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Article

Computational analysis of fusion protein of anti-HER2 scFv and alpha luffin: A new immunotoxin protein for HER2 positive cancers

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The present study deals with the computational design and analysis of a novel fusion protein based on a single chain variable fragment that binds to the extracellular domain of human epidermal growth factor receptor 2 (HER2) in breast cancer cells. Alpha luffin, a small ribosome inactivating protein (RIP), was attached to the anti-HER2 antibody fragment. I-TASSER modeling provided the full-length structure of the fusion protein. Molecular docking evaluated the molecular interactions of the complementarity-determining regions of designed fusion protein to HER2. Energy minimization and molecular dynamics simulations were conducted to refine the complexes. RMSD plot revealed reasonable stability of the fusion protein during the simulation. The free binding energy profile of complexes affirmed a favorable binding affinity of proteins in complex with HER2 using molecular mechanics Poisson-Boltzmann surface area (G-MMPBSA) algorithm. In general, this approach looks promising in the development of new fusion proteins in terms of immunotoxins with appropriate cytotoxicity.

Keywords: Cancer. Targeted therapy. Fusion protein. Molecular dynamics. Immunotoxin.

INTRODUCTION

The most frequent malignancy in women worldwide is breast cancer (BC). Due to the estimation of American cancer society, the incidence of BC cases will be increased by approximately 23.1% in the United States in 2020 (Siegel, Miler, Jemal, 2020). Similar to the other cancers, surgery, radiation therapy, and chemotherapy are the common treatments for breast cancer (Dhankhar *et al*., 2010). Conventional chemotherapeutics' success as first-line drugs for cancer treatment is limited due to the long-term side effects (Palumbo *et al*., 2013). In usual, advances in the understanding of the molecular mechanism of breast cancer have strengthened hope for developing new strategies of treatments, including immune-based and targeted therapies (Tong *et al*., 2018; Vanneman, Dranoff, 2012).

Targeted therapy has revolutionized cancer treatment and focused on specific molecules involved in cancer cells' growth and spread (Padma, 2015). Likewise, several small molecules, monoclonal antibodies, and their derivatives are highlighted as new modalities of targeted therapies (Vanneman, Dranoff, 2012). Although, numerous successful monoclonal antibodies have been approved by the FDA, there are several concerns about their serious adverse effects and inability in complete eradication of cancer cells (Adler, Dimitrov, 2012; Guan *et al*., 2015). To improve the efficiency of antibodies, the idea of "immunotoxins" (ITs) was proposed based on conjugation of a toxin component to an antibody

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(Allahyari *et al*., 2017; Dosio, Brusa, Cattel, 2011). Immunotoxins, as protein-based therapeutics, are constituted of a potent bioactive agent and a targeting moiety. Their specific binding to the targeted molecules on the surface of cancer cells would achieve a significant and selective potency of these components (Dosio, Brusa, Cattel, 2011). Small antibody molecules such as single-chain variable fragments (scFv) have shown satisfactory results in the construction of immunotoxins (Di Paolo *et al*., 2003; Shan, Liu, Wang, 2013).

The anticancer agent in ITs can be a bacterial, plant, and insect toxin that mainly belongs to the group of ribosome-inactivating proteins (RIPs). RIPs are translation inhibitory proteins with a wide range of antiproliferative, antitumor, immunomodulatory, and antiviral activities (FitzGerald *et al*., 2004; Li, Hall, 2010). Alpha luffin, a small RIP (30kDa) from the seeds of *Luffa cylindrica*, was reported to have inhibitory effects on the proliferation of cancer cell lines. *In vitro* biological assays of immunotoxins constructed with α-luffin showed anti-tumor activities against human melanoma cells. Besides, the immunotoxin based on human IL-2 and Luffin P1 which inhibited T cell proliferation, provided promising results in the treatment of autoimmune diseases (Schrot, Weng, Melzig, 2015; Liu *et al*., 2012).

In the case of breast cancer, tumor-associated antigens (TAAs) such as human epidermal growth factor receptor 2 (HER2) are the ideal antigens for being targeted. ERBB2 or HER2/neu is a transmembrane receptor tyrosine kinase, which is overexpressed in approximately 20% of breast cancers (Mitri, Constantine, O'Regan, 2012).

We explored the computational analysis of the designed fusion protein containing an anti-HER2 single-chain variable fragment (scFv) antibody molecule linked to the alpha-luffin protein.

MATERIAL AND METHODS

Design of the construct

The amino acid sequence of the variable regions of light and heavy chains of anti-HER2 Trastuzumab monoclonal antibody (DrugBank accession number: DB00072) were linked together through a 15 amino acid linker ($(Gly_4Ser)_3$). To design the fusion protein, the encoding sequence of the mature alpha luffin protein (UniProtKB-Q00465) was linked to the C-terminal end of anti-HER2 scFv molecule using $(Gly_4Ser)_3$ as a linker. Cathepsin B specific cleavage site (GFLG) was inserted immediately after the linker sequence at upstream of the α-luffin sequence to separate the functional and efficient toxin from the scFv moiety within the target cells. A conserved KDEL recognition motif, as endoplasmic reticulum (ER) retention signal, was also inserted at C-terminal end of α-luffin protein before the stop codon. The final coding construct was oriented as V_L-$(Gly_4Ser)_3$-V_H-$(Gly_4Ser)_3$-(GFLG)-alpha luffin-KDEL.

Structural Analysis of Proteins

The primary structure of the designed anti-HER2 scFv, as well as the full-length fusion protein, were evaluated using the Expasy ProtParam server (Gasteiger *et al*., 2005). The instability index was calculated to predict the protein's *in vivo* stability from its amino acid composition, while index values below 40 represent protein stability (Guruprasad, Reddy, Pandit, 1990). The grand average of hydropathicity (GRAVY) was also calculated, which displays the hydropathic characteristics of the protein and is calculated by the sum of hydrophathy values of all amino acid residues divided by the number of residues in the sequence (Kyte, Doolittle, 1982).

The secondary structure of proteins was predicted using the CFSSP (Chou & Fasman Secondary Structure Prediction) server by algorithms analyzing the secondary structures such as alpha helix, beta-sheet, and turns based on proteins of known structures (Kumar, 2013).

Homology modeling

Homology modeling was implemented as a comparative modeling method to generate 3D protein models. In I-TASSER (Iterative Threading ASSEmbly Refinement), the construction of full-length models starts from multiple threading alignments to find structural templates from the PDB database. Then, iterative fragment assembly simulations were followed

by structure refinement to generate 3D protein structures (Roy, Kucukural, Zhang, 2010). PDB formats of anti-HER2 scFv and the fusion protein were obtained by uploading the amino acid sequences in FASTA format. Subsequently, structural validations were performed using PROCHECK software, ERRAT, Verify3D, and ProSA servers to select the best models (Colovos, Yeates, 1993; Eisenberg, Lüthy, Bowie, 1997; Laskowski *et al*., 1993; Wiederstein, Sippl, 2007).

Evaluation of Binding Affinity of Proteins and HER2

The crystallographic structure of the human epidermal growth factor receptor 2 (HER2) (PDB entry: 3BE1) was used for molecular docking studies (Bostrom *et al*., 2009). To predict the structure of complexes, extracellular domain of HER2 with anti-HER2 scFv alone or the fusion protein, molecular docking was conducted using HADDOCK (High Ambiguity Driven protein-protein DOCKing) webserver (van Zundert *et al*., 2016) in which a user-friendly interface was provided to study the protein-protein interactions by defining sets of active and passive residues representing the protein interface. All docking settings were left at default values, where the standard protocol generates 1000 models from the sampling of 10000 docking solutions, and the maximum number of conformers per ligand was set to 100. Top HADDOCK structures were visualized using PyMol and analyzed by LigPlot+ software (DeLano, 2002; Laskowski, Swindells, 2011).

Molecular Dynamics Simulation

Running the molecular dynamics (MD) simulation of anti-HER2 scFv/HER2 as well as fusion protein/HER2 complexes was implemented using GROMACS version 5.1.4 (Abraham *et al*., 2015) with the original GROMOS96 force field 43A1. Complexes were solvated with SPC/E (extended simple point charge) water model in a cubic box of 10 Å marginal radius. The steepest descent algorithm was used to minimize the energy by removing any steric clashes or unusual geometries. Thereafter, the equilibration simulation was carried out for 500 ps under canonica NVT ensemble, followed by isothermal-isobaric NPT ensemble. In the last step, the production simulation was performed at 300K for 40 ns with 2 fs time step. The output trajectories were analyzed in terms of structural stability by calculating the root mean square deviation (RMSD), and the graphs were visualized using Grace software (GRACE: http://plasma-gate.weizmann.ac.il/Grace/).

The free energy of binding (binding affinity), surface accessible surface area (SASA), Van der Waals (VdW) energy, and electrostatic energy was calculated using GROMACS Molecular Mechanics Poisson-Boltzmann Surface Area (G-MMPBSA) method implemented in the GROMACS 5.1.4. The coordinates of 2000 snapshots structures were taken from 40000 frames of the MD runs. Their free energy of binding ($\Delta G_{binding}$) was computed using the equations as described by Kumari *et al*., 2014.

RESULTS

Protein structural analysis

The mature alpha-luffin protein used in this study is composed of 258 amino acid residues. The designed scFv molecule totally comprises 250 amino acid residues with a 15 amino acid linker sequence between the two variable regions. One methionine and one glycine amino acids were also incorporated at the N-terminal region of the scFv molecule to avoid frameshift changes and were followed by a poly-Histidine tag ((His6). Considering the linker sequences and motifs designed in the encoding construct, the full-length deduced fusion protein would be 531 amino acid residues in length (Figure 1).

Regarding physicochemical properties, the instability index of anti-HER2 scFv and the fusion protein was 42.34 and 34.25, respectively. It showed that the anti-HER2 scFv molecule can be classified as an unstable protein, while the fusion protein was considered a stable entity. The negative values of GRAVY for the proteins indicated a hydrophilicity pattern representing higher interaction with water for both tested molecules (Table I). CFSSP server predicted the possible secondary structure contents within tested molecules as has been shown in Table I and Figure 2.

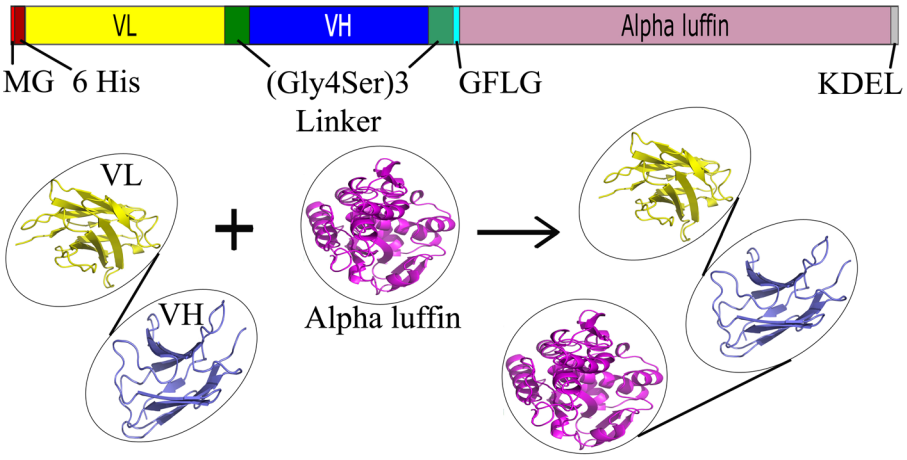

FIGURE 1 - Schematic view of the designed construct.

TABLE I - Prediction of primary and secondary structures by ProtParam and CFSSP

Proteins	Length (a.a)	Instability Index[1]	GRAVY[2]	Helix (%)	Extended sheet (%)	Turn (%)
anti-HER2 scFv	250	42.34	-0.403	24.6	62.6	12.8
Fusion protein	531	34.25	-0.223	39.5	48.2	12.3

[1] Instability index value <40 indicated that the protein is stable.
[2] Grand average of hydropathicity

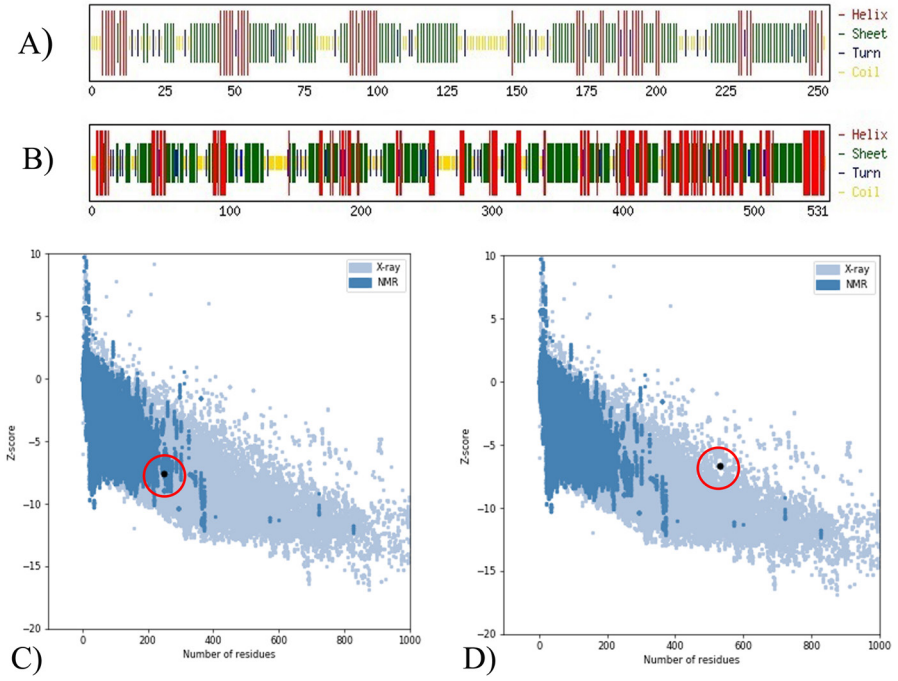

FIGURE 2 - Prediction of the secondary structures and ProSA plots. A and B) Secondary structural analysis of anti-HER2 scFv and the fusion protein, respectively; C and D) ProSA plots of anti-HER2 scFv and the fusion protein, respectively. The black spot in each plot represented the overall quality of the models compared to the experimentally determined protein (X-ray and NMR).

Domain assembling and modeling of the fusion protein

Five top 3D structure models predicted by I-TASSER were ranked by C-scores ranging from -5 to 2, and the harboring the highest C-score was selected as the best model for each tested protein (C-score of 0.57 and -2.8 as the highest values for anti-HER2 scFv and the fusion protein, respectively). Firstly, there was a convenient consistency between predicted structures and their secondary structure predictions. The observed higher frequency of helix in the structure of the fusion protein could be explained by the predominant helix secondary structure of the alpha luffin moiety. The predicted 3D structures of anti-HER2 scFv and the fusion protein are shown in Figure 3.

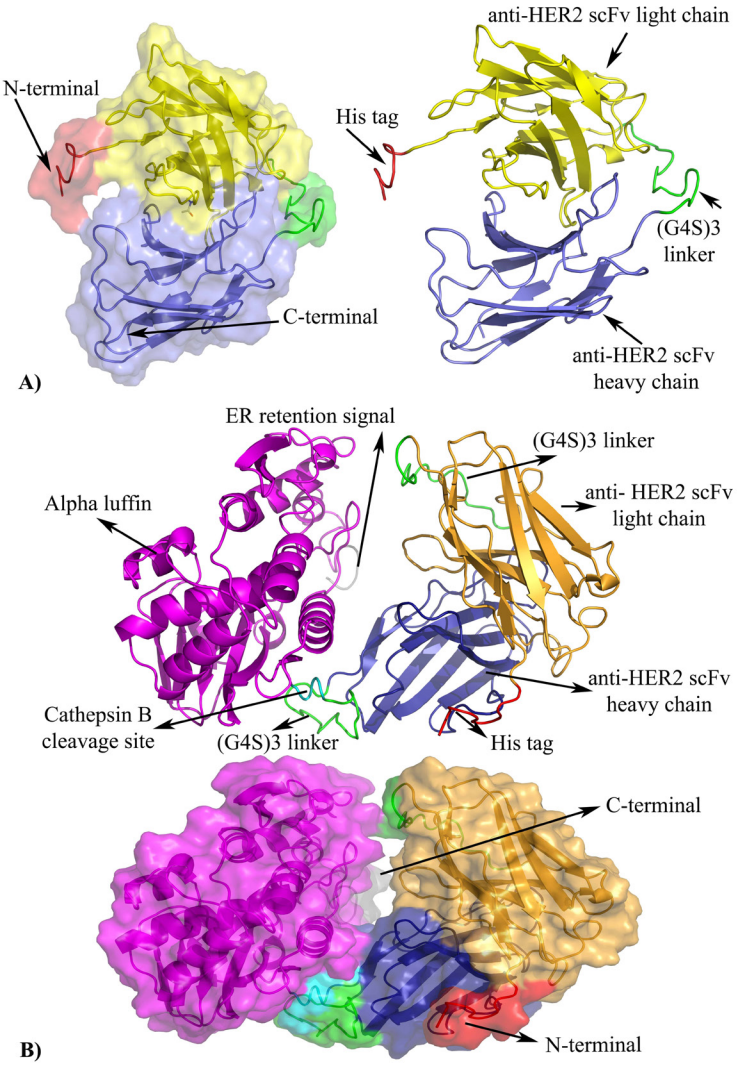

FIGURE 3 - Design of the 3D structures using PyMol: A) anti-HER2 scFv; B) Fusion protein.

Secondly, different stereochemical features of predicted structures were assessed using PROCHECK, Verify3D, ERRAT and ProSA servers. Obtained data have been summarized in Table II. TM-score represents a metric assessment of protein structural similarity with the value in (0, 1] where 1 assumed as the perfect match

between two structures (Xu, Zhang, 2010). ProSA Z-score for both proteins determined the overall model quality as if the position of black spot in the plot (Figures 2C and 2D) revealed the conformity of models with identified protein structures. Verify3D evaluated the compatibility of atomic structures (3D) with the corresponding primary amino acid sequence ((1D) where the highest score (100%) shows the best compatibility.

TABLE II - Structural validation of predicted models

Proteins	TM-Score	ProSA[1] Z-score	Verify 3D (%) [2]	ERRAT (%) [2]	Ramachandran plot quality (%)			
					Most favored	Additionally allowed	Generously allowed	Disallowed
scFv	0.8	-7.62	100.00	79.03	67.5	22.7	6.4	3.4
Fusion protein	0.8	-6.7	89.27	66.27	60.5	31.5	4.7	3.3

[1] ProSA Z-score determines the overall model quality.

[2] 100 is the best and 0 is the worst.

Furthermore, the statistics of the atomic non-bonded interactions within the predicted structures were determined by ERRAT compared to the experimentally high-resolution structures. Higher scores show a higher quality of the structures. Finally, Ramachandran plots of 3D structures developed by PROCHECK indicated that an acceptable percentage of residues were detected in the most favored and allowed regions of each model (Table II).

Binding affinity of scFv molecule and the fusion protein to HER2 and the pattern of their interaction

Molecular docking studies of anti-HER2 scFv or the designed fusion protein to the extracellular domain of HER2, as their cognate receptor, remarkably revealed similar low energy of docking, -1907.6 and -1915.6 ($KJMol^{-1}$), respectively. The visualization of complexes illustrated that proteins interacted with HER2 through the amino acids located in complementarity-determining regions (CDRs) of the scFv molecule. Complexes were analyzed to determine the interaction pattern of the two tested proteins toward HER2 using LigPlot+ software. Intriguingly, a comparable number of hydrogen bonds was involved in both complexes of anti-HER2 scFv/HER2 and the fusion protein/HER2 (12 and 11 H-bonds, respectively) (Figures 4 and 5). Whereas numerous different CDRs residues of each protein were taking part in the interaction to the extracellular domain of HER2, there was no considerable difference among their binding affinities to the target. The results are summed up in Table III and presented in Figures S1 and S2.

TABLE III - Docking and G_MMPBSA calculations

Parameters	scFv/HER2	Fusion protein/ HER2
Lowest Energy of docking ($KJMol^{-1}$)	-1907.6	-1915.6
Number of H-bonds	12	11
Number of hydrophobic bonds	70	90
Binding energy ($KJMol^{-1}$)	-54.810	-47.279
SASA energy ($KJMol^{-1}$)	-46.427	-45.686
Van der Waals energy ($KJMol^{-1}$)	-122.084	-127.183
Electrostatic energy ($KJMol^{-1}$)	80.662	131.235

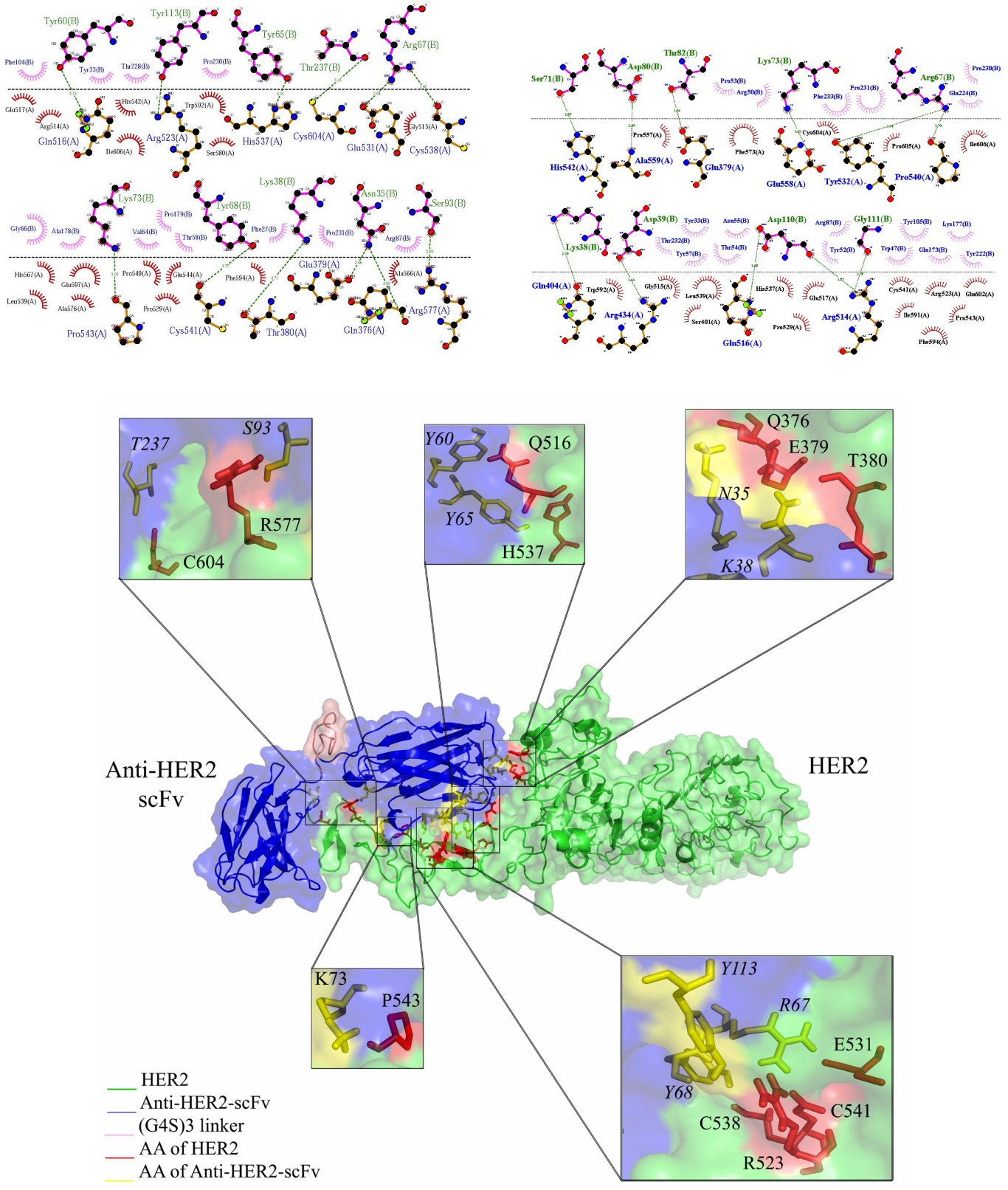

FIGURE 4 - Interaction mode of scFv molecule with the extracellular domain of HER2 protein. Surface-cartoon representation of complex resulted from molecular docking by HADDOCK. Light and heavy chains of scFv, $(G_4S)_3$ linker and HER2 are depicted in blue, pink and green, respectively. Close-up view of the stick representation of amino acids involved in the interaction of proteins has been colored in yellow and red for scFv and HER2, respectively.

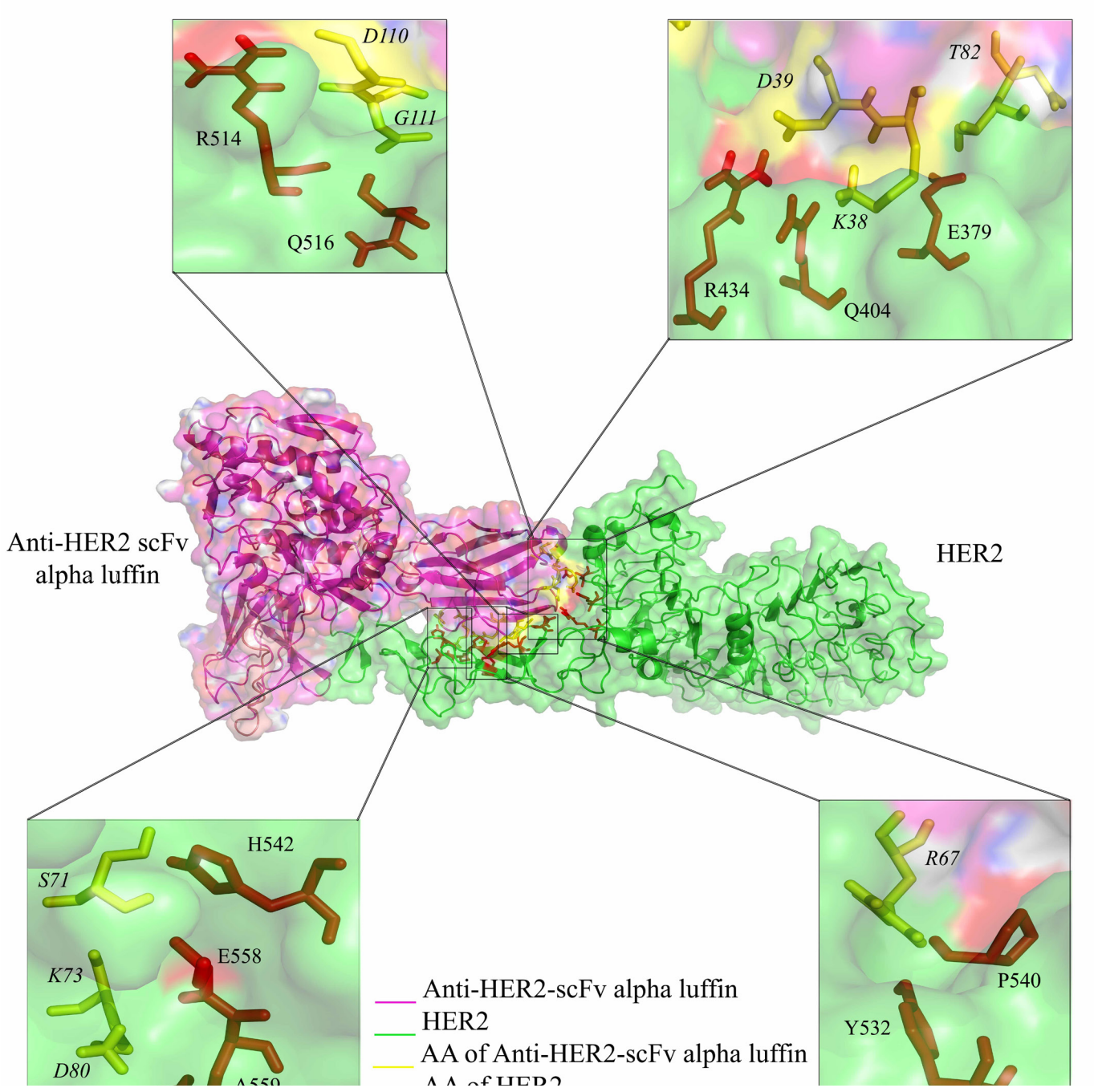

FIGURE 5 - Binding model of the fusion protein with the extracellular domain of HER2 protein. Surface-cartoon representation of complex is shown in in magenta and green for the fusion protein and HER2, respectively. Close-up view of the stick representation of amino acid residues involved in the interaction of the fusion protein and HER2 are illustrated in yellow and red, respectively.

Molecular Dynamic Simulation

The docking complexes of scFv/HER2 and fusion protein/HER2 were subjected to 40 ns MD simulations using GROMACS. MD output data were analyzed in terms of root mean square deviation (RMSD) of structures during simulations. Evaluating backbone RMSD is reliable for tracking the conformational changes of complexes during simulations. RMSD plots are depicted in Figure 6. Although, a great deal of fluctuation observed in the initial steps of simulations, it seems that system has reached the equilibrium after 10 ns and the value of RMSD for both complexes was in the same range. scFv/HER2 complex showed limited conformational changes between 0.2 and 0.4 nm, while the observed fluctuations in the fusion protein/HER2 complex during simulations could be explained by the larger size of this protein complex.

Furthermore, MD simulation trajectories were used to extract the average stable complexes to calculate free energy of binding using G_MMPBSA tool. The free energy of binding and related components (vdw, electrostatic and solvation energies) resulted from the MMPBSA estimation of scFv/HER2 as well as fusion protein/HER2 was summarized in Table III. The binding energy of scFv/HER2 and fusion protein/HER2 complexes was -54.810 and -47.279 KJMol^{-1}, respectively. SASA and van der Waals energy values of complexes were in the same range. There was a significant difference in the electrostatic energy of complexes which could be due to the different numbers of hydrophobic interactions. Although 90 hydrophobic bonds were contributed in the interaction of fusion protein to the extracellular domain of HER2 but scFv interacted to the HER2 ECD by 70 hydrophobic bonds.

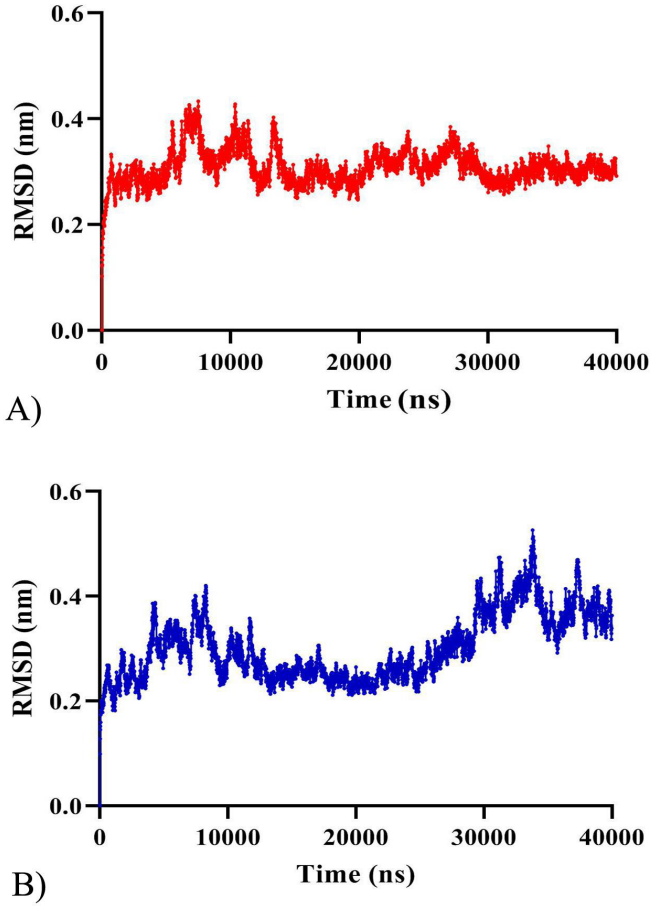

FIGURE 6 - RMSD plots versus time; A) RMSD of the backbone atoms of scFv/HER2; B) RMSD of the backbone atoms of the fusion protein/HER2.

DISCUSSION

Currently approved monoclonal antibodies (mAbs) for cancer therapy can identify and specifically bind to the target cells. However, these therapeutic proteins are hardly potent to eradicate the cancer cells. One approach to enhance their efficiency is their conjugation to a toxic protein to guarantee sufficient cell cytotoxicity (Akbari *et al*., 2017). Antibody-toxin conjugates are usually recombinant proteins that can selectively target the tumor antigens through the antibody moiety and release their cytotoxic compartment into the target cells (Nagayama *et al*., 2017). The present study was performed to analyze the efficiency of designed fusion protein containing anti-HER2 scFv antibody fragment linked to the toxic alpha-luffin protein through computational methods against the extracellular domain of HER2 protein.

Computational methods have revolutionized protein engineering. These techniques allow a broad range of biocomputing capabilities from the prediction of protein three-dimensional models, study the mutagenesis effects and characterization of protein-protein interactions to the molecular dynamics simulations (Baran *et al*., 2017). The predicted secondary and 3D structures of designed fusion protein were validated in terms of conformational state and overall quality. The structure of anti-HER2 scFv antibody molecule was made of predominantly extended-sheets with connecting turns similar to the common folding of one antibody variable fragments comprising six loop segments in complementarity-determining regions (CDRs) between the barrels and β-propellers (Baran *et al*., 2017). This structural folding in the fusion protein could be promising for its high affinity toward the specific receptor and appropriate function of the entire molecule.

To inspect the structural consequences of the interaction of designed proteins towards the extracellular domain of HER2 antigen, these proteins were subjected to molecular docking as the target agent. The interaction mode of tested proteins towards HER2 revealed the favorable capability of the fusion protein harboring the toxin moiety.

Molecular Dynamics (MD) simulations provide insight into the dynamic behavior of proteins. The top models of molecular docking were subjected to 40 ns MD simulations. The flexibility and stability of protein complexes were evaluated by computing the root mean square deviation (RMSD). Indeed, the average distance of a set of atoms in the input proteins was measured with respect to the same atoms during the simulation. Furthermore, MD trajectories and structural snapshots were analyzed to explore the binding affinity of the complexes. G_MMPBSA tool calculates the difference of binding free energy between the two conformational states of complex (free and bound states) (Chen *et al*., 2016; Kumari *et al*., 2014). The absolute value of the free energy of binding is estimated based on the interaction features of the proteins. Regarding atoms and amino acids in the binding site, different ranges of hydrophobic, hydrogen, electrostatics, and pi-pi interactions are being involved in the interactions. The overall binding free energy is computed considering the either the favorable or unfavorable impact of these interactions. The increasing number of hydrophobic bonds could explain the more positive value of the fusion protein/HER2 complex's calculated electrostatic energy compared to the scFv/HER2 complex (90 *vs.* 70 interactions).However, the number of hydrogen bonds and the values of SASA and vdW energies of the complexes were in the same range which could be interpreted as the lack of unfavorable impact of toxin moiety on deduced interactions of scFv fragment towards the extracellular domain of HER2.

Nowadays, by considering detailed information about the target agent and the lead molecule, a targeting drug molecule can be designed based on computational techniques. The overall data evidenced that the computer-aided studies could provide a bright insight into the structural and functional details of newly designed therapeutic agents by which pharmaceutical companies hope to grab numerous benefits in a shorter time (Baldi, 2010).

Anti-HER2 scFv molecule derived from Trastuzumab was linked to a small RIP type I protein, alpha luffin, to construct a recombinant fusion protein. The conformational state of the antibody-toxin conjugate was evaluated computationally. This approach looks promising to develop a new immunotoxin molecule with appropriate cytotoxicity in a targeting manner. Experimental evaluation of present fusion protein was initiated by cloning and expressing the

fusion protein in *E. coli*. Endonuclease activity on DNA and RNA samples has confirmed the correct assembly of the fusion protein (Barkhordari *et al*., 2019). However, further biological studies should be performed to investigate the targeted biological activity of the recombinant protein.

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CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

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