The concurrent mutations of C26N/N53F can reduce the antigenic propensity of nsLTP2 as an anti-tumor or viral drug carrier

short running title: The nsLTP2 mutated protein for anti-tumor or viral drug carrier

Samira Rahimirad[1], Najaf Allahyari Fard[*2], Raheleh Mirzabeygi[3], Behnam Mortazavi[4]

1. Department of Medical Genetics, Faculty of Medical Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran.
2. Department of Systems Biotechnology, Faculty of Industrial and Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran.
3. Department of Biology, Faculty of science, Nourdanesh Institute of Higher Education, Meymeh, Isfahan, Iran
4. Department of Animal Biotechnology, Faculty of Agriculture Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran.

*Corresponding author

Najaf Allahyari Fard

Email: allahyar@nigeb.ac.ir

Phone:

Postal address:

Co-author emails:

Rahimirads@yahoo.com

Raheleh0704@gmail.com

B_mortazavi@nigeb.ac.ir

Abstract

Nonspecific Lipid Transfer Proteins (nsLTP2) are small and soluble proteins, which due to their unique features have the ability of binding to lipids and some pharmaceutical compounds, are considered as good options for drug delivery systems. Their stability against proteolysis and thermal denaturation leads to allergenic reactions which limit its clinical usage. The bioinformatics approach was carried out to hydrophobicity and antigenicity analysis of *Oryza sativa* (Iranian group) nsLTP2. Using Molegro Virtual Docker software, the affinity and binding strength of several fatty acids, steroid-based anti-viral, and anti-tumor drugs with nsLTP2 were identified. Results demonstrated that there is only one transmembrane segment in the nsLTP2 protein sequence which is located in the signal peptide region, also calculating the average antigenicity propensity (AP) of amino acids showed that concurrent mutations of C26N/N53F can reduce the antigenic propensity of these proteins. Furthermore, Abacavir (MolDock Score = -119.348), DHA (MolDock Score = -152.601), and Basedoxifene (MolDock Score = -156.776) could be considered as the best antiviral, phospholipid, and anticancer ligands for it, respectively.

1. Introduction

Nonspecific Lipid Transfer Proteins (nsLTPs) are a superfamily of plant proteins considered as carriers in drug delivery system [1, 2]. These proteins are classified into two major groups based on molecular weight. NsLTP1 (9 KDa) and nsLTP2 (7 KDa) are helical proteins and have eight conserved cysteines with four disulfide bonds [3, 4]. These disulfide bonds form a stable structure to avoid drug oxidation or degradation and make an intense resistance to heat, denaturants, and proteases [5]. They contribute to controlling abiotic stress conditions during high temperatures and drought [6, 7]. An unstable drug in pharmacological therapies can be degraded against environmental changes. Plant nsLTPs can bind to a wide range of lipid molecules and it can be purified from barely [8], rice [9, 10], maize [11], wheat [12], and some fruits like peach and apple [13, 14]. The different isoforms of nsLTPs can be found in different plant species, because of a small multigene family. Also, nsLTPs can participate in pathogen resistance and developmental processes [15, 16]. nsLTP2 has some difference with nsLTP1 including: (a) different disulfide bond composition, (b) more structural stability, (c) smaller size and less sequence similarity [17]. The source of nsLTPs varies among different plant species. for instance, immunocytochemical studies showed that nsLTPs can express in the cell wall of leaves, flowers, vascular bundles, stems, siliqua, and petioles in maize, land cress, broccoli, and castor bean [11, 18-20]. However, in wheat seeds, they express in the aleurone grains [21]. NsLTP2 has a hydrophobic cavity enclosing to four disulfide bonds which is more flexible than nsLTP1s and gives the advantage to bind to a wide variety of lipids including sterols [22]. Some mutagenesis studies have been performed to strengthen the ligand binding and transfer activity. Direct mutagenesis of Leu8, Phe36, and Val49 of rice LTP2 lead to increased lipid binding and transfer activity, while the mutation of Tyr45 to Alanine caused a decreased lipid binding and transfer [23]. Almost 63 LTPs recognized as an allergen which their heterogeneous behavior can be a proof for this feature. Most of the allergens belonging to nsLTP1 family, but a few of them pertinent to nsLTP2 family. The aim of this study was to perform a mutagenesis analysis in order to alleviate antigenicity by replacing low antigenic residues with high antigenic residues and amplify ligand binding and transfer activity for pharmaceutic therapies.

2. Method

2.1 Protein constructing

The amino acid sequence of Oryza sativa (PUE92 isolate) LTP2 (Accession no. KJ174106) was obtained from the GenBank database. The protein contains 96 amino acid residues, in which 27 residues in N-terminus are known as a signal peptide. The solution structure of nsLTP2 from Oryza sativa is available in the PDB database but because of the slight differences in the sequence of Iranian rice nsLTP2, comparative modeling was performed using I_TASSER (Iterative Threading ASSEmbly Refinement), (https://zhanglab.ccmb.med.umich.edu/I-TASSER/), a hierarchical approach to protein structure and function prediction [24]. The structure of nsLTP2 (PDB id: 1l6h) was applied as a template. The signal peptide was removed from the mature protein structure. The amino acid sequence alignment of this protein and *Oriza sativa* (Indica group) nsLTP2 (PDB ID=1L6H) was performed using the Clustal Omega tool, a highly accurate web tool for multiple sequence alignment analysis. To identify similarities among protein sequences, the Clustal W with character count format was applied.

2.2 Hydrophobicity analysis

Hydrophobicity analysis of Iranian rice nsLTP2 and transmembrane areas was performed by two methods. The first method, TMHMM predicts transmembrane segment of protein sequence using database based on the Hidden Markov Model (www.cbs.dtu.dk) [25]. In the second method, hydrophobic segments were predicted by Sliding Window technique based on the Doolittle & Kyte method with the help of Protscale database (www.expasy.org/protscale)

2.3 Antigenicity prediction

The immune epitope is mainly responsible for activation of the immune system including B or T leukocyte, using proteins as promising drug carriers generally require the lowest level of immunization. IEDB is a free epitope database that consists of B or T cell tools, MHC-II binding predictions, and other analysis tools for immune epitope assessments [26]. In this study, the prediction of linear epitopes from the protein sequence was utilized. The protein sequence without signal peptide was submitted in the IEDB web server to determine high antigenic regions. Detection of high antigenic

plots was performed by seven sequence-based methods listed in the IEDB web server [27-32]. Antigenic plots based on structural method were identified by using the simulated 3D structure (Ellipro) [33]. Eventually, two amino acids were selected as the residues with the highest antigenicity effect and then replaced with amino acids that reduce the antigenicity effect to a significant amount.

2.4 Comparison of mutant protein with non-mutant protein

Both Superpose and TM-align software were applied to evaluate the structure of the mutant protein compared to the non-mutant protein and change in their binding sites. Also, the stability of the simulated mutant protein was verified by employing DynaMut (http://biosig.unimelb.edu.au/dynamut/)and STRUM (https://zhanglab.ccmb.med.umich.edu/STRUM/) web servers. TM-align is a protein structural alignment tool which is used for protein structure comparisons [34] [35]. Inputting two protein structures in PDB format is allowed, then structural similarities are applied for alignment. TM-align score differs from the min of 0 to max of 1. The input structures have an optimum match when they score equal to 1[36].

2.5 Ligand collection

PubChem along with ChemSpider software (http://www.chemspider.com), which are freely accessible chemical data sources [37, 38]. The deposited data of ChemSpider and PubChem complement each other and in total, they provide data contents for more than millions of compounds. They were utilized to download 3D structures of steroid-based anti-cancer drugs, anti-viral compounds, and fatty acid molecules. The name and the PubChem or ChemSpider ID of molecules are represented in table 1.

Table 1: Name and chemical IDs deposits in PubChem and ChemSpider data resources of intended ligands comprising three main antiviral drugs, phospholipids, and anticancer steroids drug groups.

2.6 Molecular docking

One of the professional tools for the docking process in Molegro Virtual Docker which predict the interaction between protein and ligand with high accuracy. Also, this docking platform has been

proven to properly detect cavities and the prediction of ligand binding. The binding affinity (kcal/mol) of the nsLTP2 protein to different types of drugs and phospholipids was searched through the Molegro Virtual Docker (MVD) software based on the following parameters: the number of runs 10, population size 50, crossover rate 0.9, scaling factor 0.5, maximum iteration 1500, and grid resolution 0.30. To found that how mutations could affect the binding affinity of this protein to different ligands, the obtained MolDock scores of wild nsLTP2 were compared with the mutated protein-related scores.

3. Results

The sequence alignment analysis of Iranian rice-derived nsLTP2 comprises with Indica group revealed that there was a slight difference in some amino acids including residues number S2, A12, and T51 which were glycine, threonine, and serine in India group respectively.

Table 2. Physicochemical characteristics of Iranian rice nsLTP2 (expasy.org/prot param)

```
Iranian     ASCNAGQLTVCAGAIAGGARPTAACCSSLRAQQGCFCQFAKDPRYGRYVNTPNARKAVSS    60
Japonica    AGCNAGQLTVCTGAIAGGARPTAACCSSLRAQQGCFCQFAKDPRYGRYVNSPNARKAVSS    60
            * ********* ***************************************** ********

Iranian     CGIALPTCH 69
Japonica    CGIALPTCH 69
            *********
```

Figure 1: Multiple sequence alignment analysis between Iranian and Indica derived nsLTP2 protein. The figure shows the differences between groups.

The IEDB results showed that some plots in Iranian nsLTP2 protein have high antigenicity potential in terms of sequence and structure. We selected two amino acids as the most antigenic plots (C_{26} and N_{53}) which these plots were above the threshold in seven sequence-based methods and a structural based method in IEDB. Then asparagine and phenylalanine replaced by cysteine (C26N) and asparagine (N53F) respectively. With two changes in protein, we observed a high decrease in protein antigenicity which shown in figure 1.

C26N Kolaskar & Tongaonkar Antigenicity Results

(A)

(B)
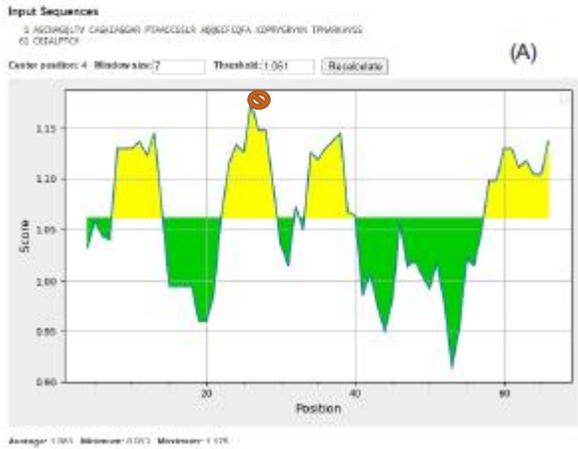
## N53F Parker Hydrophilicity Prediction Results

(C)

(D)
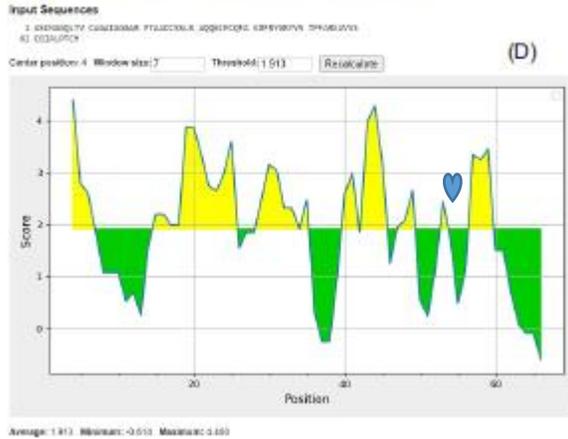
Figure 2: (A) nsLTP2 wild type (B) nsLTP2 C26N mutation. The kolaskar & Tongaonkar Antigenicity results revealed lower protein antigenicity by replacing asparagine in position 26. (C) nsLTP2 wild type (D) nsLTP2 N53F mutation. The parker hydrophilicity prediction results showed lower protein antigenicity by replacing phenylalanine in position 53.

The comparison of simulated Iranian nsltp2 protein with the mutant protein consist of two mutations in 26 and 53 positions revealed that the protein structure has no change after applying mutations (Figure 3).
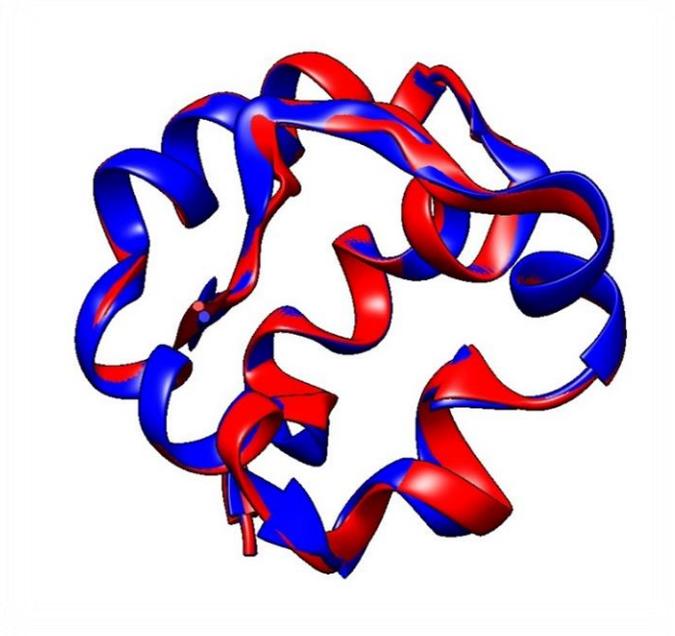
Figure 3: Comparison mutant protein (red) with non-mutant protein (blue) by TM-align.

To survey the impact of the mutation on ligand-carrying features of the nsLTP2 protein, a comprehensive analysis comprises of various ligands belongs to anti-viral drugs, steroid based-anti cancer drugs, and phospholipids were performed. The 3D model of wild type and three mutation forms of the protein was constructed using the I-TASSER server, then the docking process was performed based on the criteria mentioned in the method. Using the cavity detection algorithm of MVD, the cavity of each constructed protein was identified. As it is clear in figure 4, mutations of C26N and N53F in both single and concurrent form changed the position of the cavity compared to wild type. From these predicted cavities, nsLTP2 mutant C26N has the highest volume (30.208 Å).
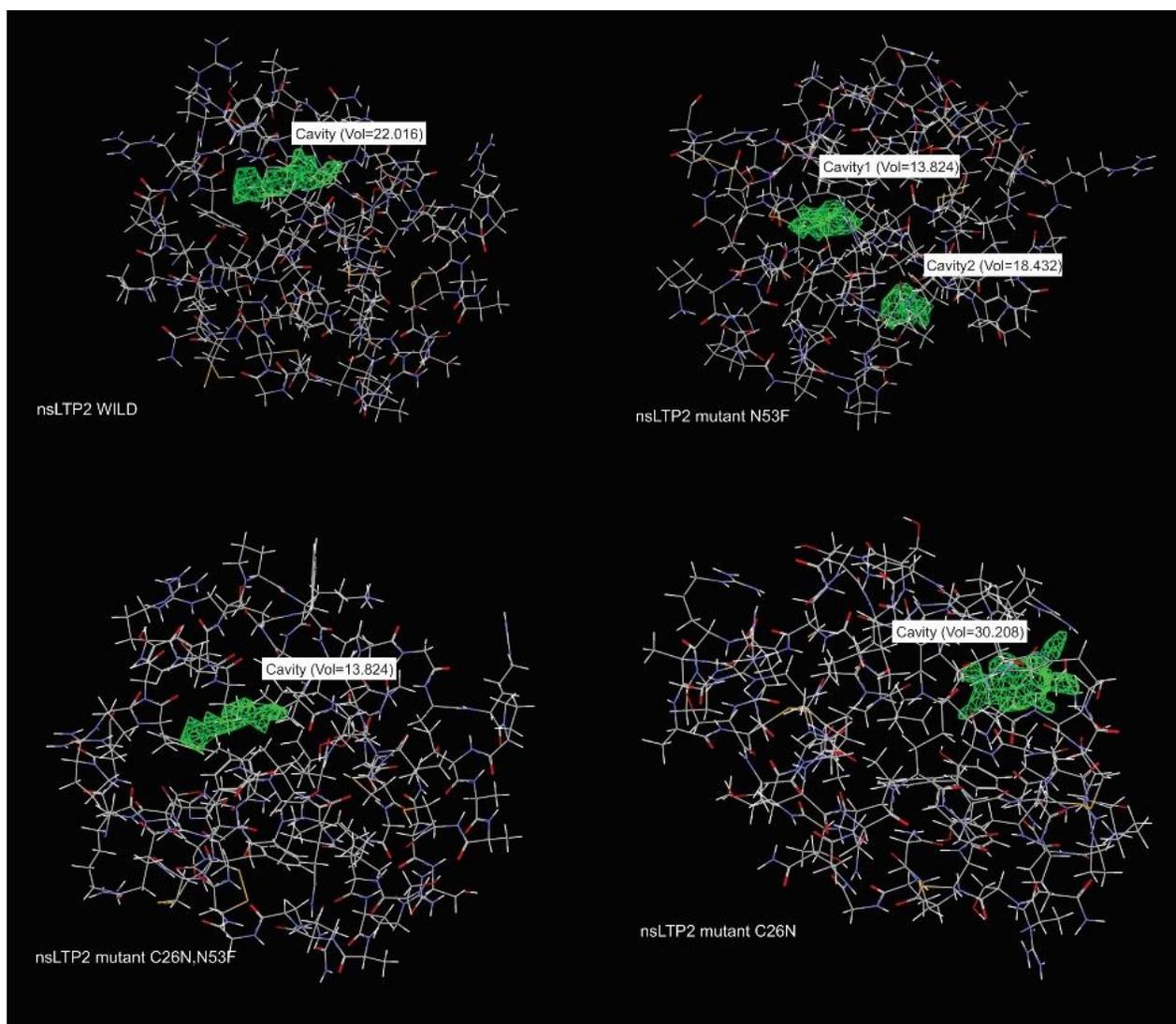
Figure 4: MVD-detected cavities in nsLTP2 (wild type, mutant type: C26N/N53F – C26N – N53F) and their calculated volume (in Å), cavity position in each protein is represented in green, carbon, oxygen, and nitrogen atoms are shown in grey, red, and blue respectively.

Docking studies of various ligands including phospholipids and antiviral drugs have been revealed their binding affinity to the nsLTP2 Indica groups. In the current study, the binding affinity of Iranian rice-derived nsLTP2 against these ligands was calculated for the first time. To anti-viral compounds, the obtained scores were between -86.7 and -130.5 kcal/mol. Abacavir known by Ziagen trade name (PubChem ID= 441300) had the lowest score which also the previous study on the nsLTP2 Indica group reported a low binding score (-152.5 kcal/mol) for this type of HIV medication. In the mentioned previous docking analysis of anti-viral ligands against Indica-derived nsLTP2, the MolDock score of

Vidarabine medication was lower than Abacavir (-155.5 kcal/mol) [39]. For phospholipids, this protein showed the highest affinity to Docosahexaenoic acid (DHA) (ChemSpider ID=393183) which belongs to an omega-3 polyunsaturated fatty acid with 22 carbons and 6 double bonds. Another docking study which was evaluated the binding affinity of nsLTP2 (Indica group) with fatty acids demonstrated the minimum score with Hydroxypalmitic acid (-128.2 kcal/mol).

Furthermore, it has been established that this protein also tends to bind steroid compounds but the affinity of this protein for anti-cancer steroid drugs has not been assessed before. The MolDock scores revealed that wild type nsLTP2 has a higher affinity to anti-cancer steroids than the other ligand groups. Docking results reported that Raloxifene (PubChem ID= 5035), under the brand name Evista, had the best affinity (-141.8 kcal/mol) in its related group.
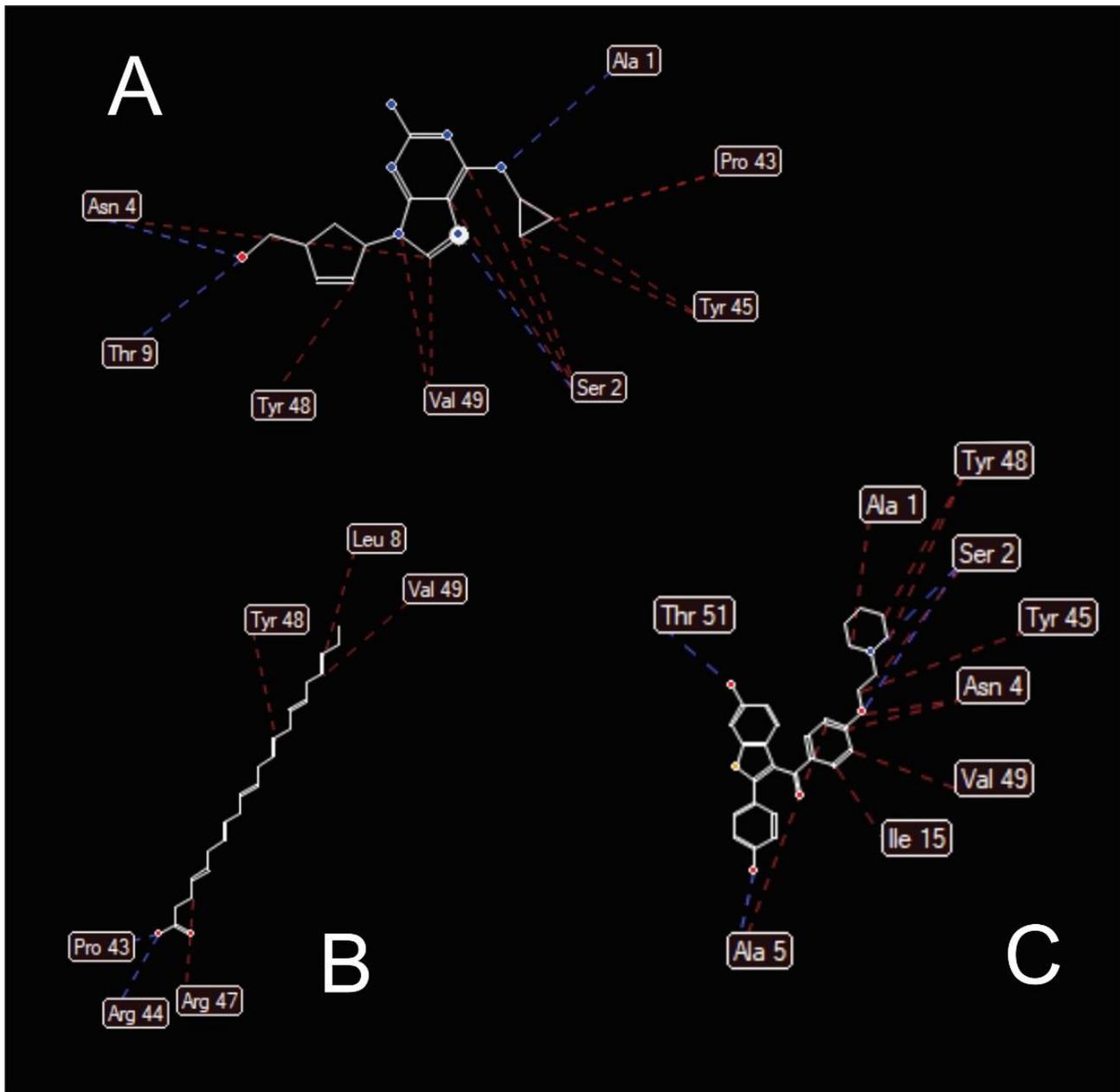
Figure 5: Hydrogen bond (Red stripes) and steric interaction (Blue stripes) of Iranian rice-derived nsLTP2 with (A) Abacavir, (B) DHA, and (C) Raloxifene as the best score ligands belongs to antiviral drugs, phospholipids, and anti-cancer steroid drugs respectively.
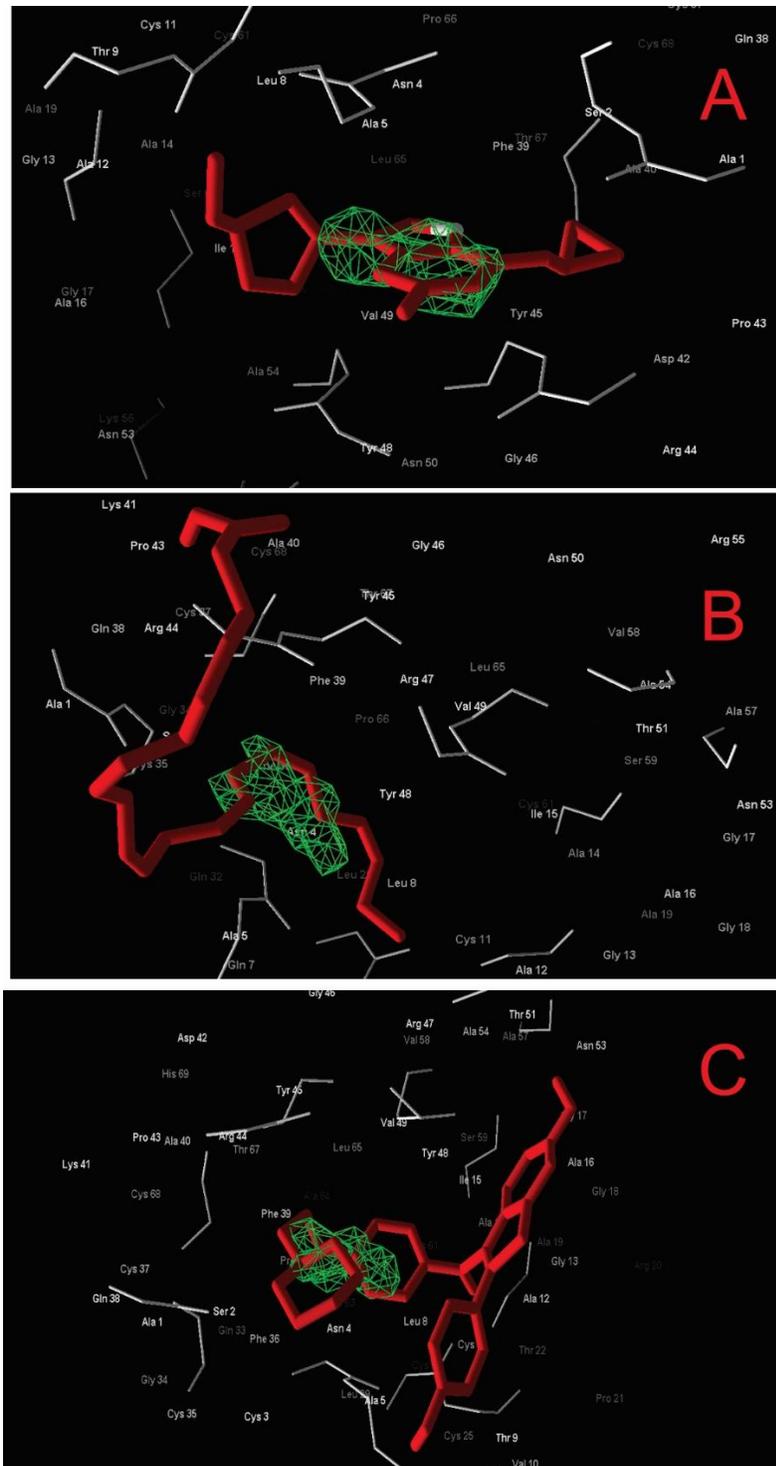
Figure 6: The position of the best score docking ligands including (A) Abacavir, (B) DHA, and (C) Raloxifene in the area of the wild type nsLTP2 related cavity. Active site residues are shown based on their name and chain ID and the ligand is represented in stick lines with fix red color. The cavity is located in green.

To decrease the antigenicity of this protein, different amino acids were searched to become nsLTP2 as a weakened stimulator of the immune system in the human body, since this protein is characterized as a potential drug carrier, the impact of the mutation on its affinity scores to various compounds would be beneficial. The collected scores of mutant nsLTP2 (C26N) demonstrated that this mutation had a noticeable role in increasing the binding affinity to antiviral drugs compared to wild type and the other mutation forms. In contrast, the N53F mutation form of nsLTP2 didn't have a significant role in improving the binding affinity to all 69 collected ligands. The docking results of concurrent mutations of C26N/N53F revealed that it had a significant role in the enhancement of the binding affinity to phospholipids. The phospholipids docking scores for the wild type were between -52.1 and -132, in contrast, the scores decreased dramatically to the range between -53.7 and -152.6 for concurrent mutations of C26N/N53F. In general, concurrent mutations of C26N/N53F in nsLTP2 had a better impact on all ligands compare to wild type and every single mutation and had the lowest score (-156.7 kcal/mol) of binding to basedoxifene (PubChem ID= 154257), a third-generation selective estrogen receptor modulator (SERM) which is used as cancer medication.

Table 3: MVD scores (kcal/mol) for 17 antiviral ligands when docked with nsLTP2 3D structures in both wild type and three mutations form comprising Cys26Asn, Asn53Phe, and concurrent mutations of Cys26Asn/Asn53Phe.

Table 4: MVD scores (kcal/mol) for 30 phospholipids when docked with nsLTP2 3D structures in both wild type and three mutations form comprising Cys26Asn, Asn53Phe, and concurrent mutations of Cys26Asn/Asn53Phe.

Table 5: MVD scores (kcal/mol) for 22 anti-cancer steroid drugs when docked with nsLTP2 3D structures in both wild type and three mutations form comprising Cys26Asn, Asn53Phe, and concurrent mutations of Cys26Asn/Asn53Phe.
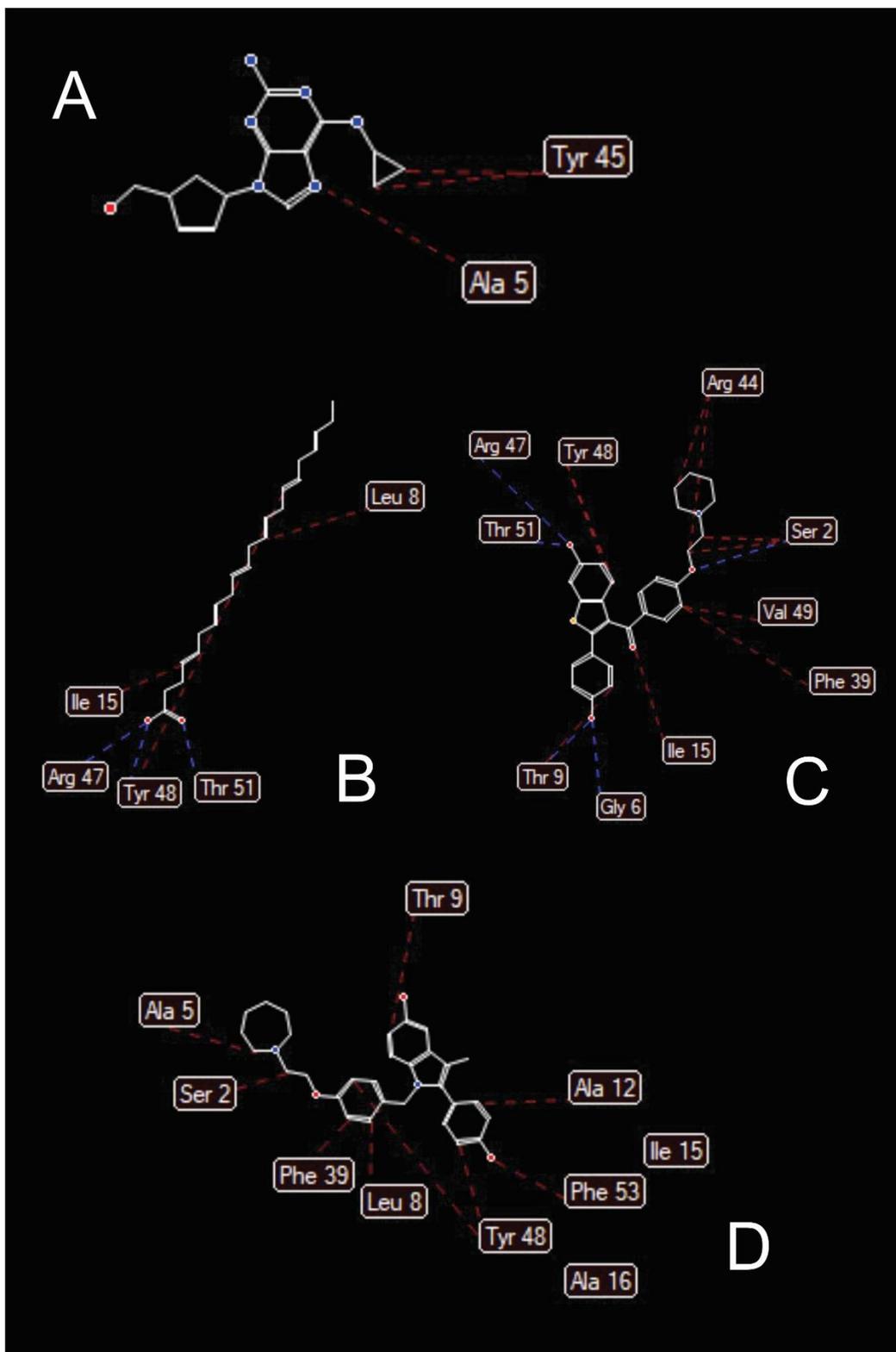
Figure 7: Hydrogen bond (Red stripes) and steric interaction (Blue stripes) of mutated nsLTP2 (C26N/N53F) with (A) Abacavir, (B) DHA, (C) Raloxifene, and (D) Basedoxifene as the best score ligands belongs to antiviral drugs, phospholipids, and anti-cancer steroid drugs respectively.
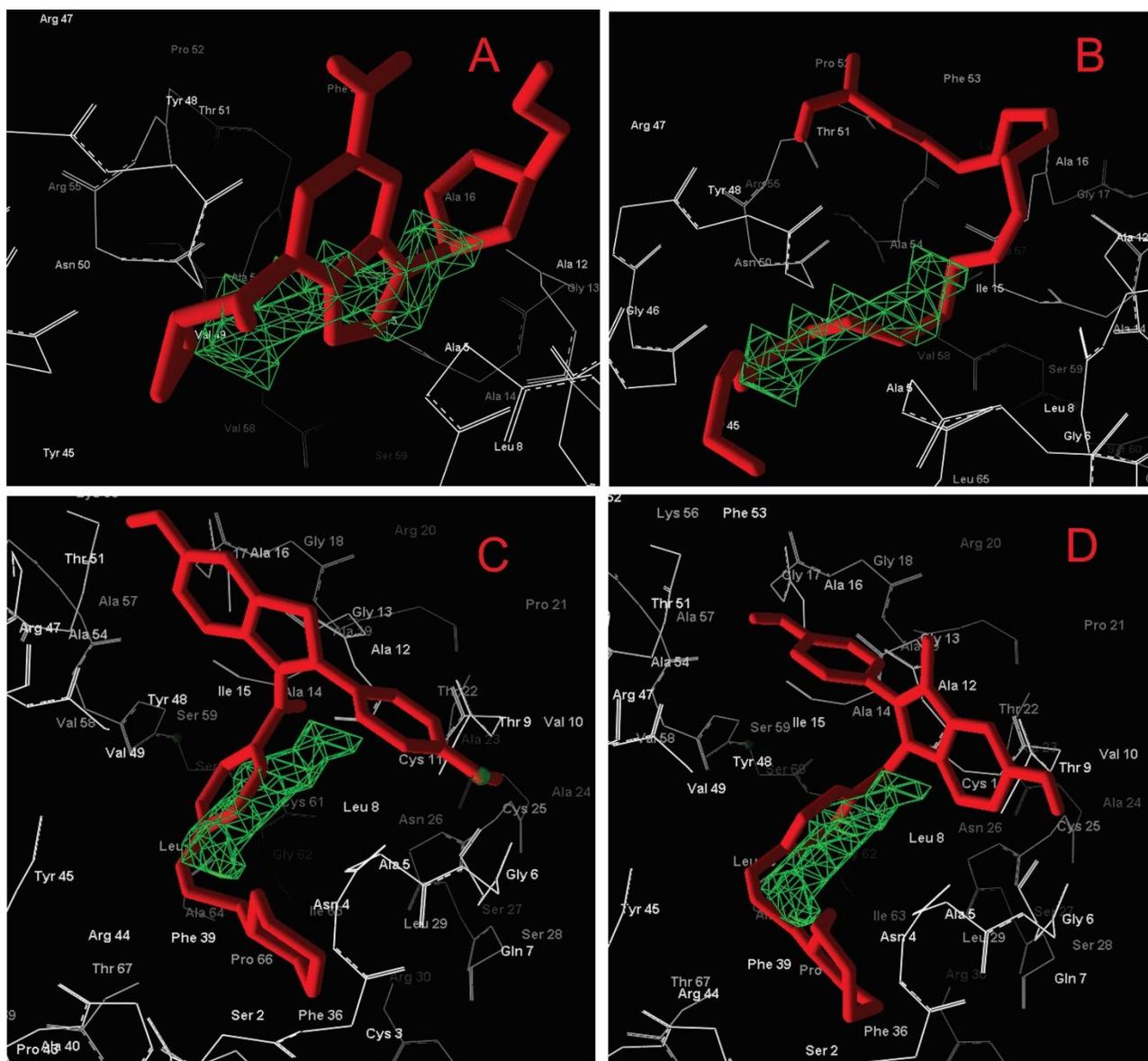
Figure 8: The position of the best score docking ligands including (A) Abacavir, (B) DHA, (C) Raloxifene, and (D) Basedoxifene in the area of the mutant nsLTP2 (C26N/N53F) related cavity. Active site residues are shown based on their name and chain ID and the ligand is represented in stick lines with fix red color. The cavity is located in green.

Docking results revealed that the molecular characteristics of each ligand affect the binding affinity to the nsLTP2 cavity site. The MolDock scores of nsLTP2 binding to various phospholipids have been dropped sharply following ligand molecular mass increasing. The lightest phospholipid, Butyricacid (Molecular mass= 88.105 Da) binds to nsLTP2 with a score of -52.1573 kcal/mol. While the binding

score of Caproicacid (Molecular mass= 116.158 Da) has decreased to -66.5219 kcal/mol. Both mentioned phospholipids bereft of double-bond in their structures. This linear relation, molecular mass increasing leads to decrease MolDock score, is established until there is no double-bond in ligand structure. Double-bond could improve the affinity binding of the ligand to nsLTP2. Comparison between GadoleicAcid (Molecular mass= 310.514 Da) with one double-bond and ArachidicAcid (Molecular mass= 312.53 Da) without double-bond in its structure showed the role of hydrogen double-bond in binding affinity enhancing. Although the ArachidicAcid is heavier than the GadoleicAcid, the MolDock score of GadoleicAcid binding to nsLTP2 was -121.997 kcal/mol and this score was about -114.574 kcal/mol for ArachidicAcid. Also, this relation is followed by the mutant protein. Furthermore, trans-double-bond might make the ligand-binding stronger than cis double-bond. Trans and Cis-palmitoleic acid (Molecular mass= 254.41 Da) binding score to wild type nsLTP2 protein were -109.911 and -104.573 kcal/mol respectively. Contrary to phospholipids, no relation between structural features of anti-viral or anti-cancer steroid drugs and affinity binding to nsLTP2 was discovered.

4. Discussion

Non-specific lipid transfer proteins (nsLTPs) are soluble proteins that have been extracted from various plants, as an illustration, wheat, rice, and barely. They are mainly involved in the formation of a hydrophobic layer to protect the plant's surface. Also, in plants, nsLTPs have a biological role in flowering. These proteins are mainly divided into nsLTP1 and nsLTP2 subfamilies. Although the molecular weight of nsLTP1 (~9 Da) is a little heavier than nsLTP2 (~7 Da), the nsLTP2 structure in more stable [22]. Also, various studies demonstrated that nsLTP2 has the ability of binding and transporting drug molecules comprises of anti-viral drugs and several steroid/phospholipids compounds.

In this study, the prediction of antigenic regions of nsLTP2 protein of Iranian rice was carried out based on seven methods. One of the features of antigenic sites in protein is the existence of hydrophobic amino acids in this section (Parker et al., 1986). Also, it has been established that locating the Val, Leu, Cyc residues on the protein surface, most likely form the antigenic sites (Kolaskar and Tongaonkar. 1990). Hydrophobicity analysis of the Iranian rice nsLTP2 protein, based on both Sliding

Window and TMHMM methods, revealed that the number of predicted transmembrane and hydrophobic regions is only one site. This region is located at the beginning of the protein sequence. The hydrophobicity hotspot is mainly concentrated at residues number 3 to 26 that are located in nsLTP2 signal peptide. Also, bioinformatics evaluation showed C26N and N53F mutations led to the reduction of antigenic properties of this Iranian rice protein. However, both two mutations not changed cavity formation. Due to the importance of this protein in drug delivery systems, it is possible to develop proteins with more appropriate structure and function through creating some changes for the reduction of antigenicity property that will have better performance drug delivery systems. Furthermore, creating mutations to the development of protein with fewer antigenicity features shouldn't affect the affinity of protein to its ligands. To assess the differences between the wild type and mutant protein's structural features, the scores related to binding to ligands including previously proved phospholipids and antiviral drugs were analyzed using Molegro Virtual Docker software. Moreover, as the nsLTP2 cable to bind steroids, in this study, we aimed to analyze the affinity of this protein to steroid-based anti-cancer agents. Overall, the 3D structure of 69 ligands belongs to the mentioned groups were downloaded.

Previously, Tousheh et al. analyzed the binding affinity of nsLTP2 (Indica group, Accession No. A2XBN5.2, PDB:1L6H) to anti-viral drugs, Nucleotides and Their derivatives using Molegro Virtual Docker. Their bioinformatics analysis revealed a high affinity of this protein to purine-analogous drugs including acyclovir (MolDock Score = -139.63) and vidarabine (MolDock Score = -155.51) as anti-viral drugs. In comparison with Iranian derived nsLTP2, it seems that the affinity score is decreased (MolDock Score =-95.1007, -98.3478 respectively) but these scores have been increased dramatically following our candidate mutations however they were not ranked in top scores. In the current study, the binding affinity of Iranian rice-derived nsLTP2 against anti-viral ligands was calculated for the first time. Abacavir known by Ziagen trade name (PubChem ID= 441300) had the lowest score among other ligands (MolDock Score =-130.537) which also Tousheh et al. study reported a low binding score (-152.5 kcal/mol) for this type of HIV medication. Although the Abacavir binding score was increased after exerting concurrent mutations of C26N/N53F or each mutation alone, it has been revealed that its binding affinity ranked top as the lowest score among all wide or mutant types.

Furthermore, the nsLTP2 has been demonstrated that has a high affinity to various phospholipids. Based on the other work on Indica-derived nsLTP2 affinity to phospholipids that also has been done by Tousheh et al., it is clear that the phospholipids-related number of carbon atoms, location of double

bonds and hydroxyl group in the acyl chain could affect the binding affinity. This feature was also going true for our analysis and The MolDock scores of nsLTP2 binding to various phospholipids have been dropped sharply following ligand molecular mass increasing. The Docosahexaenoic acid (DHA) showed the top score for wild type, C26N, and concurrent mutations of C26N/N53F in nsLTP2 (Iranian group). In this study, we analyzed the binding score of this protein to steroid-based anticancer agents for the first time and our results interestingly unravel the new potential approach to use nsLTP2 as an anticancer carrier and could serve as more efficient targeted therapy. The Basedoxifene (PubChem ID= 154257), a third-generation selective estrogen receptor modulator (SERM) is used as cancer medication especially in breast cancer and effects through inhibiting tumor progression genes including STAT3 [40]. Iranian nsLTP2 mutant types showed the highest affinity to DHA.

5. Conclusion

Briefly, our results suggest that Iranian –derived nsLTP2 with concurrent mutations of C26N/N53F could serve as the potential drug carrier for humans due to its lowest antigenicity than its wild type. Furthermore, Abacavir ($\text{MolDock Score} = $ -119.348), DHA ($\text{MolDock Score} = $ -152.601), and Basedoxifene ($\text{MolDock Score} = $ -156.776) could be considered as the best antiviral, phospholipid, and anticancer ligands for it.

Acknowledgment

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