide inhibitors based on residues involved in Hsp90 alpha HOP interaction

De-novo structure modeling of peptides using PEP-FOLD

Energy minimization using UCSF chimera

Docking of Hsp90 alpha with peptides was done by Hex 6.1

Analysis of the binding energy obtained from docking.

Amyloidogenesis prediction using FOLD-AMYLOID and modification of peptides

Docking of the modified peptides using Hex 6.1

3.2.3 Estimating the effect of peptide inhibition

Retrieval of the protein-peptide complex from HEX 6.1 docking

Docking of the complex with HOP and mutant p53 by Hex 6.1

Analysis of the total energy values changes before and after inhibition.

3.3 Methodology

3.3.1 Hsp90 alpha amino acid sequence

Retrieval of amino acid sequences of Hsp90 alpha protein from NCBI was done by entering [HSP90 ALPHA AND "homo sapiens"] in the search option and selecting protein from the database option. There were many results of the search but the NCBI Reference Sequence: NP_005339.3 was selected and the sequences were retrieved in fasta format Figure.4.

3.3.2 Homology modeling of Hsp90 alpha using PHYRE[2]

The retrieved amino acid sequence of Hsp90 alpha in fasta format was pasted in the space provided, along with the email id and project title Figure. 5. Result was sent in given email within few hours and accessed by going to the link provided.

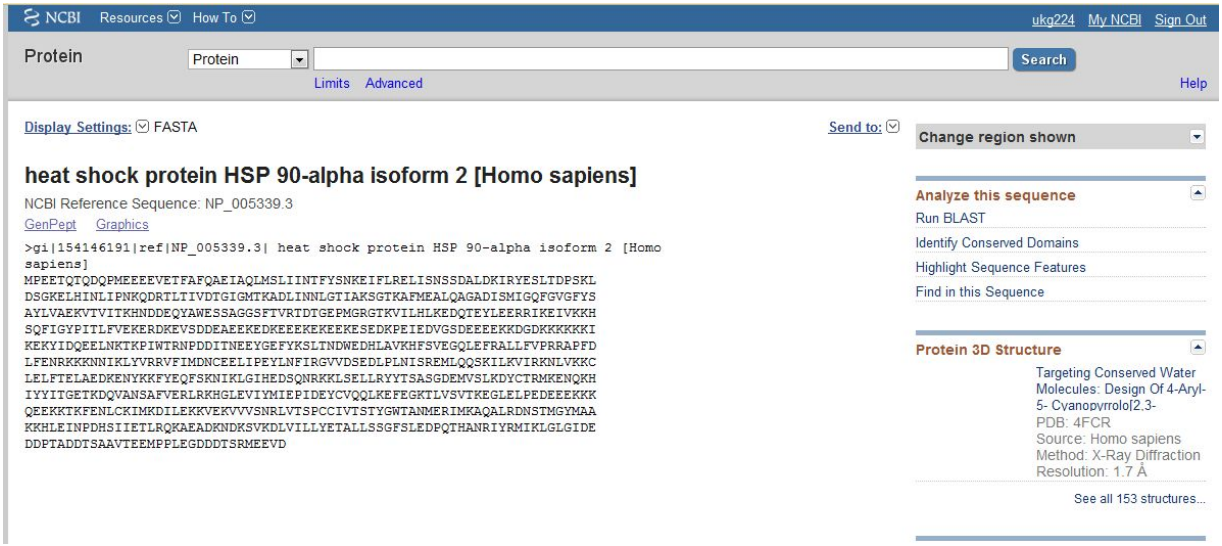


Figure 4: Retrieval of amino acid sequences from NCBI

3.3.3 Retrieval of 3D structure of p53, Hsp70, Hsp40 and HOP from PDB

For retrieving the 3D structure of proteins which are available in public databases like PDB go to the website of PDB (www.pdb.org) and in the search box type the individual names and very judiciously select the proteins. Human wild type p53 - 2OCJ, mutant p53 -2QVQ, HOP - 1ELR, Hsp70-3ATU and Hsp40-2QLD were retrieved from protein data bank (PDB).

3.3.4 Energy minimization

Prior to actual docking the 3D structure of protein was prepared in chimera by removing unwanted ions, solvents and ligands and minimizing the structure for high stability. The minimize structure functions calls add hydrogen and add charges, standard charges were added

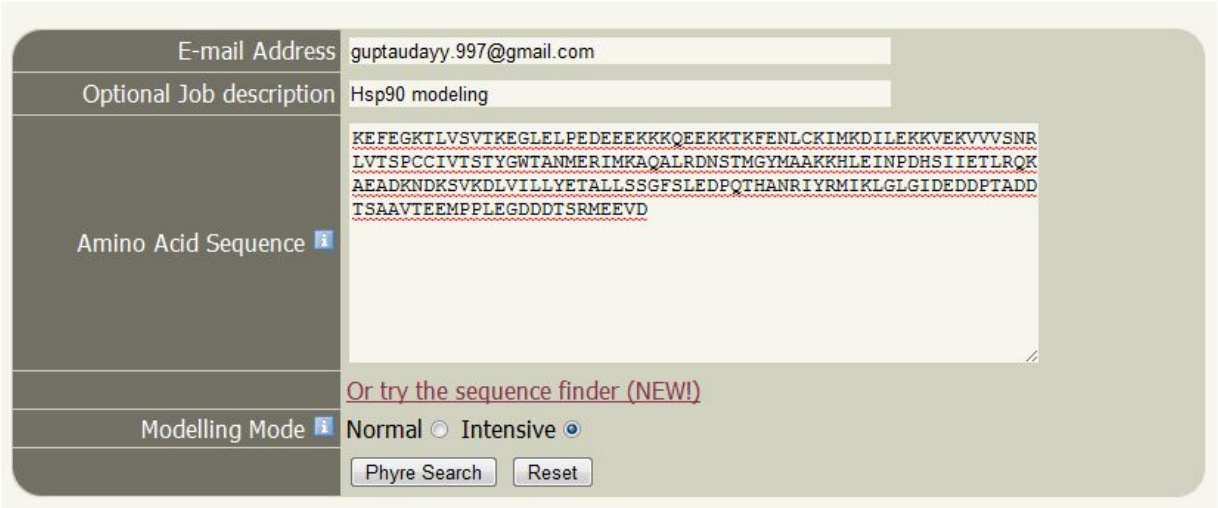


Figure 5: Submission of amino acids in PHYRE[2].

to standard residues and gasteiger charges were added to non standard residues. The standard charges were computed by the AMBER module and missing side chains were replaced by dunbrack rotamer library. The energy minimization parameters are shown in Figure 6.

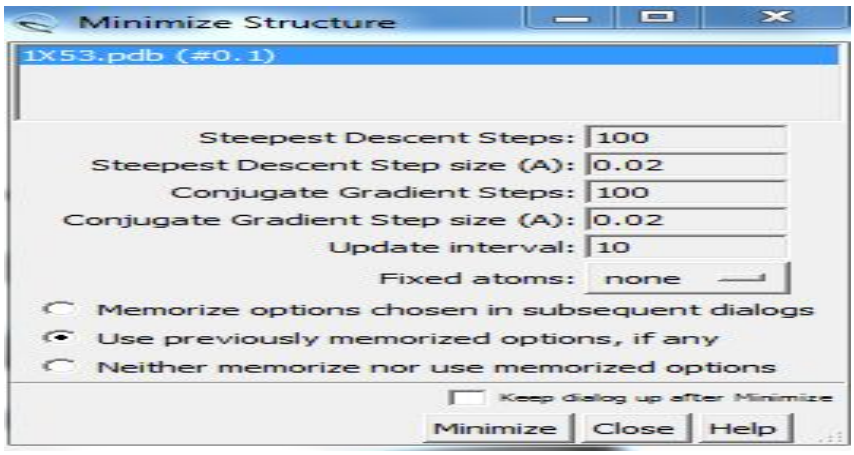


Figure 6: Energy minimization parameters

3.3.5 Design of peptides

In silico docking of Hsp90 alpha and HOP was done using Hex. The complex of Hsp90 alpha and HOP is required for proper chaperoning activity of Hsp90 alpha. The complex also brings other co-chaperones together and facilitates proper presentation of client protein. The key residues involved in this interaction was identified using LIGPLOT^{+} software a program for plotting protein-ligand(LIGPLOT) and protein-protein(DIMPLOT) interaction diagram. After docking of the Hsp90 alpha with target HOP, the residues interacting with the active site were selected for the design of new sequential peptide .Structure of proposed peptides was modeled using Mobyle server at RPRS portal using PEP-FOLD program. PEP-FOLD is a program for de-novo structure prediction of peptides. Rational peptide design and large scale prediction of

peptide structure from sequence is a challenge .PEP-FOLD is a program for de-novo modeling of 3D conformation of peptides between 9 and 25 amino acids (Maupetit et al., 2009). It builds structure only from the sequence information based on the concepts of structural alphabets (SA) .It is a two step process prediction of a limited set of SA letters at each position from sequence and then assembling of the prototype fragments associated with each SA letters using a revised version of greedy algorithm and a generic protein coarse-grained force field.

3.3.6 Peptide property calculation

For the calculation of peptide properties such as molecular weight, net charge, isoelectric point and solubility the single letter code of peptide was entered in the box from N-terminal to C-terminal without blocking.

3.3.7 Amyloid prediction by FOLDAMYLOID

Since Amyloidogenic regions in peptides are very important because such regions are responsible for amyloid formation and aggregation which might cause it to become non-functional and harmful therefore amyloidogenicity was predicted using FOLDAMYLOID program. It has been seen that only small fragment are required for amyloidogenisis and the fragments are able to form fibrils even without whole protein. Most of the fragments have elevated contents of hydrophobic amino acids residues called type1 and regions rich in ASN and GLN called type 2. FOLDAMYLOID predicts amyloidogenisis on the basis of sequence in fasta format. This program is based on hydrogen bond statistics and based on using expected

characteristics scales either expected packing density or the probability of formation of hydrogen bonds.

3.3.8 Molecular Docking by Hex 6.1 software

Hex 6.1 is an Interactive Molecular graphics program developed by Dave Ritchie for estimating docking calculations and displaying docking modes of pairs of protein and ligand molecules. Hex is used as the docking tool which calculates intermolecular “energies” by adding up all intermolecular interactions (e.g. van der Waals, electrostatic) that occur between a ligand and protein target. For a typical Hex docking open the Hex window load receptor and ligand separately necessary editing can be done many options are provided like change scene, change origin etc. Then click the docking control panel select shape +electrostatics because it is close to reality keeps other parameters default and run docking, usually it takes 2 to 4 hours to finish. After docking completion save the file in .pdb format for future analysis and note down the binding energy.

CHAPTER 4

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

4.1 Modeled structure of Hsp90 alpha

The Hsp90 alpha structure was modeled, using the PHYRE^2 homology modeling. Homology modelling is the most reliable method of structure prediction; other methods are Threading and ab-initio modelling. For Homology modelling Template identification is a crucial step, requiring sequence identity more than 25%. The model is shown in Figure 7. The modeled structure contains C-terminal domain. The spiral looks in the structures are α- helices and the arrow ones are β-sheets.

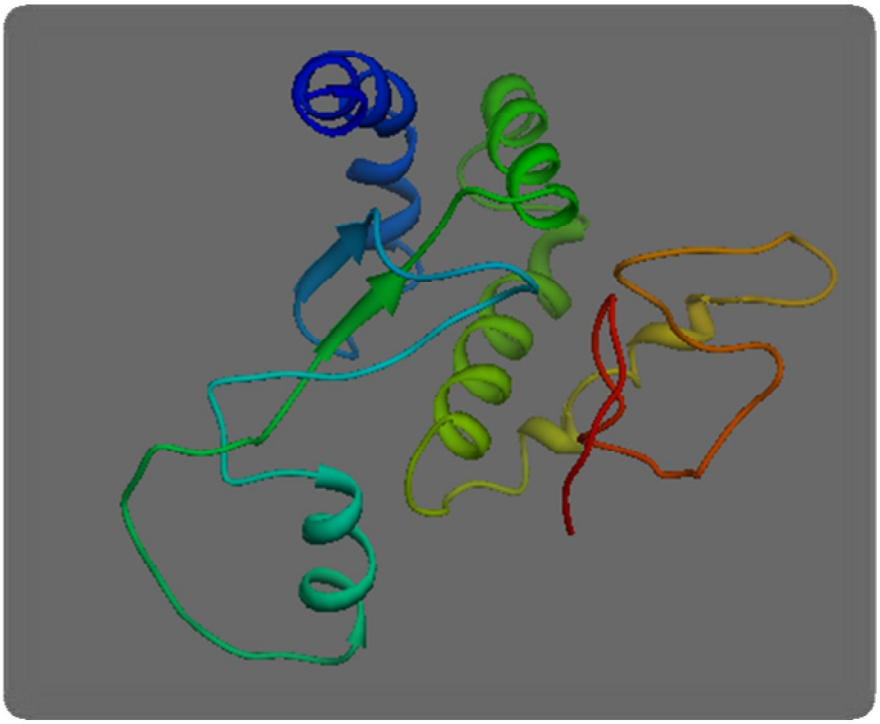
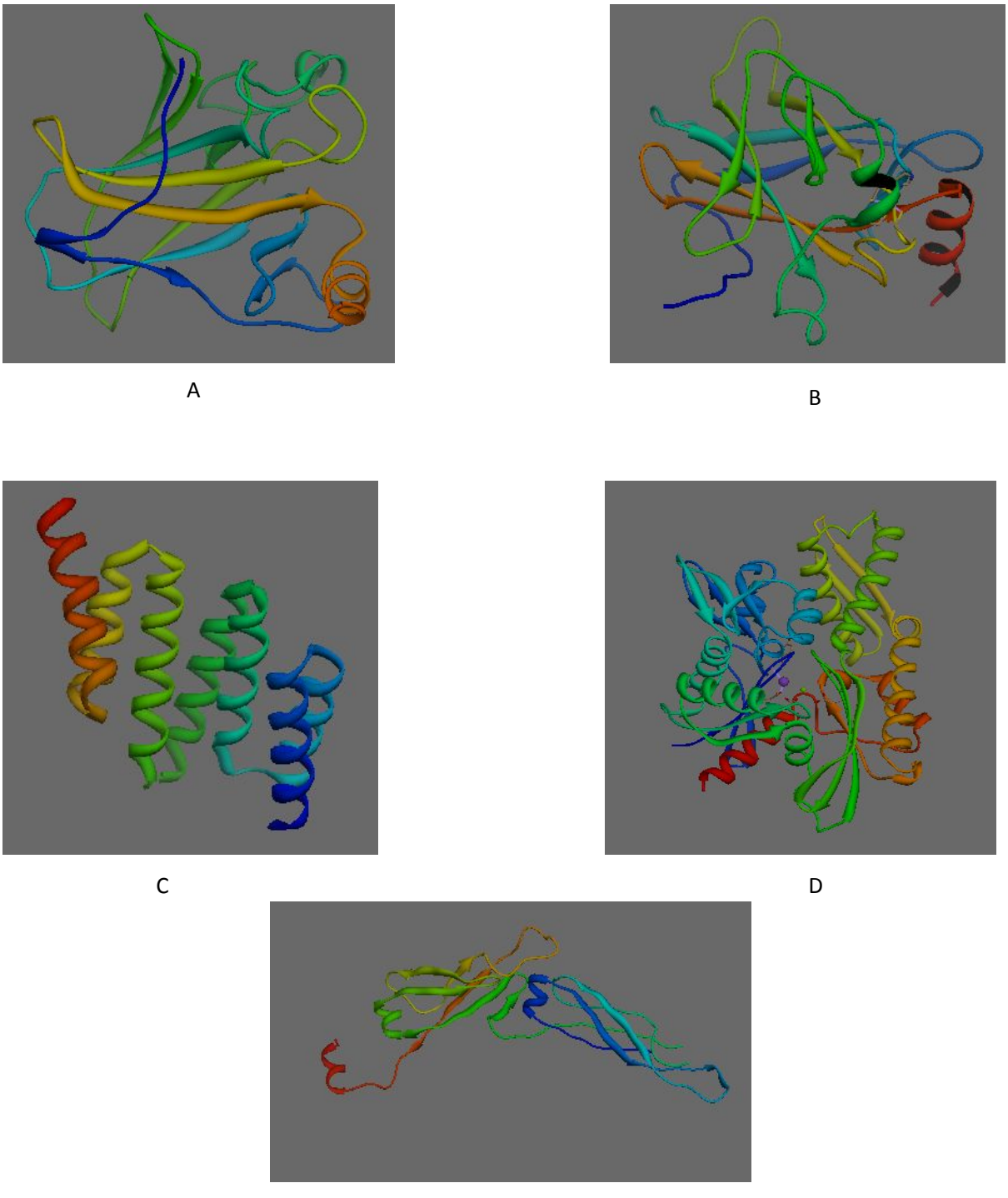


Figure 7: 3D structure of Hsp90 alpha C-terminal domain after energy minimization

4.2 3D structure of p53, HOP, Hsp70 and Hsp40



A B C D F

Figure 8: 3D structure of wild p53 (A), mutant p53 (B), Hop(C) Hsp70 (D) and Hsp40 (E) Hsp90 after energy minimization.

4.3 Study of protein-protein interactions

Protein-protein interaction study was performed with Hsp90 alpha and its co-chaperones Hsp70, Hsp40 and HOP along with client protein p53. First Hsp90 alpha alone was interacted with wild type and mutant p53, followed by sequential complex preparation and this complex was used to interact with wild type and mutant p53. The results of the interaction are tabulated in Table 1 Table 2 and Table 3 respectively. From the interaction studies it can be inferred that Hsp90 alpha binds strongly to HOP(Figure 9) than other co-chaperones and complex formation is important for proper chaperoning activity of Hsp90 alpha as complex binds more strongly to client proteins than Hsp90 alpha.

Table 1: Interaction of Hsp90 alpha with wild and mutant type p53

Protein	Client Protein	Docking Energy(Kcal/mol)
Hsp90 alpha	Wild type p53	-647.67
Hsp90 alpha	Mutant type p53	-721.49

Table 2: Interaction of Hsp90 alpha and co-chaperones

Protein	Co-chaperones	Docking energy(Kcal/mol)
Hsp90 alpha	HOP	-1179.80
Hsp90 alpha	Hsp70	-666.49
Hsp90 alpha	Hsp40	-613.86
Hsp90 alpha+HOP	Hsp70	-656.56
Hsp90 alpha+HOP+Hsp70	Hsp40	-615.46

Table 3: Interaction of multichaperone complex Hsp90 alpha with wild and mutant p53.

Complex	Client protein	Docking energy(Kcal/mol)
Hsp90 alpha+HOP+Hsp70 +Hsp40	Wild type p53	-759.44
Hsp90 alpha+HOP+Hsp70 +Hsp40	Mutant type p53	-926.49

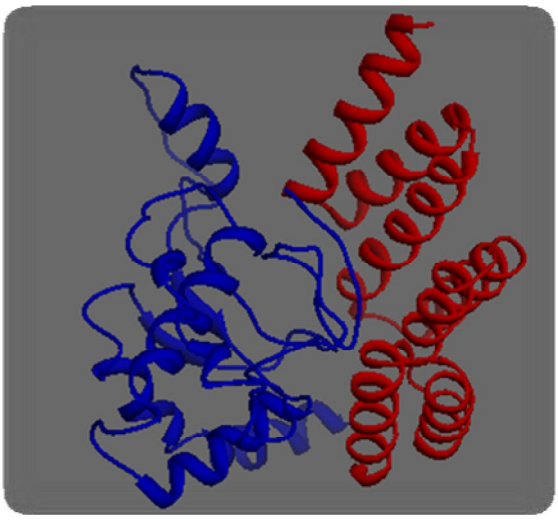


Figure 9: 3D structure of Hsp90 HOP complex

4.4 Dimplot Analysis (for protein-protein interactions)

Protein –protein 2D interaction maps was plotted by using DIMPLOT module of LIGPLOT [+]. Hydrogen bonding and distance between residues is represented by dotted line , arc represent Hydrophobic interaction, Hsp90 alpha residues are depicted by maroon color and the pink color depict the co-chaperone /client proteins residues(both Polar and non-polar).

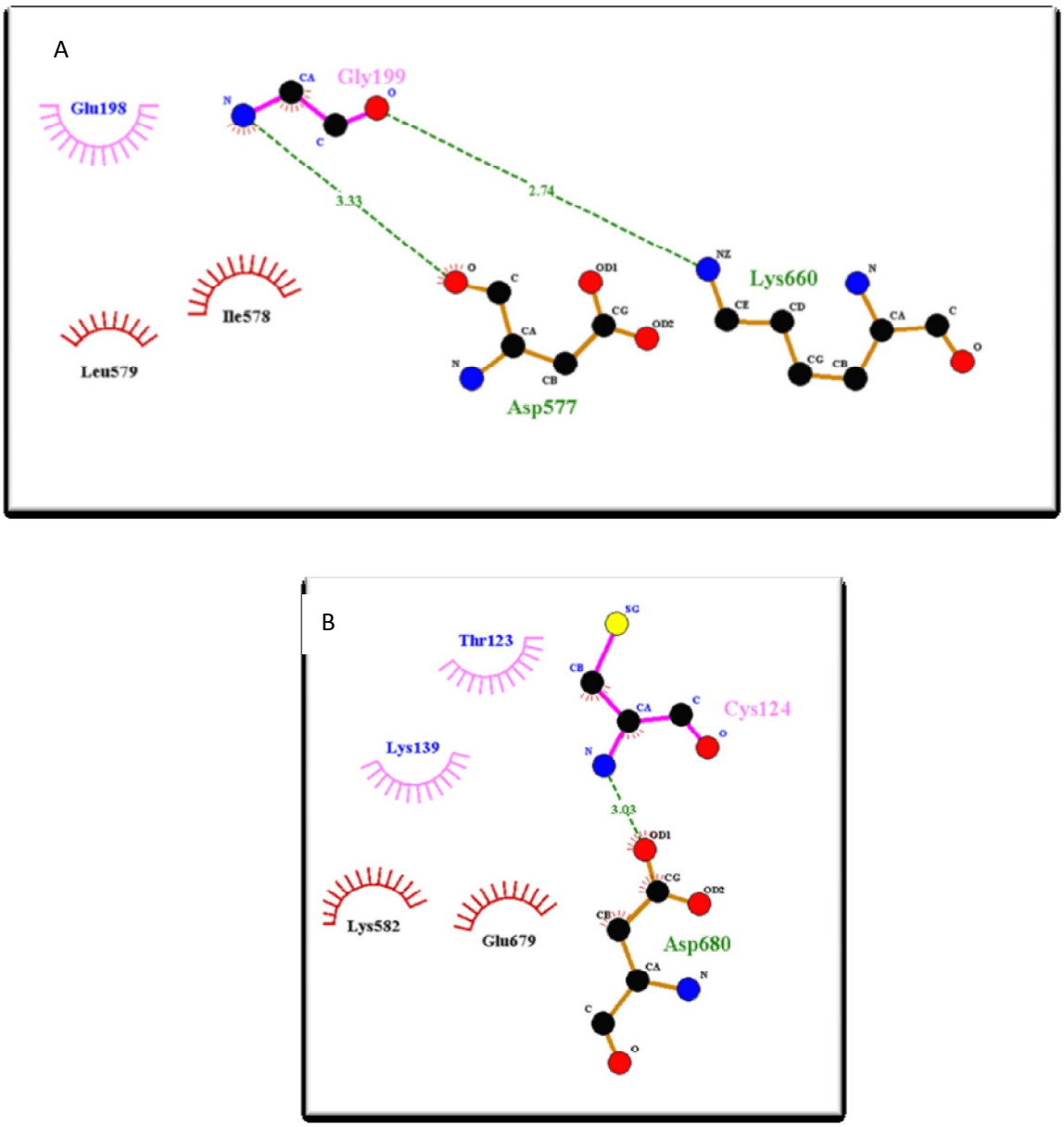


Figure 10 A and B: The dimplot analysis of Hsp90 alpha with mutant p53

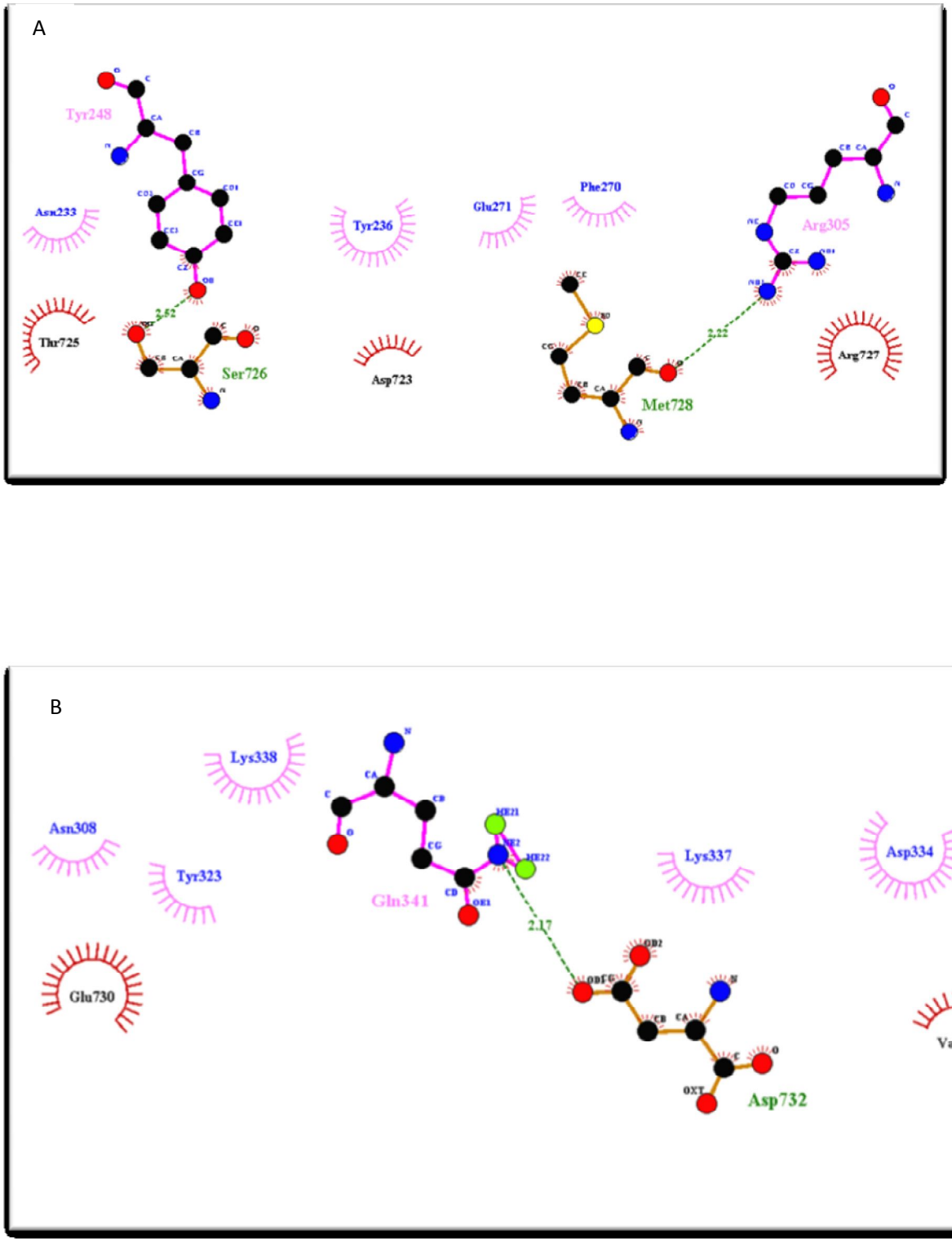


Figure 11 A and B: The dimplot analysis of Hsp90 alpha with co-chaperone HOP

The results of Dimplot analysis are tabulated below

Table 4: The residues involved in binding of Hsp90 alpha-Mutant type p53 generated and the distance measured by Dimplot.

Hsp90 alpha residue	Mutant p53 residue	Distance in angstrom
Asp577	Gly199	3.33
Lys660	Gly199	2.74
Asp680	Cys124	3.03

Table 5: The residues involved in binding of Hsp90 alpha- HOP generated and the distance measured by Dimplot.

Active residues of Hsp90 alpha	Residues of HOP	
	Hydrophobic	Hydrogen bonding
Asp723	Tyr236	-
Thr725	Asn233	-
Ser726	-	Tyr248
Arg727	Arg305	-
Met728	-	Arg305
Glu730	Ala304,Asn308,Tyr323	-
Val731	Asp334	-
Asp732	Lys337,Lys338	Gln341

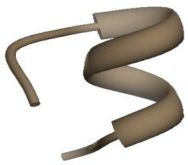
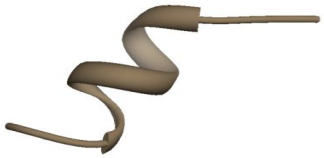
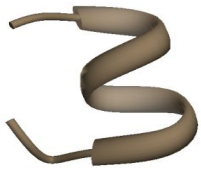
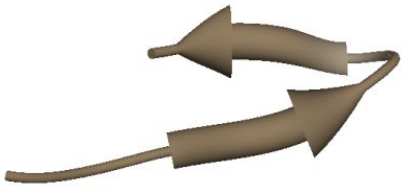
The hydrophobic residues of Hsp90 alpha involved in the interaction with mutant type p53 are Lys581, Ile664, Lys654, Asn655, Pro681, Ser658, Lys657, Asp661, Glu580, Leu579, Ile578, Gln682, Arg690, Lys693, Lys615, Ser677, Thr683, Lys582 And Glu679 and the hydrophobic residues of mutant type p53 are Ala138, His233, Leu201, Asn200, Ser116, Glu224, Val197,

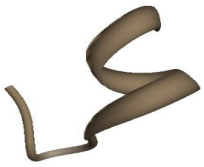
Arg196, Asn235, Glu198, His115, Ser227,Gly226, Val225, Lys120, Ser121, Leu114, Thr140, Lys139 and Thr123. The residues involved in hydrogen bonding are put down in Table 4.

4.5 Design of peptides

Peptides were designed based on the 1ELR, TPR2A domain of Hsp organizing protein and Hsp90 alpha interact to form a complex. This complex is required for proper chaperoning activity of Hsp90 alpha. The complex also brings other co-chaperones together and facilititate proper presentation of client protein like p53 etc. Initially a set of ten peptides of twelve amino acids was designed by Mobyle server taking into consideration all the interacting residues of the HOP and by random altering of the desired location of amino acid residues within the peptide (Table 6). Table 7 lists the molecular weight, solubility, and other properties of modeled peptides. For de-novo structure prediction of the designed peptide single letter amino acid sequence was entered in fasta format in the PEP-FOLD program. List and structure of designed peptide are given in Table 6.

Table 6: List and structure of designed peptides

S.no	Peptide	Structure
1	KYFKYAGRIANS	
2	KYAGRIANSYFK	
3	GRIANSYFKKYA	
4	ANSKYFKYAGRI	
5	KAYARIGNSYFK	
6	AGRIANSKYFKY	

7	INSAYKFKYARG	
8	KAYAAAGNSYFK	
9	KAYARIGNSGGG	
10	RKFSAAIGYNKY	

4.6 Hex 6.1 docking results (protein – protein/peptide)

Various energies of the designed peptide after docking with Hsp90 alpha are listed in Table 8. Comparative analysis of peptides interprets that new peptide (INSAYKFKYARG) as shown in Table 8; possess high binding affinity. However this peptide suffered from amyloidogenicity as predicted by FOLD AMYLOID program (Figure 14 (A)). Amyloidogenicity prediction of designed peptide INSAYKFKYARG showing regions of Amyloidogenic in regions 5-9.The region which shows amyloidogenicity is YKFKY.

Table 7: Properties of the designed peptides

Peptide	Molecular weight	Isoelectric point	Net charge at pH=7	Solubility
KYFKYAGRIANS	1417.63g/mol	10.33	3	good
KYAGRIANSYFK	1417.63g/mol	10.33	3	good
GRIANSYFKKYA	1417.63g/mol	10.33	3	good
ANSKYFKYAGRI	1417.63g/mol	10.33	3	good
KAYARIGNSYFK	1417.63g/mol	10.33	3	good
AGRIANSKYFKY	1417.63g/mol	10.33	3	good
INSAYKFKYARG	1417.63g/mol	10.33	3	good
KAYAAAGNSYFK	1290.44g/mol	9.94	2	poor
KAYARIGNSGGG	1150.26g/mol	10.4	2	good
RKFSAAIGYNKY	1417.63g/mol	10.33	3	good

To remove amyloidogenicity the “F” residue was substituted with M, A, L, E, K respectively because these residues have higher affinity to be in helix confirmation. Amyloidogenicity prediction of modified peptide INSAYKLKYARG showed no regions of Amyloidogenic (Figure 14(B)) .The modified peptides again went through the process mentioned above. Table 9 shows the list, Table 10 properties of the modified peptides and Table 11 binding energy with Hsp90 alpha. The structure of Hsp90 alpha and inhibitor peptide complex is given in Figure 10. Peptide INSAYKLKYARG (Figure 11) showed to bind with high affinity.

Peptide property calculator showed that INSAYKFKYARG peptide having isoelectric point of 10.33 with a net charge of 3 at neutral pH. In silico solubility of the peptide in pure water was checked and was found to be good. Solubility is calculated based on the iso-electric point,

number of charged residues and the length of peptide. Residues of designed peptide involved in binding with Hsp90 alpha was plotted using LIGPLOT^{+}. 2D Diagram of the interaction is shown in Figure 13 and the results are tabulated in Table 12.According to the results Glu730 and Val731 are the two key residues participating in hydrogen bonding and stabilizing the interaction. Various other residues involved in hydrophobic interactions are also tabulated in Table 12.

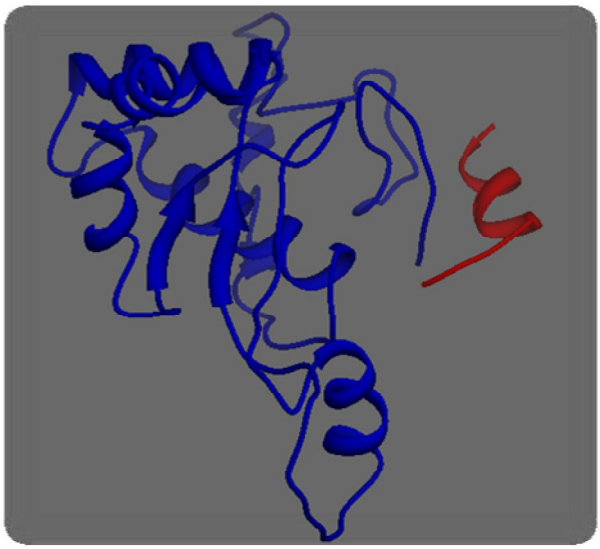


Figure 12: 3D structure of Hsp90 peptide inhibitor complex

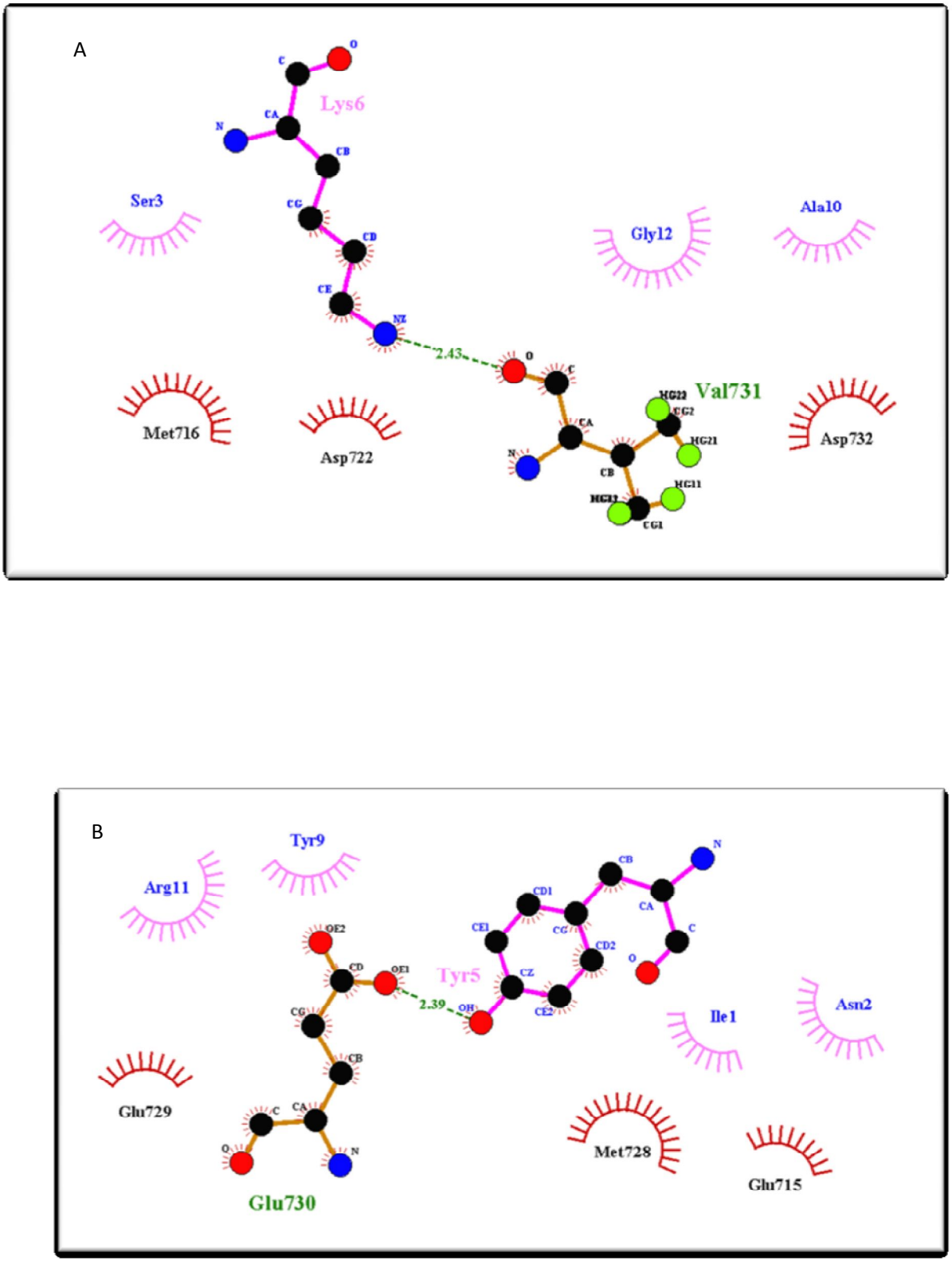
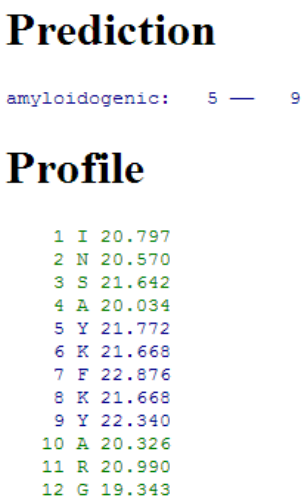


Figure 13 A and B: The dimplot analysis of Hsp90 alpha peptide inhibitor complex.

A



B

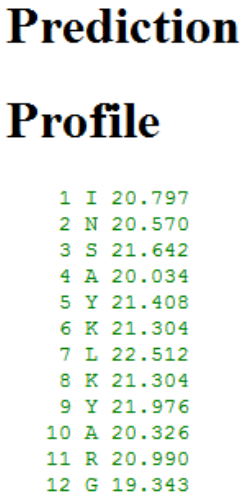


Figure 14: (A) Amyloidogenicity prediction of designed peptide INSAYKFKYARG showing regions of Amyloidogenic in blue and (B) Amyloidogenicity prediction of modified peptide INSAYKLKYARG showing no regions of Amyloidogenic

Table 8: Docking calculation between Hsp90 alpha and the designed peptides

Protein	Peptide	Energy(kcal/mol)
Hsp90 alpha	KYFKYAGRIANS	-824.22
	KYAGRIANSYFK	-823.04
	GRIANSYFKKYA	-746.73
	ANSKYFKYAGRI	-819.08
	KRIGAYFKYANS	-678.81
	AGRIANSKYFKY	-815.99
	INSAYKFKYARG	**-850.57**
	KAYAAAGNSYFK	-716.06
	KAYARIGNSGGG	-736.48
	RKFSAAIGYNKY	-657.09

Table 9: List and structure of modified peptides

S.no	Peptide	Structure
1	INSAYKMKYARG	
2	INSAYKAKYARG	
3	INSAYKLKYARG	
4	INSAYKEKYARG	
5	INSAYKKKYARG	

Table 10: Properties of the modified peptides

Peptide	Molecular weight	Isoelectric point	Net charge at pH=7	Solubility
INSAYKMKYARG	1410.65g/mol	10.33	3	good
INSAYKAKYARG	1341.53g/mol	10.33	3	good
INSAYKLKYARG	1383.62g/mol	10.33	3	good
INSAYKEKYARG	1399.57g/mol	9.94	2	good
INSAYKKKYARG	1398.63g/mol	10.54	2	good

Table 11: Docking calculation between Hsp90 alpha and the modified peptides

Protein	Peptide	Energy(kcal/mol)
Hsp90 alpha	INSAYKMKYARG	-776.29
	INSAYKAKYARG	-770.81
	INSAYKLKYARG	**-871.81**
	INSAYKEKYARG	-659.58
	INSAYKKKYARG	-835.52

Table 12 Residues of designed peptides interacting with Hsp90 alpha as predicted by Dimplot analysis

Residues of Hsp90 alpha	Residues of designed peptide	
	Hydrophobic	Hydrogen bonding
Glu715	Asn2	-
Met716	Ser3,Lys6	-
Asp722	Lys	-
Met728	Ile1,Asn2,Tyr5	-
Glu729	Arg11	-
Glu730	Tyr9,Arg11,Gly12	Tyr5
Val731	Gly12	Lys6
Asp732	Ala10	-

4.7 Effect of inhibition on binding affinity of Hsp90 alpha, HOP and p53

Docking studies showed good affinity between Hsp90 alpha, HOP and p53 .HOP is very important for organization of Hsp90 alpha and Hsp70 for proper presentation of client proteins viz, p53 etc. Results showed that the affinity for mutant p53 is more than wild type p53, hence it can be inferred that Hsp90 alpha is the main culprit which protects mutant p53 from being degraded. To study the effect of inhibition Hsp90 alpha/peptide inhibitor complex was docked with HOP and p53

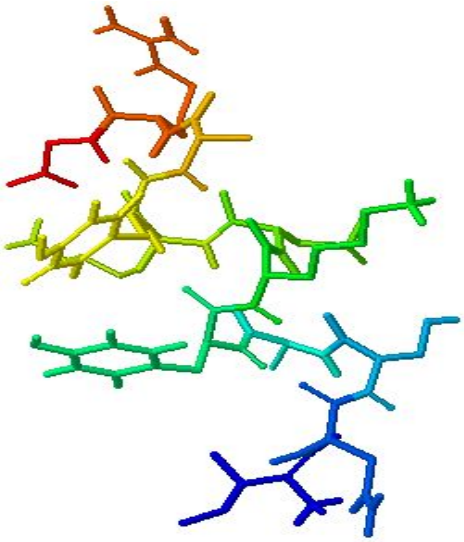


Figure 15: 3D structure (wireframe) of the peptide inhibitor (INSAYKLKYARG) showing highest affinity colored according to groups.

and the results analyzed. Results showed in Table 12 summarize all docking calculations, showing the differences of energy before and after inhibition.

Table 13: Docking calculation between Hsp90 alpha, HOP and mutant p53 after Hsp90 alpha inhibition

Complex	Protein	Energy
Hsp90 alpha	Mutant p53	-721.49
Hsp90 alpha	HOP	-1179.80
Hsp90alpha+INSAYKLKYARG	Mutant p53	-606.49
Hsp90alpha+INSAYKLKYARG	HOP	-626.56

Before inhibition the binding energy between Hsp90 alpha and mutant p53 was -721.49 and after inhibition it changed to -606.49, similarly the energy between Hsp90 alpha and HOP before inhibition was -1179.80 and after inhibition changed to -626.56. As it can be seen that the energy value changed to less negative this means that the complex is less stable after inhibition. Thus the objective to design a peptide inhibitor disrupting the interaction between Hsp90 alpha and HOP has been achieved computationally.

4.8 Discussion

3D structures for various domains of Hsp90 alpha and its co-chaperones have been resolved and provide unique and important target for creation of new anticancer drugs. It can also be a Starting structure for virtual screening for identification of new lead compounds and their optimization. Many leads and drugs have been discovered by structure based virtual screening techniques using the N terminal domain of Hsp90 alpha. N terminal domain is the widely studied domain. Very few works have been done using Hsp90 alpha C terminal domain which has been shown to have second ATP binding site and mediates in binding to co chaperone for proper chaperoning activity. In the present study C terminal domain of Hsp90 alpha was modelled using PHYRE Human wild type p53 - 2OCJ, mutant p53 -2QVQ, HOP - 1ELR, Hsp70-3ATU and Hsp40-2QLD were retrieved from protein data bank (PDB). The structures were minimized using UCSF CHIMERA. Protein-protein interaction study was performed with Hsp90 alpha and its co-chaperones Hsp70, Hsp40 and HOP along with client protein p53. Results showed that Hsp90 alpha binds strongly to HOP than other co-chaperones and complex formation is important for proper chaperoning activity of Hsp90 alpha as complex binds more strongly to client proteins than Hsp90 alpha. Peptides were designed to disrupt the interaction between

Hsp90 alpha and HOP. Structures' having minimum binding energy is reported to take less time to bind and binding is more efficient as compared to the structure having more binding energy. Hence the peptide having the minimum binding energy towards Hsp90 alpha was selected as the best antagonistic peptide against Hsp90 alpha. Since peptide may form aggregates their amyloidogenicity was also predicted using PEPFOLD and modification was done accordingly. Among the various screened peptides, INSAYKLKYARG (Figure 15) showed high specific binding activity to the Hsp90 alpha. The peptide solubility was predicted and showed good solubility. Inhibition studies showed that the peptide reduced the affinity of Hsp90 alpha towards HOP and mutant p53

CHAPTER 5

CONCLUSION AND FUTURE PERSPECTIVES

5.1 Conclusions

Experiments have shown that protein-protein interactions and protein –ligand interactions play a crucial role in biological functions. Same is also true for Hsp90 alpha. Hsp90 alpha does not work alone but a host of other co-chaperones are required for proper chaperoning activity. Hsp organizing protein (HOP) is one of the most important co-chaperones which bring Hsp70 and Hsp90 alpha together for proper presentation of client proteins. In silico protein-protein interaction studies showed that Hsp90 alpha has higher affinity for HOP than other co-chaperones. Hsp90 alpha in the multi-chaperone complex interacts more favorably with client proteins than Hsp90 alpha alone. Based on the above findings, peptide inhibitor were designed for specifically inhibiting Hsp90 alpha and HOP interaction that disrupts the formation of super complex. An important problem with peptides are tendency to aggregate which was also predicted and subsequently peptides were modified keeping the helix structure constant by substituting with amino acids having highest propensity for helix. The results showed that the peptide sequence INSAYKLKYARG showed highest energy of -861.81kcal/mol.

5.2 Future perspectives

The work done so far and all the results obtained are based on promising and reliable softwares. The process of drug designing is very lengthy and an expensive process. In silico techniques has proved to be a very reliable technique for designing of new drug candidate and assessing their activity. The in vitro and in vivo studies thus save a lot of initial cost. In this work, a new peptide (INSAYKLKYARG) was designed for disrupting the interaction of Hsp90 and HOP, so that the complex will not be formed necessary for chaperoning activity of Hsp90. The results

were solely based on docking calculations based on the fact that the more negative energy of interaction more stable is the interaction. Future work should be to study the whole system under molecular dynamics simulation so that the behavior in real systems can be assessed. After simulation in vitro and in vivo studies can be done.

CHAPTER 6

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