DESIGNING OF A POTENT ANALOG AGAINST DRUG RESISTANT HIV-1 PROTEASE: AN INSILCO STUDY

[1]MADHUR MISHRA AND [1]MOHAMMED HARIS SIDDIQUI*

[1]Department of Biotechnology, Microbiology and Bioinformatics, Integral University, Lucknow, 226026, India.
Email: mohdharis.siddiqui@gmail.com, mohammedharis_siddiqui@yahoo.co.in

ABSTRACT

The HIV-1 protease enzyme is conscientious for the post translational processing of the viral Gag and Gag-Pol polyproteins to yield the structural proteins. The necessity of this enzyme (HIV-1 Protease) for the maturation of provirus and completion of virus life cycle makes it a promising target for Anti-Retroviral therapy. Fortunately for HIV virus and unfortunately for humans the viral reverse transcriptase enzyme lacks proofreading activity and has a very poor processivity.Due to lack of proofreading activity there is approximately 10 to 100 folds increase in probability of error by the enzyme because of which the virus quickly becomes resistant to drugs. In the work presented herein factors affecting drug stability have been predicted with the help of three proteins whose PDB ID's are 1HPV (wild), 2B7Z (mutated), and 1TW7 (multidrug resistant or cross resistant). After structural analysis studies it was found out that the flap domain remains in its closed conformation in wild protease (1HPV) and subsequently it shifts towards its open conformation, resulting in the increase in the rate of mutation subsequently leading the flap tip to become more flexible and in a semi open state. The results clearly indicate that the area of the active site binding pocket increases from 581.9Å to 996.5Å with the increase in the rate of mutation. It was further inferred upon that the mutation in some of active site residues (V82A, I84V) led to the random loss in the hydrophobicity which fell down from 1.732 to 0.356 inside the binding pocket of the active site. At last we arrived to the conclusion that greater is the active site binding pocket area, the more flexible is the semi open flap and the random loss in hydrophobic environment leads to the protease becoming resistant to all the available drugs. A derivative of Indinavir (IND1) has been designed and evaluated considering all factors affecting drug stability and on the basis of the results obtained it was clearly found out that the newly designed derivative gives better insilco results to qualify as a drug lead than the available standard drug Indinavir against mutated protease.IND1 showed least minimum binding energy of -8.80 Kcal/mole, least Ki value of 0.35 µM, maximum number of hydrogen bonds(7), good ADME properties and less toxicity than Indinavir which are the prerequisites for a drug lead to be potent and effective. Finally we conclude that our study opens up the hidden potential of insilico tools to design new analogs for the efficient and effective treatment of harmful and infectious diseases.

Keywords: Dimer stability, Gag-Pol polyproteins, Post translational processing, Proofreading, Processivity.

INTRODUCTION

HIV1 has single stranded, positive-sense RNA genome of about 9.7 kilobases. Each HIV1 virus has two strands of RNA genome and each strand has a copy of the nine genes of the virus[1]. These nine genes broadly encode classified structural, catalytic, regulatory, and accessory proteins (Table 1)[2].

Table 1: Nine major Genes of HIV and their related gene products

Protein class	Gene	Gene product
Structural	gag, env	P17(matrix), p24(capsid), p7(nucleocapsid), gp120, gp41
Catalytic	pol	Protease, Reverse transcriptase, Integrase
Regulatory	tat, rev	Tat, Rev
Accessory	vpu, vif, vpr, nef	Vpu, Vif, Vpr, Nef

Navia et al. from Merck laboratories were the first group to obtain a crystal structure of HIV protease[3] (Fig.1).HIV protease has two identical 99 amino acid chains (chain A and chain B) with each chain containing the characteristics D-T-G active site sequence at position 25 to 27[4].HIV protease is an aspartyl protease which functions as a homodimer with only one active site (D25-T26-G27) which is C2-symmetric in its free form. More than 140 structures of the HIV-1 protease, its mutants and enzymes complexed with various inhibitors have been reported so far[5]. Each monomer has an extended β-sheet (GLY rich loop) known as flap domain that constitute in part the substrate binding and one of the two essential aspartyl residue, Asp25 and Asp25' which appear on the bottom of the cavity[6].The two subunits (of two 99 amino acid chains) are linked by a four stranded anti parallel β-sheet involving both the amino and the carboxy terminal of each subunit. Upon binding, both subunits form a long cleft where the catalytically important aspartic acids (D25) are located in a coplanar configuration on the floor of the cleft[7,8]. HIV-1 protease (HIV PR), an essential enzyme is needed in the proper assembly and maturation of infectious virions[9].

Protease inhibitors

HIV protease enzyme processes gag (p55) and gag-pol (p160) polyprotein products into functional core proteins and viral enzymes[10,11]. During or immediately after budding, the polyproteins are cleaved by the protease enzyme at nine different cleavage sites to yield the structural proteins (p17, p24, p7, and p6) as well as the viral enzymes reverse transcriptase, integrase, and protease[12]. The Protease inhibitors (PIs) are well known to block the protease enzyme thereby inhibiting its activity. When protease is blocked, HIV makes copies of itself that cannot infect new cells. The enzyme protease acts as a "scissor" to cut up the string into the protein for each virion (Fig. 2A).The protease inhibitors prevent protease from performing this activity[9] (Fig. 2B). There are six FDA-approved protease inhibitors (PIs):[4] Saquinavir (First developed protease inhibitor[13], highly active peptidomimetic PR inhibitor giving 90% IC_{90} at 6 nM)[14], Ritonavir (Second developed PR inhibitor[7] giving IC_{90} of 70 to 200 nM[15]), Indinavir (Third developed protease inhibitor[16] giving IC_{95} of 50 to 100nM[17]), Amprenavir (IC_{50} of 80 to 360nM[18]), Lopinavir (give in combination with ritonavir), and Nelfinavir (IC_{50} value of 9 to 60 nM)[19].

Indinavir

Indinavir, developed by Merck & Co., is the third HIV protease inhibitor licensed in the United States. Indinavir is a potent and selective inhibitor of HIV-1 and HIV-2 proteases with *Ki* values of 0.34 and 3.3 nM, respectively. The drug acts as peptidomimetic transition state analogue and belongs to the class of protease inhibitors known as HAPA (hydroxyaminopentane amide) compounds[16]. Indinavir provides enhanced aqueous[7] solubility and oral bioavailability and in cell culture exhibits an IC95 of 50 to 100 nM[17] In the presented work indinavir has been taken for making derivative whose trade name is criixivan, available in 200,300,400mg capsules, recommended dose 800mg every 8 hrs Take 1 hour before or 2 hours after meals; may take with skim milk or low-fat Meal With RTV (IDV 800mg + RTV 100– 200mg) BID Take without regard to meals, serum half life is 1.5-2 hrs, stored at Room temperature (15º–30ºC/59º–86ºF), Protect from moisture[20] (Fig. 3).
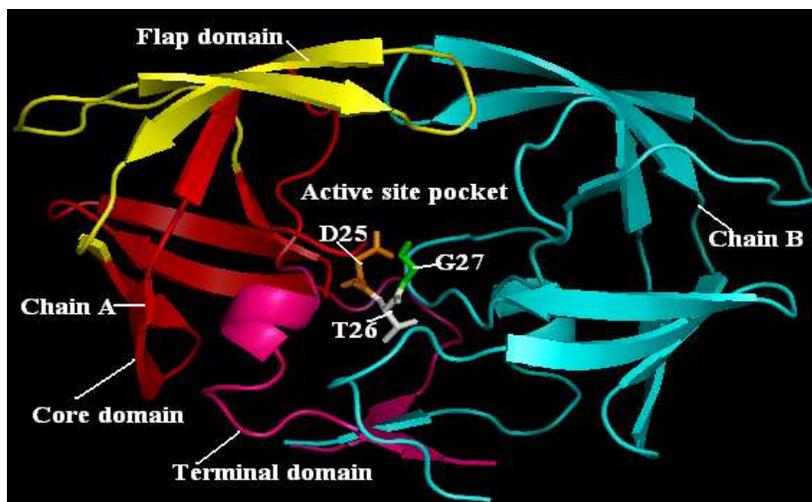
Fig. 1: HIV 1 protease with its active site, flap domain, core domain and terminal domain.
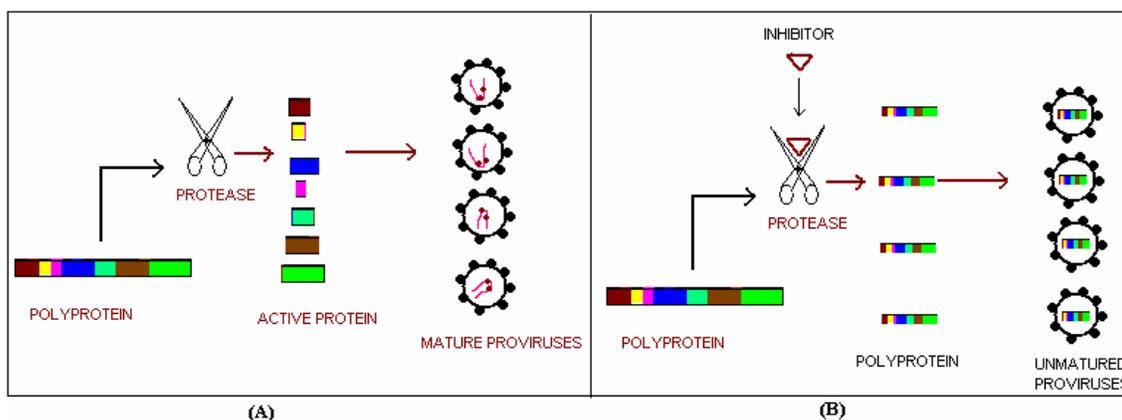
Fig. 2(A): Mechanism of action of protease on polypeptides which subsequently cuts polypeptides and forms matured viruses. (B) Action of inhibitor's on protease resulting in the formation of immature viruses.
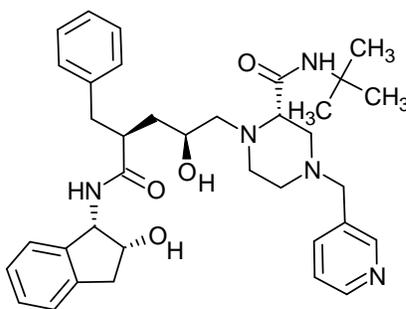
Fig. 3: Structure of indinavir.

MATERIALS AND METHODS

Selected proteins used in this study

For the prediction of structural changes, active site binding pocket area and changes in hydrophobic milieu inside active site binding pocket, three separate structures of HIV1 protease have been used.1HPV (wild), 2B7Z (drug resistant) and 1TW7 (cross resistant) has been retrieved from RCSB Protein Data Bank (http://www.rcsb.org) with the help of HIV Stanford Database (http://hivdb.stanford.edu) which

provides the following level of inferred drug resistance: susceptible, potential low-level resistance, low-level resistance, intermediate resistance, and high-level resistance. In this presented work 1TW7 is used only for structural analysis.

Prediction of structural changes

There are three softwares which were used for structural analysis of HIV1 protease. PyMOL software (Developed by- Warren Lyford DeLano and commercialized by DeLano Scientific LLC, South San Francisco,

California, U.S.A) was used for visualization, alignment between protein PDB, and analysis of docking results. Open uncomplexed structure of wild protease 1HPV (pdb id) was opened in PyMOL followed by open crystal structure of mutated protease 2B7Z (pdb id).This was followed by giving the command in command line (align 1HPV, 2B7Z) and press enter. If both the structures are different from each other then RMSD value (Root Mean Square Deviation) is obtained. Higher RMSD value means greater structural variability between both the structures. Once again 2B7Z was aligned with 1TW7 and RMSD value was predicted.

Chimera is used for superimposing the proteins structures. All the three uncomplexed structures of protease enzyme 1HPV, 2B7Z and 1TW7 were opened and superimposed to each other (Reaches Tool option – Structure comparison – MatchMaker).The gradual changes in structures with respect to the rate of mutation or rate of drug resistance were predicted.Molegro Molecular Viewer was used for visualization and distance calculation between two amino acid residues.PDB Ligand Explorer 3.9 (Powered by MBT) was used for visualization of hydrophobic interaction and hydrogen bond (Energy, Distance, and Binding pattern) between drug molecule and amino acid residues of protease enzyme.

CASTp analysis

CASTp (Computed Atlas of Surface Topography of proteins) is an online tool for the prediction of binding site and active sites. CASTp (http://sts.bioengr.uic.edu/castp/) was used for measuring the area of active site binding pocket of 1HPV (Wild), 2B7Z (Mutated) and 1TW7 (Multi drug resistant) protease enzyme. Uncomplexed PDB files of 1HPV, 2B7Z and 1TW7 were uploaded and prediction was done for area of the active site binding pocket of each PDB file.

Prediction of mutated sites

ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) is an online tool (Performs pair wise alignment and multiple sequence alignment) developed by EMBL-EBI (European Bioinformatics Institute) which was used for the prediction of mutated residues their location or site in the FASTA sequence of protease enzyme. All the three FASTA sequences of protease enzyme were submitted and prediction was carried out to find out the site of mutation and mutated residues.

ExPASy ProtScale

ExPASy ProtScale (http://web.expasy.org/protscale/) is an online tool used for the prediction of the hydrophobic values of amino acid residues of the protease. The hydrophobic milieu inside the active site pocket is measured by ProtScale tool. The FASTA sequences of the protease enzyme (1HPV) were pasted in the box provided followed by their submission.The tool plots the hydrophobic values of each amino acids present in the sequence in the form of graph as well as individual amino acids also. Hydrophobic values of residues occurring inside the active site pocket of all three sequences were predicted.

Preparation of derivative

As presented in this work Indinavir is a novel HIV1 Protease inhibitor which was used as lead compound for the designing potent derivative. Mainly three steps have been considered in the strategy adopted behind the designing of the derivative (IND1) of Indinavir drug

a) Amino acid residues present around the Indinavir (D25, T26, G27, A28, D29, D30, T31, V32, M46, I47, G48, G49, I50, T80, P81, V82, N83, I84) were visualized in wild complexed crystal structure (1HPV).

b) Those residues were predicted which make the interaction (hydrophobic and hydrogen bond) with Indinavir (V32A, M46I, V82A, I84V) and play an important role in the drug-receptor binding affinity but are altered due to mutation.

c) Atoms and groups of Indinavir have been modified based on their size, orientation and hydrophobic character of altered amino acid residues.

DrugBank database (http://www.drugbank.org) is a unique bioinformatics and chemo informatics resource which contains whole information of drugs. The PDB and MOL file of indinavir was retrieved from drug bank database[21].

ACD/ChemSketch is the powerful all-purpose chemical drawing and graphics package from ACD/Labs developed to help chemists to quickly and easily draw molecules, reactions, and schematic diagrams and calculate chemical properties.ChemSketch is used here for designing derivative (IND1) of indinavir, generating SMILE notation and chemical formula of newly designed derivatives. Smile format of IND1 (Derivative) was uploaded in an online tool CORINA (http://www.molecular-networks.com/online_demos/corina_demo) for the generating 3D structure. CORINA is widely used online program for the conversion of 2D format in to 3D format of ligand.

In-Silico ADME and toxicity prediction

Smile formats of Indinavir and IND1 (derivative) were submitted in an online tool ADME/Tox WEB (http://pharma-algorithms.com/webboxes/). The tool predict out ADME and Toxicity of both drugs. Another online source is used for toxicity prediction Organic chemistry portal avialable at www.organicchemistry.org and ChemSilico Secure Web Server (https://secure.chemsilico.com/index.php) used for the prediction of Blood Brain Barrier (BBB). Lipinski rule of five was performed with the help of Lipinski filter facility available on line at Supercomputing Facility for Bioinformatics and Computational Biology, Indian Institute of Technology, New Delhi, India[22].

Docking simulation

Docking experiments were performed using the Auto-Dock Tool 4.0, suite of automated docking tools. It is a protein-ligand docking tool in which a genetic algorithm is used to find the binding conformation of ligand. It is most commonly used docking tool used and referred in the scientific literature.The Docking process involves four main steps 1) Receptor preparation 2) Ligand preparation 3) Docking using a search algorithm 4) Analysis of binding conformation of ligand.

In the work presented herein docking has been done between Indinavir and 1HPV (wild), Indinavir and 2B7Z (mutated), IND1 (Derivative) to 2B7Z.First of all the non essential molecules like water and ligand were removed from both protein PDB files, then both protein PDB files and ligand PDB files have been energy minimized and saved as in a pdbqt format. The Grid has been set at the centre of active site pocket, which covers all the residues occuring inside the active site pocket with 52 * 46 * 66 points in x, y, z direction and -2.028, -3.722, 0.194 grid centre.The parameters are saved as grid parameter file (.gpf) and followed by autogrid run.In third step docking parameter files (.dpf) were prepared in which genetic algorithm has been selected,the value of which remains as default. These values determine the optimal run parameter which depends up on nature of ligand and proteins (receptor).10 generations were set for each GA run (10).This was followed by saving the parameters as docking parameter file (.dpf) and followed finally by the Autodock run. Docking simulation has been repeated for three times with similar parameters to improve the precision level of results.It should be taken care that similar results should be obtained after each repeats.The generated results were visualized in PMV (Python Molecular Viewer) and PyMOL. The interactions were studied in terms of minimum binding energy (Kcal/mol), Ki (Inhibition constant) value in μM and number of hydrogen bonds formed with active site residues.

RESULTS

Structural deformation due to mutation

In structural analysis RMSD (Root Mean Square Deviation) has been calculated between the wild and mutated protease structure. When alignment was done between the 1HPV-2B7Z, 2B7Z-1TW7 and 1HPV-1TW7 it is observed that 2B7Z showed less structural variation from 1HPV with 0.470 RMSD (Fig. 4A), while 1TW7 showed high structural variation from 2B7Z with 1.631 RMSD (Fig. 4B) and 1TW7 also showed high structural variation from 1HPV with 1.716 RMSD (Fig. 4C).

Where,

Distance has been measured by Molegro viewer

In the following analysis it has been observed that in wild protease flap tip remains in a closed conformation but after mutation the closed

conformation shifts towards a semi open conformation. Major structurally deformed sites have been shown by yellow circle (Fig. 4C)

Analysis of active site binding pocket area

In a protease enzyme there are various pockets present but an active site pocket has important role as it is in the active site that a polyprotein chain enters for the post-translational processing. Active site binding pocket area of 1HPV, 2B7Z and 1TW7 has been predicted. 1HPV has lower active site pocket area 581.9 Å with 965.9 volume (ID 21) (Fig. 5A) while 1TW7 has greater active site pocket area 996.5 Å with 2675.9 volume (ID 16) (Fig. 5C) and 2B7Z has moderate active site pocket area 745.5 Å with 1231.5 volume (ID 19) (Fig. 5B). With the help of following analysis it has been observed and can be inferred

upon based on the results obtained that drug resistance increases with the increase in the area of the active site pocket.

Prediction of mutated sites

Position of mutated residues in peptide chain (FASTA sequence) has been predicted with the help of online tool ClustalW2. With the help of this tool multiple sequence alignment has been done between the FASTA sequences of 1HPV, 2B7Z and 1TW7. Mutated sites are represented by colon (:) (Altered amino acid more different in chemical nature form wild amino acid) and dots (.) (Altered amino acid slightly different in chemical nature form wild amino acid) (Fig. 6A). Another more precise way for visualization of MSA results is also represented by Jel-view (Fig. 6B).
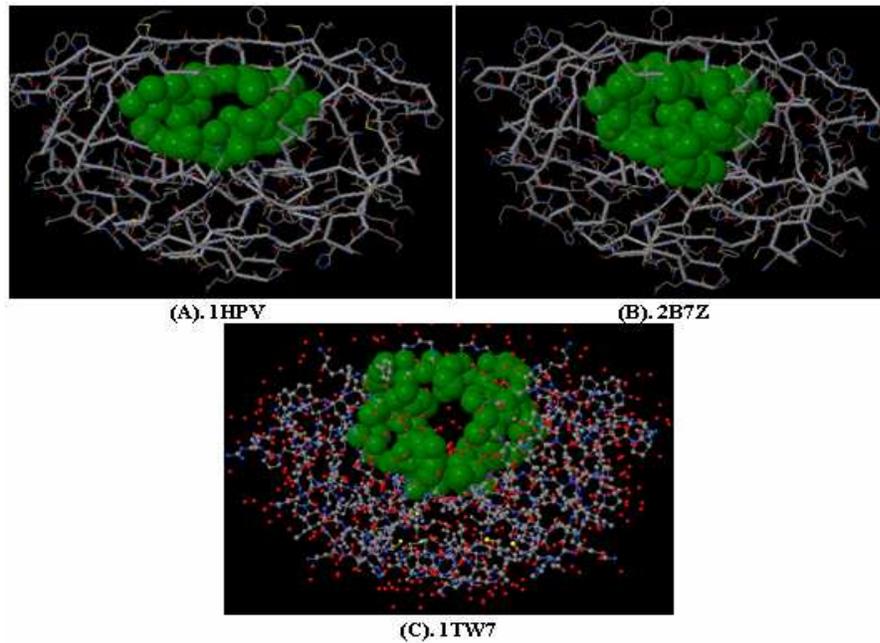
Fig. 5: (A) Active site pocket area (Green balls) in 1HPV (Wild). (B) Active site pocket area in 2B7Z. (C) Active site pocket area in 1TW7.
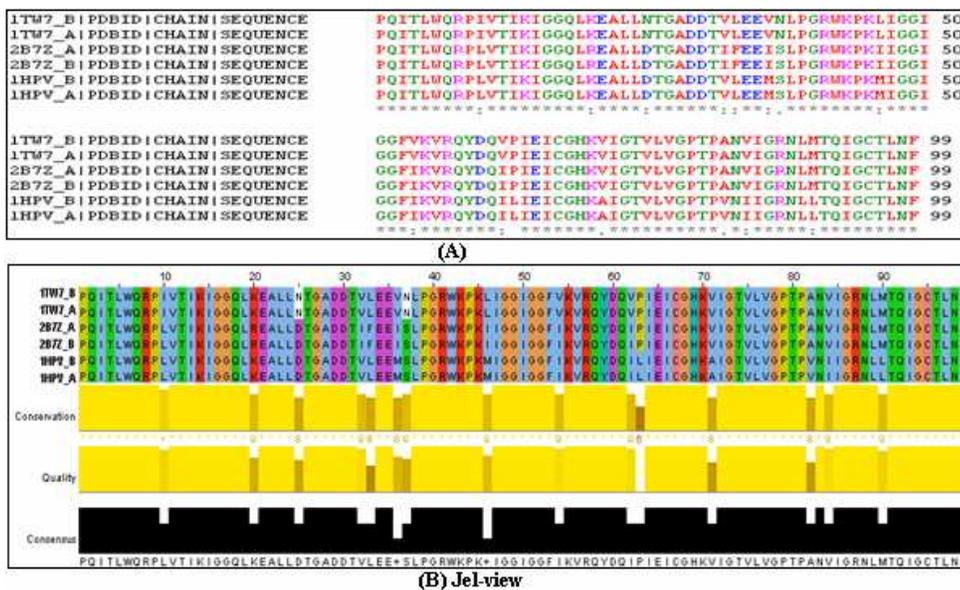
Fig. 6: (A) Normal view of multiple sequence alignment. (B) A jel-view of multiple sequence alignment.

Hydrophobicity analysis

Hydrophobic analysis has been done for wild (1HPV) and mutated (2B7Z) protease. In this analysis a single chain has been considered, because protease is a homodimer (chain A and chain B) in which each monomer subunit have similar structure and similar amino acid sequences. So, for the prediction of gross hydrophobic score of chain A and chain B, the predicted hydrophobic score of chain A has been multiplied by two to obtain the total score.Hydrophobicity of amino acids which were involved in making the active site pocket and few residues (V32, V82, and I84) which facilitate drug binding have been predicted. After the submission of the FASTA sequence, the tool generates a table of hydrophobic score as well as a graph in which hydrophobic score of amino acid is plotted on the basis of incoming

hydrophobic score of amino acids(table 3). In this plot the hydrophobic score has been taken at 'Y' axis and position of amino acids at X axis (Fig: 7). Predicted hydrophobic score of amino acids (which occurs inside the active site pocket) has been given in Table 3. The total hydrophobic score (chain A) of amino acids which are present around the active site pocket in wild protease (1HPV) was found to be 0.866, so the gross hydrophobic score (inside active site pocket including both chain A and B) becomes 1.732 (0.866 * 2 = 1.732),while in case of mutated protease (2B7Z) the total hydrophobic score (chain A) was found to be 0.178 thereby making the gross hydrophobic score (inside active site pocket including both chain A and B) of 0.356 (0.178 * 2 = 0.356).Therefore in this analysis it was observed that the gross hydrophobic score reduces in mutated protease (2B7Z) which indicates that loss of hydrophobic milieu inside the active site pocket.

Table 3: Predicted hydrophobic score of amino acid which were involved in making active site pocket.

Position of A.A.	H.P. Score 1HPV	H.P. Score 2B7Z	Position of A.A.	H.P. Score 1HPV	H.P. Score 2B7Z
T26	- 0.044	- 0.044	I47	0.033	0.322
G27	- 0.322	- 0.322	I48	0.422	0.711
A28	- 0.278	- 0.244	G49	0.911	1.2
D29	- 0.278	- 0.356	P81	1.067	0.767
D30	- 0.278	- 0.356	V82A	0.566	0.256
T31	- 0.589	- 0.667	N83	0.100	- 0.2
V32I	- 0.333	- 0.122	I84V	- 0.111	- 0.411
Total H.P. Score				0.866	0.178
Gross H.P. Score				1.732	0.356
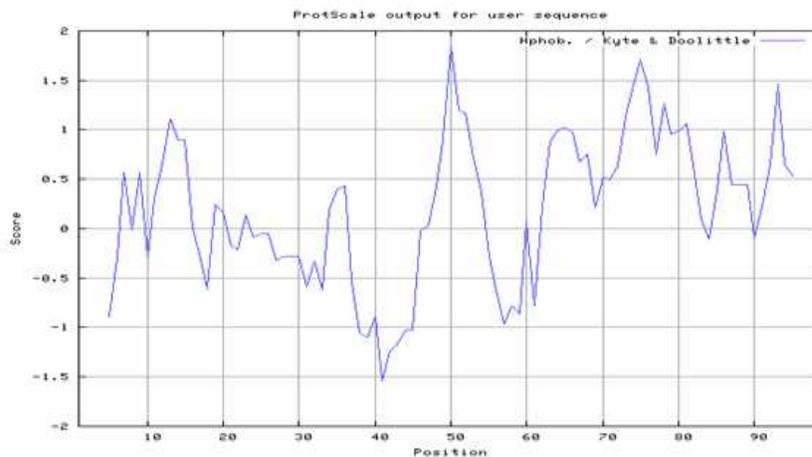
(A) 1HPV
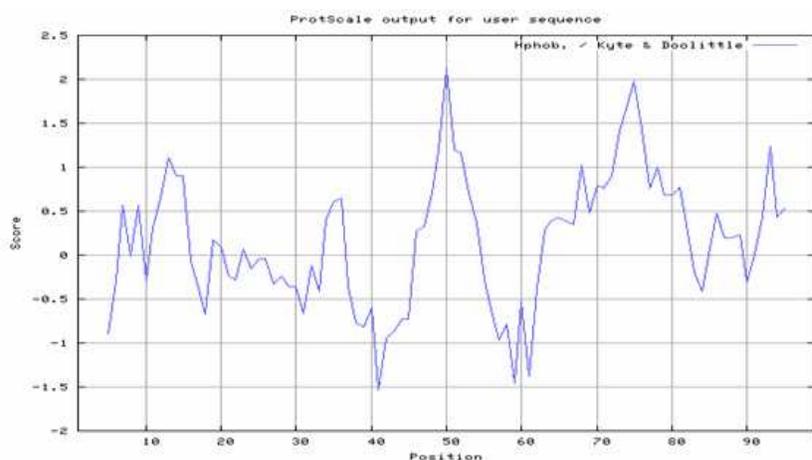
(B) 2B7Z

Fig. 7(A): Predicted graph of wild protease (1HPV). (B) Predicted graph of mutated protease (2B7Z).

ADME and toxicity test of indinavir and derivative (IND1)

ADME (Absorption Distribution Metabolic Excretion) properties and toxicity of Indinavir and IND1 (Derivative) has been predicted with the help of the said online tools. In this test it has been observed that newly designed derivative IND1 showed good ADME and very less toxicity than the previously available standard drug Indinavir (Table 4).IND1 did not show mutagenic, tumorigenic, irritant and reproductive effects rather it showed good protein binding property and less blood brain barrier penetration (BBB).

Docking analysis

Docking simulation of Indinavir and IND1 (derivative) with wild (1HPV) and mutated (2B7Z) protease was performed and the effectiveness of results was expressed in terms of minimum binding energy (Kcal/mole), Ki value (inhibitory constant) and number of hydrogen bonds formed. After the docking analysis it has been observed that the indinavir showed -6.08 Kcal/mole minimum binding energy, 35.21 µM Ki value and 5 hydrogen bonds with 1HPV (wild protease) while it showed -5.82 Kcal/mole minimum binding energy, 53.78 µM Ki value and 4 hydrogen bonds with 2B7Z (mutated protease). When docking interaction between IND1 (derivative) and mutated protease (2B7Z) was carried out, IND1 showed better results than indinavir. IND1 showed least minimum binding energy -8.80 Kcal/mole and 352.96 nM (0.35 µM) Ki value with 7 hydrogen bonds (Visualized in PyMOL) as well as least minimum binding energy was obtained during each run (10). On the basis of least minimum binding energy, less Ki value and number of hydrogen bonds IND1 can be taken forward as a more effective drug than standard indinavir.

Table 4: Predicted ADME properties and toxicity values of indinavir and IND1.

Properties	Indinavir	IND1	Properties	Indinavir	IND1
Oral Bioavailability	0.110	0.303	Mutagenic	No	No
Protein Binding	94.55	96.44	Tumorigenic	No	No
Logs			Irritant	No	No
Stomach	-1.81	-2.63			
Duodenum	-3.54	-5.00			
Ileum	-3.92	-4.19			
Blood	-3.99	-3.33			
Colon	-4.01	-2.73			
Water Solubility	0.0594	0.033	Reproductive Effect	No	No
AMES test	0.009	0.006	Drug Likeness	6.97	3.24
Lipinski Five			Drug Score	0.53	0.49
Molecular weight	613	563			
Log P	0.828	3.607			
H-Donor	4	4			
H-Acceptor	5	4			
Molar Refractivity	140.8	128.0			
Toxicity			Blood Brain Barrier	-0.84	-0.87
Blood	0.97	0.98			
Cardiovascular System.	0.98	0.77			
Gastrointestinal System	0.99	0.96			
Kidney	0.96	0.91			
Liver	0.98	0.90			
Lungs	0.88	0.40			

Where,

Oral bioavailability to Toxicity properties are predicted by ADME/Tox WEB (http://pharma-algorithms.com/webboxes/).
Mutagenic to Drug score properties are predicted by Organic chemistry portal (www.organicchemistry.org
Blood brain barrier (BBB) properties are predicted by ChemSilico Secure Web Server (https://secure.chemsilico.com/index.php).

Table 5: Docking studies of indinavir and IND1 with wild (1HPV) and mutated (2B7Z) protease.

Name of Compound and protein	Run	Minimum binding energy (Kcal/mole)	Ki value µM	Number of H-Bonds (PyMOL)	Residue name
Indinavir docked with 1HPV	1	-6.08	35.21	5	GLY126/Ob-IND1/O31, ASP124/Ob-IND1/O31, ASP124/Ob-IND1/O24, ILE149Nb-IND1/O41 GLY48/Ob-IND1/O17
Indinavir docked with 2B7Z	1	-5.82	53.78	4	ASP30/Oa-IND1/O31, ASP25/Oa-IND1/N6, ASP25/Oa-IND1/N3 GLY47/Oa-IND1/N14
IND1 docked with 2B7Z	10	-8.80	0.35	7	GLY27/Ob-IND1/O6, ASP29/Na-IND1/O44, GLY27/Oa-IND1/O44, ILE50/Na-IND1/O37, ILE50/Nb-IND1/O28, ILE50/Ob-IND1/O28 ILE50/Na-IND1/O28

Where,

a* Show chain A and b* show chain B, (2) shows two hydrogen bonds forms.

DISCUSSION

The first step by which retroviruses develop this resistance has been thought to be a result of the relatively low fidelity of reverse transcriptase enzyme. Because the DNA polymerase does not have a proofreading activity, base mismatch errors are integrated into viral DNA before being integrated into the host cell genome. This proviral DNA then works as a template for all new viral transcripts, passing along any mutations which were integrated during the initial reverse transcription event. Although only one or two reverse transcription products are successfully integrated into each host cell genome, a huge number of mutations can accumulate in a very little time. This is made to be possible by the large pool of infected host T-cells that turn over extremely rapidly[23].

It is observed that at least 10^9 new cells are infected each day in a typical HIV-infected patient during the latent or steady state stages of infection[24] and that every point mutation that could possibly occur along the length of the viral genome does occur that too at a staggering frequency of between 10^4 and 10^5 times daily. In addition to reverse transcriptase infidelity, mutations may be introduced into the viral genome through host cell RNA polymerase II transcription[23].

Drug resistance in HIV protease is commonly observed after three major events:-

1) Flap tip becomes semi open after mutation[25].

2) Active site pocket area increases due to structural deformation in the wild protease.

3) Loss of hydrophobic character of those residues which make good hydrophobic interaction with the drug molecule as well as loss of hydrophobic milieu inside active site pocket of wild protease.

After the analysis of different ligand bound conformations of HIV protease we came to the conclusion that some key mutations alter the equilibrium between the closed and open conformation of the protease. These differences may alter the dissociation rate and drug binding affinity[25]. With the help of NMR studies it is observed that flap domain has greater impact on drug binding affinity. Closed conformation of the flap helps in maintaining the geometry of active site binding pocket area as well as it provides good support to drug at the time of binding. Some mutations (V82A/I84V) alter the closed conformation of the flap protease and resulting in the shifting towards semi open conformation. Dissociation of product and the release of the drug molecule would probably be affected by the rate of flap opening as well[25]. Thus we can say that the flap conformation play a major role in regulating both the association rate and the dissociation rate. On the basis of structural analysis between wild (1HPV), mutated (2B7Z), and highly mutated (1TW7) HIV protease it has been observed that binding affinity of drug continuously decreases with increases the rate of flap opening.

On the basis of RMSD values (greater RMSD value means greater structural variation and lower RMSD value means lower structural variation) which were obtained after structural alignment it has been observed that, structural deformation and rate of opening of flap tip increases with increases the rate of mutation in 1HPV (No mutation), 2B7Z (mutation in 10 residues), 1TW7 (mutation in 12 residues). In the structural alignment analysis it has been observed that closed conformation of flap domain shifts towards open conformation (Fig. 4C).In order to prove the phenomena of shifting of closed conformation towards open conformation, distance has been measured from Asp25 (Active site) to Gly 51 (Flap tip). Measured distance values obtained in increasing order 12.97 (1HPV), 13.27 (2B7Z) and 18.50 (1TW7) with respect to the rate of mutation (Table 2).

The pocket area of the active site plays an important role in the drug packaging. In wild type protease active site pocket area is less than mutated protease so the drug molecule is packed properly inside the active site pocket. Another reason behind tight packing of drug molecule in wild protease is that, the active site residues remains closer to drug molecule, resulting the formation of strong hydrophobic interaction and hydrogen bonding between the drug and active site pocket's amino acid residues. While in case of mutated protease active site pocket area become large enough resulting in drug molecule being not packed properly and easily comes out from active site pocket. Some residues (V32, V82 and I84) play important role in drug binding due to strong hydrophobic interaction with drug molecule. After mutation these residues are replaced or changed by such type of residues which have less hydrophobic character (A82, V84) than wild type residues leading to the loss of hydrophobic interaction or formation of weak hydrophobic interaction with drug molecule.

In case of V82A mutation, both the methyl (CH_3) groups of Valine (Constant H.P value = 4.80 and 117 Mr Value) are exposed inside the active site pocket which provide more surface area than Alanine (Constant H.P value = 1.80 and 89 Mr value) for making strong hydrophobic interaction with drug molecule in wild type protease (Fig. 9B). While Alanine (A82) has a single methyl(CH_3) group which is exposed outside from active site pocket because of which it forms weak or no hydrophobic interaction with drug molecule due to less surface area as well as increasing distance from drug molecule in mutated protease (Fig. 9B).

Similarly (I 84) Isolucine (Constant H.P value = 4.50 and 131 Mr Value) has more hydrophobic character than Valine in wild protease. Isolucine provides wider surface area inside the active site pocket than Valine resulting in the formation of weak hydrophobic interactions or loss of hydrophobic interaction due to less surface area (Fig. 9B).

In case of V32I mutation the incoming residue after mutation, is Isolucine which has more hydrophobic character than Valine. But Isolucine disturbs the geometry of active site pocket due to its large side chain. After visualization and distance analysis (18.53Å (1HPV) and 20.27Å (2B7Z) between V32Ia to V32Ib, where a and b denote chain A and B) it was observed that isolucine escaped inside from its actual position and away from active site pocket (Fig. 9A) and is capable for providing only single methyl (CH_3) group for hydrophobic interaction with drug molecule in mutated protease. While in Valine both the methyl (CH_3) group of side chain inside the active site pocket were exposed in the wild protease, leading to the inference that increment in hydrophobic character is not favoured by good hydrophobic interaction thereby responsible for disturbing the geometry of active site pocket.

(A)

(B)

(C)

(D)
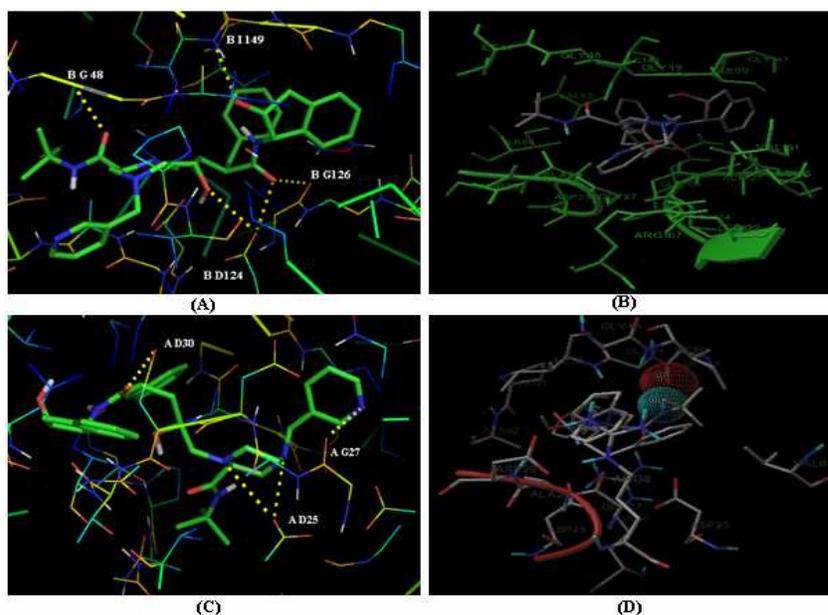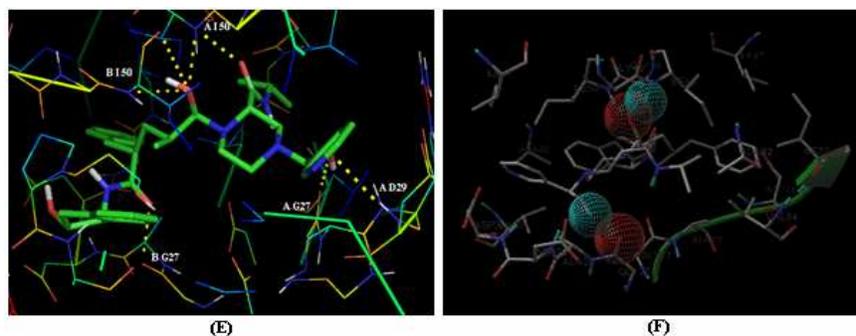
Fig. 8(A): Docked structure of Indinavir and 1HPV, visualized in PyMOL (Yellow dotted line show hydrogen bonds) (B) Docked structure of Indinavir and 1HPV, visualized in PMV(C) Docked structure of Indinavir and 2B7Z, visualized in PyMOL (D) Docked structure of Indinavir and 2B7Z, visualized in PMV (Cyan and red circle show hydrogen bond) (E) Docked structure of IND1 and 2B7Z, visualized in PyMOL (F) Docked structure of IND1 and 2B7Z, visualized in PMV.
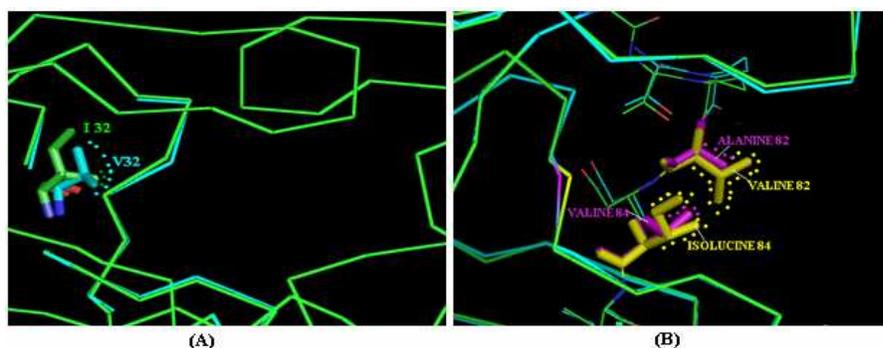
Fig. 9: It shows (A) Showing the escaped position of Isolucine (After mutation) and exposed area of Valine and Isolucine (B) Dotted line showing exposed area of V82A and I84V inside the active site pocket.

At the time of making new derivative IND1 mainly hydrophobicity and location of surrounding residues has been considered. We have induced some modifications in indinavir in which two major justifications have been considered (Fig 10).

1) Removal of those groups and atoms which did not participate in any interaction with an intention to reduce the molecular weight of derivative as compared to indinavir (indinavir has molecular weight more than 500 Daltons).

2) Increasing the hydrophobicity of those groups and atoms which remain near to the mutated residue or residues which after mutation take the place of wild residues and have less hydrophobic character (V82A, I84V) than wild residues in their native state.

Mostly anti-protease inhibitors do not follow the Lipinski's rule of five. In comparison with Indinavir which showed the variation in molecular weight 613 (500) and molecular refractivity 140.828 (45-130) our designed derivative IND1 followed strongly Lipinski's rule of five with the exception that it only showed a slight variation in molecular weight 563 but less than Indinavir. IND1 also showed good ADME properties and very less toxicity than Indinavir.The docking results of Indinavir with wild protease (1HPV) gave 5 hydrogen bonds were observed with GLY126/Ob-IND1/O31, ASP124/Ob-IND1/O31, ASP124/Ob-IND1/O24, ILE149Nb-IND1/O41 and GLY48/Ob-IND1/O17. While dock results of Indinavir and mutated protease (2B7Z) gave 4 hydrogen bonds were observed with ASP30/Oa-IND1/O31, ASP25/Oa-IND1/N6, ASP25/Oa-IND1/N3 and GLY47/Oa-IND1/N14. When dock results of IND1 and mutated protease (2B7Z) has been visualized, 7 hydrogen bonds were observed with GLY27/Ob-IND1/O6, ASP29/Na-IND1/O44, GLY27/Oa-IND1/O44, ILE50/Na-IND1/O37, ILE50/Nb-IND1/O28, ILE50/Ob-IND1/O28 and ILE50/Na-IND1/O28 (Table 5).
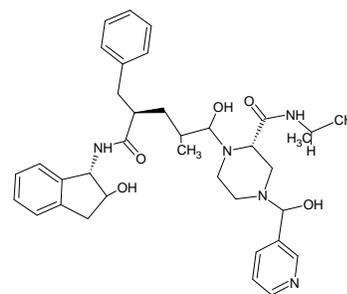
Fig. 10: 2D structure of IND1 (Derivative).

IND1 showed least minimum binding energy (-8.80 Kcal/mole), least Ki value (-35µM) and maximum number of hydrogen bonds (7) forms with receptor sites. Least minimum binding energy of IND1 than indinavir clearly indicates the increased rate of association of drug molecule with the receptor site, least Ki value (concentration of drug required for inhibition) indicates that lower concentration of IND1 required for inhibition than indinavir and maximum number of hydrogen bonds with receptor site (mutated) indicates the stronger binding of IND1 with receptor site than Indinavir (Table 5). IND1 showed also best binding pattern inside the active site pocket than indinavir (Fig 8E).On the bases of docking simulations, IND1 may act as a good anti protease inhibitor for mutated protease than standard Indinavir.

CONCLUSION

Based on the results of the insilico analysis we observed that three factors, semi open flap domain, greater active site pocket area and loss of hydrophobicity inside the active site pocket are basically the

major factors responsible and subsequently leading to the drug resistance in wild protease. Some modifications have been incorporated considering all three factors in previously available drug Indinavir.The study performed herein is the first of its kind suggesting that the newly designed novel derivative of indinavir (IND1) showed best minimum binding energy, less Ki value, maximum number of hydrogen bonds with receptor site and best binding pattern than the indinavir. IND1 showed good ADME properties and less toxicity than the indinavir.Our structural analysis may be explored in an effective way by researchers who are engaged in designing anti-protease inhibitors as well as our insilico results clearly suggest that IND1 is highly effective drug (anti protease inhibitor) against mutated protease. The study also opens the hidden potential of insilico bioinformatics tools for quick and cheap preliminary screening of novel drugs for their efficacy before moving towards the costly and time consuming wet lab studies.

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