Deciphering the molecular mechanism of altered interaction of second and third generation HIV-1 protease inhibitors atazanavir and lopinavir with wild type,ASP30ASN and LEU76VAL mutant Protease enzyme via *in silico*-based approach

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Abstract

Protease (PR) inhibitors uplift the efficiency of the highly activated anti-retroviral therapy (HAART) regimen against HIV treatment. However, emergence of drug resistance strains against PR inhibitors is the major issue in the treatment of HIV infected patients. The present study focuses on to understand the resistance mechanism of ASP30ASN and LEU76VAL mutant PR enzyme against second and third-generation PR inhibitors at molecular level. The results provided the comprehensive analysis of alterations in atazanavir,lopinavir, darunavir and tipranavir interactions with ASP30ASN and LEU76VAL mutant PR enzymes at molecular level corresponding with the experimental reports. Atazanavir and darunavir interactionwith PR enzyme gets affected by ASP30ASN mutation and mutant PR enzyme remains in open conformation during MD simulation. This might result in resistance of ASP30ASN protease against atazanavir and darunavir. Whereas, lopinavir and tipranavir binding with ASP30ASN mutant PR enzyme becomes strong and exhibits high stability. The drugs atazanavir, darunavir and tipranavirmaintains its hydrophobic interaction with substrate binding region even in the presence of LEU76VAL mutation and exhibits high stability.However, the lopinavir drug was affected by electrostatic modifications of the active site created by LEU76VAL mutation and loses its contact with substrate binding region. Also, lopinavir binding with LEU76VAL mutant protein increased the flap domain dynamics and stabilizes the dimer confirmation of PR enzyme against LEU76VAL mutant. As a consequence, resistivity was developed against lopinavir. This detailed information obtained from the present study will shed some light for future drug designing against HIV PR enzyme.

Key words: HIV-1, wild type, ASP30ASN and LEU76VAL mutant protease enzyme, DFT calculations; molecular dynamics simulation; MM-GBSA free energy calculations.

1. Introduction

The human immune deficiency virus (HIV) belongs to the retrovirus family causes acquired immune deficiency syndrome (AIDS) that leads to death. In worldwide around 38.4 million people are living with the virus, most of them avail anti-viral drugs that improves their quality of life. These anti-viral drugs can be classified into entry level inhibitors, reverse transcriptase (RT), protease (PR) and integrase (IN) inhibitors. These classes of drugs are come under the category of highly activated anti-retroviral therapy (HAART) to achieve the viral suppression level [1] in HIV infected patients. Especially, introduction of PR inhibitors in HAART regimen improves the clinical outcomes of AIDS related treatment [2]. These noteworthy drugs were developed based on the structure of PR enzyme. The structure and function of PR enzyme was determined in the late of 1980s. The PR enzyme process the gag and gag-pol polyproteins into matured proteins during the final stage of viral life cycle. Inhibition of HIV protease activity leads to the formation of inappropriate protein components which results in the development of impotent HIV virions. For this reason, it was quickly recognized that molecules prohibiting the enzyme activity were the outstanding drug candidates for the treatment of AIDS. Whereas, the PR enzyme belongs to aspartate protease family has simple homodimer structure with each subunit has 99 amino acids. The N-terminal β-strands comprises of residues 1-35 including catalytic triad (Asp25-Thr26-Gly27) followed by broad loop (36-42). The second half of the enzyme contains β-strands including flap regions (43-85) followed by helix (86-94) and c-terminal amino acids (95-99). The catalytic triad forms rigid network via the interactions with the neighbouring amino acids and it is called as “fireman’s grip” [3]. Further the detailed investigation of flap opening and closing mechanism is necessary to understand how the substrate/inhibitors bind with the active site and further process takes place. Hence the flap domain poses three states including open, semi-open and closed confirmation and vice versa during substrate/inhibitors binding. After

the successful completion of PR structural analysis, structure-based inhibitors were developed and so far, ten inhibitors have been approved by food and drug administration (FDA) of USA. They are classified into three generation of inhibitors based on the treatment protocol [4]. However, emergence of drug induced mutation of PR enzyme leads to the resistant viral strains' development against approved drugs but not for viral substrates/polyproteins. Hence these mutant PR enzyme causes drug failure and it is a major pitfall in HIV-1 treatment. To design the "resistant-repellent" drugs detailed investigation of mutant PR enzyme structures is required. Based on resistance, these mutations were classified as major and minor which increases the susceptibility to viral poly proteins whereas the PR inhibitors almost lost. Therefore, the present study focused on examining the role of second and third-generation inhibitors atazanavir, lopinavir, darunavir and tipranavir with respect to wild type, ASP30ASN and LEU76VAL mutant PR enzyme. Second line drugs were peptidomimetic drugs while atazanavir designed with aza-hydroxyethylene core, phenyl pyridyl at P1 and benzyl group at P1′ of substrate; whereas, lopinavir was designed with hydroxyethylene core phenoxyacetyl at P2 and cyclic urea in P2′ position of substrate. Third line drugs Darunavir is a peptidomimetic drug derived from the first-generation inhibitor amprenavir. The substitution of bis-tetrahydrofuran group in the P2-site enhances its inhibition activity than amprenavir. Tipranavir is a non-peptidomimetic drug with dihydropyrone scaffold. They were derived based on the strategy that they have reduced amount of peptidic backbone features but regaining the central hydroxyl group. Both molecules are rich in hydrophobic group to have interaction with the active site hydrophobic amino acids and also have polar group to interact with the catalytic residue Asp25. The peptide like carbonyl in the earlier inhibitors of PR was replaced by Sulphonamide group [1, 2]. The ASP30ASN mutation modulates the confirmation of active site and disrupts the inhibitor binding. Reports proclaimed that the ASP30ASN mutation has no effect [5, 6] whereas, LEU76VAL mutant decrease the susceptibility of lopinavir and increase the same

for atazanavir [7]. Initially DFT studies has been carried out to understand the structure, stability and reactivity nature of second- and third-line drugs in gas phase. Further, molecular docking and molecular dynamics simulation have been carried out to obtain the structure, conformational modifications, stability and binding nature of all molecules with wild type, ASP30ASN and LEU76VAL mutant PR enzyme complexes. Free energy calculation results carry the detailed information about the coulomb, hydrogen bonding, lipophilic and Van der Waals binding free energy value of the wild type and mutant complexes. As a consequence, the present study provides a clear picture about the role of ASP30ASN and LEU76VAL mutant in the binding affinity of the drugs atazanavir, darunavir, lopinavir and tipranavirtowards the PR enzyme.

2. Computational details

2.1. Molecular optimization and molecular docking

The molecular structures of second- and third-generation drugs have been obtained from pubchem database. Optimization of these molecules was carried out by density functional theory (DFT) method using the basis set 6-311G** incorporated in GUASSIAN03 software package [8-10]. The highest occupied molecular orbital (HOMO), lowest unoccupied molecular orbital (LUMO) and molecular electrostatic potential (MEP) maps were got from using GaussView6 package [11]. The crystal structures of wild type (4HLA), ASP30ASN (2Q64) and LEU76VAL (3PWM) mutant PR enzyme were retrieved from protein data bank [12-14]. Further the optimized structures were converted into pdbqt form using Auto Dock Tools. In addition, polar hydrogens, united atom Kollman charges, solvation parameters and fragmental volume parameters were added to the wild type, ASP30ASN and LEU76VAL mutant protein structures and saved in pdbqt form. Auto Grid was used for the preparation of the grid map using a grid box. The grid size was set to $30 \times 30 \times 30$ xyz points with the default grid spacing and grid centre was designated at dimensions (x, y, and z): for wild type, ASP30ASN and LEU76VAL mutant PR enzymes

were 11.57, -22.14 and 0.294; -12.125, 22.1 and 24.62; 14.8, 12.81 and 18.611, respectively. From the ligand, protein pdbqt files and grid box properties configuration files AutoDockVina was employed for docking. AutoDockVina provides iterated local search global optimizer [15-18]. The results less than 1.0 Å in positional root-mean-square deviation (RMSD) was clustered together and represented by the output with the most favourable free energy of binding. The pose with lowest energy of binding or binding affinity was extracted and aligned with receptor structure was saved as complex pdb file and used for further analysis [19]. The lowest binding energy values of thewild type, ASP30ASN and LEU76VAL mutant complexes are -9.7, -9.8, -11.3 (atazanavir and darunavir), and -10.2, -10.4, -12.4 (lopinavir and tipranavir) kcal/mol, respectively.

2.2. Molecular dynamics and free energy calculations

MD simulation was completed using OPLS_2005 force field implemented in Desmond package implemented in Schrödinger suite [20, 21]. The buffer was set with orthorhombic periodic boxes of volume 10 $Å^3$ was constructed using TIP3P water association system. Charged ions (Na^+/Cl^-) were placed isotopically to neutralize the Ewald charge summation. Minimization of the system has been carried out by steepest descent and conjugated gradient methods and canonical ensemble (NVT) was maintained while heating the system from 0 to 300K at 200 ps. Finally, the MD production has been carried out with isothermal-isobaric ensemble (NPT), constant temperature (300 K) and pressure (1 bar) up to 100 ns for all the complex systems were performed in 2 fs time step. RMSD and root mean square fluctuation (RMSF) [22, 23] of atazanavir, darunavir, lopinavir and tipranavir-wild type, ASP30ASN and LEU76VAL mutant complexes were observed to predict the strength of the intermolecular interactions and conformational changes. The principal component analysis (PCA), details of cross correlation map (DCCM) plots, solvent accessible surface area (SASA) and inter and intramolecular hydrogen bond analysis were created with the CPPTRAJ and R-package software [24, 25].The interaction of second- and third-line

inhibitors with wild type, ASP30ASN and LEU76VAL mutant were analysed using PyMOL software [The Pymol Molecular Graphics System Version 2.0, Schrodinger LLC] and Discovery Studio Visualizer [BIOVIA, San Diego, CA, USA].The binding free energy (MM/GBSA) of the complex structures were calculated from MD simulation trajectories by Prime application available in Schrodinger software package [26-28].In order to obtain the free energy landscape (FEL) of the complexes, the free energy value was calculated using $\Delta G = -RT\ ln\ (P_{ij}/P_0)$ where P_{ij} is the population of structures and P_0 is the maximum population.Hence theFELvalues of the complexes were obtained from in-house script. The corresponding scatter plots were generated using *origin* software package.

3. Results

3.1. Global descriptors of molecules atazanavir and lopinavir

The MEP and HOMO-LUMO plots of atazanavir, darunavir, lopinavir and tipranavir molecules are shown in Figure S1. The corresponding global descriptors of the molecules retrieved from HOMO-LUMO energy values are listed in Table 1.

In MEP plot the electrophilic regions are shown in red colour and the nucleophilic regions are in blue colour. Whereas, the electrophilic region designates the reactive regions of the molecules. The positive electron density is shown in red colour and the negative electron density is shown in green colour of HOMO-LUMO plots. Based on global hardness and HOMO-LUMO band gap energy values all the molecules seem stable and atazanavir has the lowest chemical reactivity and high kinetic stability than all other molecules. The lower LUMO energy of atazanavir and tipranavir, stands for nucleophilic natureprefers to bind with positively charged amino acids. Whereas darunavir and lopinavir were electrophilic by default and prioritize positively charged amino acids to interact. The chemical potential defines the propensity of electrons to escape from the fundamental state, whereas lopinavir possess lower energy value than other molecules. Electronegativity is the measure of tendency to attract electrons by an atom in a chemical bond, hence atazanavir and darunavir has more electrophilic than other two molecules and they might form more number of intermolecular interactions. The electrophilicity index is the measure of toxicity of the molecule based on the energy flow within the molecule. Tipranavir has more electrophilic index (~0.14) than atazanavir (~0.09), darunavir (~0.08) and lopinavir (~0.07). Even though, all the values are moderately low and they are less toxic in nature.

3.2. RMSD, RMSF, Rg and DCCP plots of second- and third-line drugs in complex with wild type, ASP30ASN and LEU76VAL mutant PR enzyme.

In molecular docking the interaction of the drugs with wild type and mutant (ASP30ASN and LEU76VAL) PR proteins are almost similar and did not show notable conformational changes in the protein (Data not shown). The RMSD and RMSF plots of all the molecules in complex with wild type, ASP30ASN and LEU76VAL mutant PR enzyme is shown in Figure S2A and B, respectively. The corresponding RMSD values of all the complexes are below 2 Å proposes the high stability of the system. Especially, tipranavir-wild type, ASP30ASN and LEU76VAL mutant complexes possess RMSD value in the range of ~1.5 Å, respectively. Whereas the RMSD value of darunavir-ASP30ASN mutant PR enzyme complex after 35 ns MD simulation reached around 2 to 2.3 Å. Further detailed analysis indicates that the wild type complexes have RMSD value of the range 1.5 Å. Both ASP30ASN and LEU76VAL mutation of PR enzyme plays different role in drug binding except tipranavir. Proportionately the RMSD values of atazanavir-ASP30ASN seems lower than atazanavir-LEU76VAL mutant PR enzyme complex. For darunavir and lopinavir based complexes the ASP30ASN mutation increased the RMSD value whereas, LEU76VAL mutation reduced the same.

The RMSF values of atazanavir, darunavir and tipranavir-wild type, ASP30ASN and LEU76VAL mutant PR enzyme complexes are within the range of 2 Å except for darunavir-ASP30ASN mutant PR complex. Thecorresponding RMSF values of this complex reached 2 Å to 3.7 Å, around flap region amino acids and substrate binding domain. The active site of atazanavir, darunavir lopinavir and tipranavir wild type PR enzyme complexes undergoes low fluctuations. Hence, the ASP30ASN and LEU76VAL mutation hikes the RMSF values of

active site amino acids present in the atazanavir-PR enzyme complex. Except lopinavir-wild type PR enzyme complex the flap domain of all wild type complexes exhibits low RMSF values. The ASP30ASN mutation hikes the flap domain RMSF values andon the other hand, the LEU76VAL mutation increased the RMSF values of substrate binding region of all the complexes. The Rg values of all the complexes are shown in figure S3 about the range of 17-18 Å, respectively. This stable range denotes the high compactness and low secondary structure modifications during MD simulation.

3.3. PCA and DCCM plots

The PCA and DCCM graphs of second and third-generation drugs-wild type, ASP30ASN and LEU76VAL mutant complexes are shown in Figure 1 and S4(A-D), respectively. To extract the main concerted and functionally relevant motion from MD trajectory PCA plots has been utilized. Using PCA the global and collective motion of a ligand-receptor complex can be mined from local and fast motion. The corresponding PC1, PC2 and PC3 values of all the wild, mutant-type complexes and apo-form are listed in Table 2. The first three eigenvectors revealed dominant motion with a higher eigenvalue. The apo-form of wild type and Leu76Val PR enzyme has higher mobility than ligand bounded form, indicates the binding of drug affects the overall mobility of the PR enzyme. The molecules atazanavir and tipranavir controls the overall motion of the wild type PR enzyme than darunavir and lopinavir in comparison with the apo form. Whereas the molecules darunavir, lopinavir and tipranavir controls overall mobility of the Leu76Val mutant PR enzyme than atazanavir in comparison with apo-form. On the contrary, Asp30Asn mutation, exhibits lower motion than ligand bounded form. In particular atazanavir and darunavir increase the overall mobility of the complexes than lopinavir and tipranavir. These changes denote that mutation highly disrupts the mobility of PR enzyme during MD simulation.

The DCCM plots of atazanavir, darunavir, lopinavir and tipranavir-wild type, ASP30ASN and LEU76VAL mutant complexes are shown in Figure S4 (A-D). The DCCM plots gives the clear picture about the correlation of amino acids with respect to ligand/drug molecule. The cyan colour indicates the presence of pair wise correlation residues and the pink colour denotes the presence of anti-pair wise correlation residues with respect to drug molecule. In the case of atazanavir and darunavir molecules, the ASP30ASN mutation leads to the higher occurrence of anti-pair wise correlating residues than the drugs lopinavir and tipranavir. The LEU76VAL mutant contributes some impact on the flap domain of atazanavir and substrate binding region of both atazanavir and darunavir than all other molecules.

3.4. Intermolecular interactions, binding free energy calculations and FEL analysis:

The intermolecular interactions between second-generation drugs and wild type, ASP30ASN and LEU76VAL mutant PR enzyme complexes are shown in Figure 2-6.The corresponding intermolecular distance values are listed in Table 3. In atazanavir-wild type and ASP30ASN mutant PR enzyme complexes, atazanavir forms strong hydrogen bonding and hydrophobic interactions with the catalytic triad amino acids Asp25 and Gly27; active site amino acids Arg8, Ala28 and Asp29; and flap domain residues Ile47 and Gly48. In atazanavir-LEU76VAL mutant complex, the drug losses its contact with active site and substrate binding region amino acids. As a consequence, the molecule moves towards the flap region and causes open confirmation.

Darunavir formed strong interactions with catalytic domain, flap region and substrate binding domain of wild type and LEU76VAL mutant proteins. However, in ASP30ASN mutant, darunavir did not interacted with mutated amino acid Asn30 and substrate binding region amino acids. Nevertheless, darunavir formed strong interaction with the flap domain residues in ASP30ASN mutant protein. Unlike ASP30ASN, in wild type and LEU76VAL mutant PR enzymes it formed strong hydrogen bonding interactions with Asp30. In addition, hydrophobic interactions with substrate binding region amino acids Pro81, Val82 and Ile84 are observed.

The lopinavir-wild type PR enzyme complex formed strong interactions with active site and flap domain amino acids and not with catalytic triad residues.Such effect is reflected in RMSD and RMSF plots of the same complex. Whereas, the ASP30ASN and LEU76VAL mutant causes reverse effect in lopinavir binding that leads to decrease in RMSD and RMSF values. In ASP30ASN mutant PR, lopinavir formed strong intermolecular interactions with catalytic triad, active site and flap domain amino acids whereas these interactions got weaker due to LEU76VAL mutation. Especially, in ASP30ASN mutant PR enzyme complex, the

intermolecular distances of amino acids Asp25 and Ile50 are around 1.6 and 3.0 Å, and inLEU76VAL mutant complex the distances become 2.3 Å with lopinavir.

Therefore, due to LEU76VAL mutation the molecule lopinavir moved towards flap domain from catalytic triad during MD simulation and the flap domain loop structure gets modified into energetically less favoured parallel beta sheets. The corresponding secondary structure modifications of the complexes during docking and 100 ns MD simulations are shown in Figure 5.

The tipranavir molecule formed similar kind interactions with the amino acids of wild type, ASP30ASN and LEU76VAL mutant PR enzyme. However, tipranavir lost its interactions with substrate binding domain of ASP30ASN mutant PR enzyme during MD simulation. There are no significant changes in interactions with LEU76VAL mutant complex. The LEU76VAL mutation weakens the hydrophobic contacts of tipranavir with substrate binding region amino acids.

The binding free energy values of all the complexes are listed in Table 4. The ASP30ASN mutation affects the total binding free energy of all the complexes. In order to evident the other results the darunavir-ASP30ASN mutant PR enzyme complex has the highest binding free energy (-51.5 kcal/mol). The coulomb and hydrogen bonding interaction energy of atazanavir and lopinavir based complexes gets increased in the order of wild type, ASP30ASN and LEU76VAL mutant PR enzyme complexes. In special case, the coulomb interaction energy value of lopinavir-LEU76VAL mutant PR enzyme complex is excessive which is about 1.3 kcal/mol. The total binding free energy of tipranavir-LEU76VAL mutant complexes seems to be the lowest among all other complexes(-93.3 kcal/mol). This phenomenonattributes that the molecule binding may not be affected by the LEU76VALmutation.Overall, the total binding free energy value of darunavir ASP30ASN mutant complex seems higher than other complexes. Despite that, the LEU76VAL mutant decreases the covalent interaction energy of darunavir-PR enzyme complex than all other

LEU76VAL mutant complexes. Interestingly, the ASP30ASN mutation causes low covalent interaction energy in darunavir-ASP30ASN mutant complex than all other complexes.

The FEL plots of apo form and all the complexes are shown in Figure 7. The minimum free energy values of apo form wild type, ASP30ASN and LEU76VALPR enzyme structures during MD simulation indicates the LEU76VAL mutation increase the overall energy of PR enzyme and the agreeing values are 3.2, 3.0 and 4.0 kcal/mol, respectively.In the case of wild type PR enzyme, the minimum free energy value of apo form and ligand-based complexes are almost similar except darunavir and lopinavir. Whereas, these two molecules reduced the free energy value of the enzyme. The relative values of apo form, atazanavir, darunavir, lopinavir and tipranavir-PR enzyme complexes are 3.2, 2.6, 3.2, 2.8 and3.1 kcal/mol, respectively. The minimum free energy value of ASP30ASN mutation in apo form and the ligand-based complexes are 3.0, 3.2, 2.9, 3.0 and 3.9 kcal/mol, respectively. This value signifies that binding tipranavir with this mutant PR enzyme requires more energy. The minimum free energy values of LEU76VAL mutant PR enzyme gets reduced more due to binding of drugs. The corresponding values are 4.0, 3.2, 3.3, 3.4 and 3.2 kcal/mol, respectively.

3.5 SASA and hydrogen bonding analysis

SASA is a key metric in MD simulations used to measure how much of a molecule's surface is exposed to the solvent. It is often used to study protein folding, binding interactions, and conformational changes. High SASA value indicates the complex undergoes more exposure towards solvent and low SASA denotes less revelation.

The apo form of wild type PR enzyme has low SASA value than ligand bounded form and mutated case. The value indicates that, ligand binding and mutation affects the overall conformation of the PR enzyme. In addition, SASA values of each drug molecule has been investigated and the corresponding plots are shown in Figure S5. The SASA value of atazanavir, darunavir and lopinavir wild type PR enzyme complexes are in the range of 10800-11200 $Å^2$and for tipranavir based complex the value ranges around 10400 to 11000 $Å^2$, respectively. Similarly, SASA values of apo form, atazanavir and darunavir ASP30ASN PR enzyme complex shares similar solvent exposure region, 10800-11600 $Å^2$, respectively. The lopinavir based complex shows less solvent exposure than others whereas, tipranavir based complex has an uneven exposure during MD simulation. The drug binding reduces the SASA values of LEU76VAL PR enzyme, the molecules darunavir, lopinavir and tipranavir displays compact form and the values ranges between 10000-10600 $Å^2$, respectively. For atazanavir based complex the molecule displays slightly increased area (10750-11000 $Å^2$).

The intramolecular hydrogen bonding analysis plot shown in figure S6, reveals that mutation and drug binding disrupt the network of interaction within the protein. The LEU76VAL mutation, impacts less on intramolecular network than ASP30ASN mutation.

The intermolecular hydrogen bonding between drugs and wild, ASP30ASN and LEU76VAL mutant PR enzyme complexes are shown in figure S7. In wild type PR enzyme complexes, atazanavir exhibits more interactions than other three molecules. In the case of ASP30ASN mutant PR enzyme complex, atazanavir and lopinavir molecules displays more interactions

than others. The tipranavir-LEU76VAL mutant PR enzyme complex exhibits less interactions than other three complexes.

4. Discussion

4.1. Atazanavir-wild type, ASP30ASN and LEU76VAL mutant PR enzyme complexes

Atazanavir has low toxicity than all other protease inhibitors. Although, high ionization potential value and electrophilic index of atazanavir was reflected in its EC_{50} which is in the range of 2.6 to 5.3 nM. Whereas, the drug darunavir has the EC_{50} value of 17 nM and the respective ionization potential and electrophilic index values seems low [29-31]. The ASP30ASN mutation increased the fluctuations of active site and flap domain amino acids in atazanavir-PR enzyme complex and does not affect the substrate binding region. As a consequence, anti-pair wise cross correlating residues are prominently present in the same complex that leads to the increase in motion of the system and causes less stability of the complex. Further, the less stability may accompany with the declination of susceptibility of atazanavir towards ASP30ASN mutant PR enzyme. In atazanavir-wild type PR enzyme complex the central hydroxyl (OH) group formed strong hydrogen bonding interaction with Asp25 (2.8 Å) and for ASP30ASN (3.6 Å) however inLEU76VAL mutations the interaction got weaker. However, atazanavir formed hydrophobic interactions with the substrate binding regions of wild type, ASP30ASN and LEU76VAL mutant PR enzyme.

Reports proclaim that LEU76VAL mutant causes hyper susceptibility of atazanavir towards PR enzyme [32-34]. Nevertheless, the underlying mechanism is not yet recognised. The unknown mechanism of high susceptibility of atazanavir towards LEU76VAL mutant PR enzyme has been revealed in the present study using 100 ns MD simulation of atazanavir-LEU76VAL mutant complex and is as follows: Leu76, forms Van der Waals interactions with Asp30, Thr74, and with the side chains of Lys45 and Ile47 in the first β-strand of the two-stranded flap and behaves as a central component of flap-substrate binding region interface. Also, it connects the flap and active site residues via exclusive hydrophobic interaction

network. The major goal of LEU76VAL mutation is to reduce the hydrophobic contacts between substrate binding region and flap domain to lead the open conformation formation and dissociation of dimer [35-39]. Yet, atazanavir betrays the LEU76VAL mutational effects via strong hydrogen and hydrophobic bonding with the flap domain residues Gly48, Gly49 and Ile50; and hydrophobic interaction with the substrate binding region amino acids Thr80, Pro81 and Val82. Such interactions result in lower hydrophobic interaction energy for atazanavir binding with the LEU76VAL mutant PR enzyme (Table 4). In this way atazanavir retains the dimer stability and closed conformation; subsequently the susceptibility of atazanavir becomes high in the case of LEU76VAL mutant PR enzyme.

4.2. Darunavir-wild type, ASP30ASN and LEU76VAL mutant PR enzyme complexes:

The lower HOMO energy value of darunavir indicates the large reactive nature of the molecule when react with electrophiles. Therefore, darunavir is more favour for catalytic amino acid Asp25 and other negatively charged amino acids present active site. Darunavir has no observed cytotoxicity up to 1000 μM concentration [3]and the respective electrophilicity index is also quite low. Electrophilicity value predicts that energy flow within the molecule, whichwas low in darunavir in comparison to atazanavir and tipranavir, indicates the lower toxicity of the molecule [4]. The changes in RMSD and RMSF values of Darunavir-ASP30ASN complex indicates conformational modifications in the complex system. Especially the RMSF value of amino acids Gly48-Phe53 and Pro81 of both subunits seems higher than other amino acids. These amino acids fall around flap region and substrate binding region specifies the effect of ASP30ASN mutation disturbing the binding of darunavir. The ASP30ASN mutation modifies the orientation of Asp30 to face away from the binding pocket and hinders its access to PR inhibitors [5]. The DCCM plots represent that ASP30ASNand LEU76VAL mutant PR enzyme encourages formation of anti-pairwise correlation. This denote the higher resistivity of both mutant PR enzymeover darunavir and supports the experimental reports[6, 7]. The ASP30ASN mutation causes the shrinking of

active site pocket volume and that contribute to the movement of darunavir towards the flap domain [8]. Further investigating these phenomena in detail via intermolecular interaction analysis explores that darunavir almost lost its contact with catalytic and substrate binding site that leads to semi-open conformation of flap domain and prohibits the access of darunavir to the catalytic domain of ASP30ASN mutant protein. The MM-GBSA free energy studies also confirmed that the rise of electrostatic and van der Waals interaction energy values refers to the resistive mechanism of ASP30ASN against darunavir [9]. Further, by analysing the intermolecular contacts of darunavir-LEU76VAL mutant PR complex from MD simulation studies confirmed that the LEU76VAL mutation disrupted the hydrogen bonding network of Leu76 with the binding pocket amino acids especially, Asp25, Asp30/Lys45, Asp30, Thr31/Thr74 and lowered the internal hydrophobic contacts [10]. The hydrophobic interactions of darunavir with the substrate binding region was dislocated and it was confirmed by the presence of anti-pairwise correlating residues and changes in hydrophobic and van der Waals interaction energy of the darunavir-LEU76VAL mutant complex.

4.3. Lopinavir-wild type, ASP30ASN and LEU76VAL mutant PR enzyme complexes:

The lopinavir drug has higher chemical reactivity and chemical potential than atazanavir. Also, lopinavir is nucleophilic in nature. The RMSD of lopinavir-ASP30ASN mutant PR enzyme gets increased and the corresponding value gets decreased for LEU76VAL mutant complex with respect to wild type. The high RMSD value of earlier one is due to the high fluctuations of active site and flap domain residues whereas the low RMSD value of later is due to nominal fluctuations of the same than wild type. Occurrence of anti-pair wise cross correlating residues is nearly same for wild type and ASP30ASN mutant PR enzyme and less for LEU76VAL mutant. In relationship with the RMSD, RMSF and DCCM plots ASP30ASN mutant complex have higher mobility and less stability than wild type and LEU76VAL mutant complexes.Despite, the intermolecular interaction between hydroxyl

group of lopinavir with Asp25 becomes stronger (~1.6 Å) than wild type and LEU76VAL mutant complexes. By this way lopinavir is stable against ASP30ASN mutation.

According to reports the LEU76VAL mutant was developed to resist lopinavir, however binding of lopinavir with the LEU76VAL mutant active site was found to be not affected by the mutation in the earlier study [35]. Also, it has been postulated that the decrease in potency of lopinavir might be due to altered flap opening and closing dynamics. Due to reduced hydrophobic interaction and higher dimer dissociation constant, the LEU76VAL mutant enzyme was found to be less stable [14]. Insights in the structural properties through intermolecular interactions reveals that due to LEU76VAL mutation the large side chain of leucine has been modified with small side chain of valine. Owing to the emergence of free space in that region; the side chains of charged residues Asp30, Lys45 and Gln58 are shifted towards the binding pocket and alters the electrostatic properties of the active site [7, 40-44]. In accordance, in the present study, coulomb interaction energy of lopinavir-LEU76VAL mutant PR enzyme complex was found to be elevated than all other complexes (Table 4). Although such interaction energy barrier occurs, the interaction of lopinavir with active site and flap domain region might rescue the dimer stability and flap movement. From this present study the proposed drug resistance mechanism is as follows: The α-helix secondary structure conformation of substrate binding region (B-chain: Arg87-Leu89) has been converted into more stable loop confirmation [45]. By this way lopinavir might rescue the dimer stability. Consequently, the loop conformation of flap domain (A-chain) visualized in docking studies was changed into energetically less favoured β-sheets during MD simulation in the presence of lopinavir. This may lead to altered flap opening and closing dynamics in the presence of lopinavir [46]. Further, flap opening may cause substrate binding inside the active site and drug inhibition failure may occur. Overall, the LEU76VAL mutation might counterfeit the lopinavir inhibition by altered flap mobility as well as lopinavir binding might decrease the dimer instability of the LEU76VAL mutant [35].

4.4. Tipranavir-wild type, ASP30ASN and LEU76VAL mutant PR enzyme complexes:

The electrophilicity index of tipranavir is higher than all other drugs of the present study. There is no observed cytotoxicity concentration of tipranavir up to 100 μM [11] which is very low in comparison with darunavir (~1000 μM). While lower LUMO energy value of tipranavir denotes molecular reaction with nucleophiles. Hence the drug forms strong intermolecular interaction with the residue Arg8 during MD simulation. The RMSD and RMSF values were also quite low than other drugs investigated in the present study. This indicates that tipranavir is one of the strong inhibitors among the second and third generation drugs and remains stable even in the presence of ASP30ASN and LEU76VAL mutant PR enzyme. The DCCM plots also confirms that the development of anti-pairwise correlation residues towards tipranavir was very less than other drugs (Figure S4). The PCA plots and the corresponding PC values of tipranavir-wild type, ASP30ASN and LEU76VAL mutant complexes were also seems to be smaller (Table 2). Hence the mobility of the tipranavir based complex was low during MD simulation and suggest the higher stability of the same. The intermolecular interactions reveal that tipranavir forms strong hydrogen bonding and hydrophobic interactions with the active site, flap domain and substrate binding domain residues of both ASP30ASN and LEU76VAL mutant PR enzyme up to 100 ns MD simulation. Whereas, other third line drug darunavir loosed its contact with active site due to ASP30ASN mutation. Reports proclaimed that LEU76VAL mutation increases the susceptibility of tipranavir towards PR enzyme[7]. In order to support the experimental results, the tipranavir-LEU76VAL mutant PR enzyme complex has very less anti-correlation residues, least mobility in PC analysis, strong intermolecular interactions with active site, flap domain and substrate binding domain residues, lowest binding free energy and van der Waals interaction energy, respectively. As a consequence, tipranavir rigidify the flap domain of wild type and mutant PR enzyme than other second and third line drugs and supports the

experimental results [12]. Hence the drug shall not move away from the active site of wild type, ASP30ASN and LEU76VAL mutant PR enzyme and may prohibits the enzyme activity.

5. Conclusion

The molecular level resistance mechanism of some second and third-generation PR inhibitors has been investigated using DFT, molecular docking, MD simulation and free energy calculations.The ASP30ASN mutation interferes the binding of atazanavir and darunavir with the active site, flap domain and substrate binding region to cause open confirmation therefore may results in resistivity of PR enzyme. In MD simulation for 100 ns, RMSD and RMSF plots indicates that ASP30ASN mutation highly impacted darunavir interaction. Especially the flap domain undergone more fluctuations and led to semi-open conformation in darunavir-ASP30ASN mutant PR complex. The intermolecular interactions and free energy values also confirmed that darunavir gets affected by mutation, which is due to the higher reactivity of darunavir as calculated from global descriptors. Hence the new drug should be highly hydrophobic to regain these contacts to make the complex more stable. However, the ASP30ASN mutation tightens the binding of lopinavir and tipranavir with the above said regions and influenced theirhigher susceptibility towards PR enzyme. LEU76VAL mutant plays differently in the binding of second and third-generation drugs atazanavir, lopinavir, darunavir and tipranavir with PR enzyme. The mutation causes hyper susceptibility for atazanavir, darunavir and tipranavir and resistivity for the lopinavir. The above said three drugs betrays the effect of LEU76VAL mutant PR enzyme via strong bonding with the flap domain and substrate binding region amino acids. This leads to stable atazanavir, darunavir and tipranavir-LEU76VAL mutant PR enzyme complex and improves drug activity. While, in lopinavir-LEU76VAL mutant PR enzyme complex; the electrostatic properties of the active site have been altered due to mutation and it was confirmed via high coulomb interaction energy for lopinavir.Also, lopinavir binding may altered the flap mobility dynamics and increase the dimer stabilization of LEU76VAL mutant PR enzyme thereby would increase the

resistance of LEU76VAL mutant towards lopinavir. Hence these evidences of resistance mechanism obtained from present study is crucial for further drug designing strategy.

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