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Original Article

Computational Design and Evaluation of scFv-DSH2a as a PSMA-Targeted Immunotoxin for Prostate Cancer Therapy

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HIGHLIGHTS

- A novel immunotoxin (scFv-DSH2a) was designed to target PSMA in prostate cancer.
- Bioinformatics tools confirmed its stability, solubility, and non-allergenicity.
- Molecular docking showed strong binding affinity to PSMA.
- Molecular dynamics simulations confirmed complex stability over 100 ns.
- scFv-DSH2a is a promising candidate for targeted prostate cancer immunotherapy.

ABSTRACT

Prostate cancer is one of the most prevalent malignancies in men and a leading cause of cancer-related mortality. The prostate-specific membrane antigen (PSMA), which is highly overexpressed in prostate tumors and internalized upon ligand binding, represents a promising target for immunotoxin-based therapies. In this study, a novel chimeric immunotoxin, scFv-DSH2a, using bioinformatics tools, was designed to selectively target PSMA-expressing cells. The construct was generated by fusing the single-chain variable fragment (scFv) of the J591 antibody to the cytotoxic DELTA-stichotoxin-Hmg2a (DSH2a) via a flexible linker. Structural modeling and refinement were performed using GalaxyWEB and Galaxy Refine, and the model was validated through PROCHECK and ProSA-web. Physicochemical properties were assessed using ProtParam, solubility predicted via SOLpro, and allergenicity evaluated using AllerTOP v.2.0. Protein–protein docking with PSMA was conducted using ClusPro, and molecular dynamics simulations were performed with GROMACS to assess complex stability. The designed immunotoxin exhibited favorable stability (instability index: 32.72), solubility (probability: 0.624), and non-allergenic properties. Docking results revealed strong binding affinity to PSMA, and molecular dynamics simulations confirmed structural stability over 100 nanoseconds, with low root mean square deviation (RMSD) and root mean square fluctuation (RMSF) values and consistent radius of gyration. These findings suggest that scFv-DSH2a is a promising candidate for targeted immunotherapy in prostate cancer, offering specificity, stability, and potential safety for future therapeutic development.

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Introduction

Prostate cancer often remains asymptomatic until advanced stages and may present with distant metastases (Abbasi and Basiri, 2018). Prostate cancer treatment differs across the various stages of the disease and is influenced by the tumor grade. While an optimal treatment has yet to be discovered, there is an urgent need for new therapies, particularly for patients with high-grade malignancies who face limited treatment options (Buzlea et al., 2023). Emerging cancer treatment approaches focus on developing drugs that precisely target cancer cells, while minimizing harm to healthy tissues. Monoclonal antibodies (mAbs) represent a novel class of cancer therapies, designed to identify and bind specifically to target cells (Singh et al., 2018).

Although mAbs demonstrate significant effectiveness, they seldom succeed in fully eliminating cancer cells. Several strategies have been employed to improve their potency, with one of the most promising being the conjugation of antibodies with chemicals or toxins (Pastan et al., 2006). Toxins are more potent than mAbs in targeting cancer cells, as just one toxin molecule can destroy a cancer cell. As a result, immunotoxins hold significant potential as candidates for prostate cancer treatment in this therapeutic approach (Buzlea et al., 2023).

In cancer treatment, effective targeting of cancer cells is a key aspect. Toxins are able to effectively eliminate host cells, while antibodies can recognize and target specific cells. Consequently, if a conjugate of an antibody and toxin is produced, it can specifically target the cancer cell and eliminate it with high efficiency and precision. However, the large size of antibodies may prevent these molecules from crossing the host membrane, which could limit their use in cancer treatment. Using a single-chain variable fragment (scFv) molecule instead of a full antibody can alleviate this limitation to a large extent. Using smaller antibody fragments as the targeting component reduces immunogenicity and increases the penetration of the immunotoxin into solid tumors (FitzGerald et al., 2004; Buzlea et al., 2023).

The scFv is a type of monovalent antibody where the variable light (VL) and heavy (VH) chains are linked by a flexible peptide, enabling it to bind specifically to its target antigen (Pastan et al., 2006). Due to its small molecular weight, scFv possesses good elasticity, low antigenicity, ease of production, high cell permeability, tissue compatibility, and strong binding affinity, making it a suitable candidate for use in various immunotoxins. As a result, scFv has been developed for the targeted delivery of toxins to cancer cells (Ahmad et al., 2012).

The ScFv of J591 is a single-chain variable fragment originating from the monoclonal antibody J591, which targets the prostate extracellular membrane antigen known as PSMA (Prostate-Specific Membrane Antigen). The PSMA is a membrane glycoprotein whose expression increases tenfold in prostate cancer. After interacting with an antibody, this antigen is internalized by the cell, making it a promising mechanism for delivering cytotoxic agents directly into prostate tumor cells. PSMA expression is not dependent on androgen levels and tends to rise as the disease advances. Elevated levels of PSMA are found in the neovasculature associated with tumors, while normal vasculature does not exhibit such high expression (Parker et al., 2013).

The toxin DELTA-stichotoxin-Hmg2a (DSH2a), also referred to as HMgIII, is derived from the sea anemone *Heteractis magnifica*, a member of the Stichodactylidae family found in the Indian and Pacific Oceans. Sea anemones are known to be a valuable source of cytotoxic proteins. The DSH2a (HMgIII) toxin has a molecular weight of roughly 19 kDa and forms pores with approximately 1 nanometer in size in the cells' membrane (Wang et al., 2000; Kem and Dunn, 1988).

Since no research has yet explored the scFv-DSH2a construct in the context of prostate cancer, this study aims to develop this chimeric immunotoxin using bioinformatics tools. The construct will feature a PSMA-specific scFv as the targeting element, combined with the toxic DSH2a component. A 3D model of this chimeric protein has been created, and its structure, stability, solubility, and binding affinity to PSMA have been predicted and assessed through bioinformatics methods.

Materials and Methods

Construct design of a novel immunotoxin

For conducting the study and designing the immunotoxin under investigation, we obtained the sequence of DSH2a from UniProt (UniProt ID: Q9U6X1) and the sequence of scFv J591 (including the VL and VH sequences) (Ho et al., 2017). We used the sequence GSTSGGGSGGGSGGGGSS as a flexible linker. By integrating the antibody (VL-VH J591) and the toxin, using this flexible linker, we constructed a pharmaceutically desired recombinant structure (Wu et al., 2001; Samavarchi Tehrani et al., 2021).

Secondary structure evaluation

At this stage, the secondary structure of the scFv was examined both on its own and in combination with DSH2a using the SOPMA server (https://npsa.lyon.inserm.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html).

This tool assesses the proportions of alpha helices and extended strands. Extended strands are a structural pattern identified when specific amino acids participate in more than one secondary structure or when main-chain amino acids form hydrogen bonds with amino acids in a secondary structure. Moreover, it identified beta turns, which usually occur when the protein chain needs to change direction to connect two other elements of secondary structure, with beta turns being the most common, and random coils. It uses homology-based methods to predict the percentage of secondary structures (Singh et al., 2016). After connecting DSH2a to the scFv via a short linker, the proportions of each secondary structure were evaluated in the final construct.

Analysis of physicochemical properties and solubility

The ProtParam server (https://web.expasy.org/protparam/) was utilized to analyze several physicochemical properties of the immunotoxin, such as the instability index, aliphatic index, grand average of hydropathicity (GRAVY), extinction coefficient, net charge, and molecular weight (Gasteiger et al., 2005; SamavarchiTehrani et al., 2021). Additionally, SOLpro (http://scratch.proteomics.ics.uci.edu/) was employed to evaluate the solubility of the construct, as predicting protein solubility is crucial for the successful expression of engineered proteins (Magnan et al., 2009).

Building a 3d model, refinement and validation of the modeled structure

GalaxyWEB (https://galaxy.seoklab.org/) is an online platform designed for predicting and refining protein structures. To predict the structure, the chimeric protein sequence must be submitted in FASTA format. A distinguishing feature of this server compared to other protein structure servers is that it identifies unreliable regions where template information is unavailable or inconsistent and refines them using ab initio methods (Ko et al., 2012).

After inputting the chimeric protein sequence, the GalaxyWEB server generated five top-ranked refined models, from which we chose Model 1 for further analysis. To enhance the predicted models, we utilized the Galaxy Refine web server, which employs a refinement technique that includes side-chain rebuilding and repacking. The model with the highest percentage of residues in the favored region of the Ramachandran plot and the best GDT_HA score was selected for additional evaluation. The GDT_HA score ranges from 0 to 1, with higher scores indicating greater similarity between the test protein and the reference structure. Subsequently, we validated the 3D model using the ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php) and PROCHECK servers (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/), which assess the stereochemical properties of the protein structure, enabling us to identify the most accurate and geometrically optimal structures with minimal errors (SamavarchiTehrani et al., 2021). The quality of the chosen model was evaluated using PROCHECK both prior to and following the refinement process.

Allergenicity prediction

The potential allergenicity of the chimeric structure was assessed using the AllerTOP online bioinformatics server (https://www.ddg-pharmfac.net/AllerTOP/). This server utilizes a non-alignment-based method to predict allergens by analyzing the amino acid composition of proteins (Dimitrov et al., 2013). The sensitivity, specificity, and accuracy values for AllerTOP are 0.876, 0.780, and 0.828, respectively (Dimitrov et al., 2014).

Protein–protein docking analysis

The docking of the chimeric protein with PSMA was carried out using the ClusPro server (https://cluspro.bu.edu/login.php), where the designed immunotoxin served as the ligand and PSMA acted as the receptor. This server is capable of predicting protein-protein interactions and the binding affinity of the ligand model (Kozakov et al., 2017). For the analysis, the PSMA and the refined 3D structure of the chimeric protein scFv + DSH2a (used as the ligand) were submitted in PDB format, and the server's default settings were employed.

Molecular dynamics (MD) simulation study

To evaluate the stability of the strongest interaction from the immunotoxin–PSMA design, a complex simulation was performed for 100 nanoseconds using GROMACS version 2021.2 (Ghesmati Z). Water molecules (TIP3) were included, with a distance of 1.3 Å maintained between the edges of the biomolecule and the boundaries of the water box. The potassium chloride (KCl) salt ions were added at a concentration of 0.15 M, which approximates physiological ion levels, to electrically neutralize the system. Energy minimization was performed using the steepest descent method until the maximum force fell below 1000 kJ/mol/nm.

Equilibration was carried out in two stages. The first stage involved an NVT ensemble, maintaining a constant number of particles, volume, and temperature. The duration of this phase depends on the system's composition, but in an NVT setup, the temperature must stabilize at the desired value; if stabilization is not achieved, additional time is needed. Typically, a duration of 50-100 ps is adequate. Once the temperature was stabilized, the second stage of equilibration focused on

stabilizing pressure (and, consequently, density) before data collection. This pressure equilibration was conducted under an NPT ensemble, where the number of particles, pressure, and temperature remain constant, with pressure managed using the Parrinello-Rahman barostat.

Finally, to briefly review the results and confirm that our simulation was successfully executed, we used the gmx check command-line tool. The stability of the designed immunotoxin–PSMA complex was assessed by evaluating parameters, such as 1) root mean square fluctuation (RMSF) that shows the flexibility of each amino acid, indicating which amino acid has the most impact on the movement of the protein molecule, 2) root mean square deviation (RMSD) that shows the average deviation between corresponding atoms of the two proteins. The smaller RMSD indicates more similarity between the two structures. An acceptable RMSD range is 2 Å when the model overlaps with the template. However, RMSD alone cannot be the sole criterion for evaluating the constructed model. The RMSD graph shows changes in protein structure over the simulation time, which can be used to assess the stability and equilibrium of the structure throughout the simulation and radius of gyration (R_g).

Results and Discussion

Construct design of a novel immunotoxin

To construct the desired immunotoxin sequence, we used amino acids 35 to 211 of the DSH2a toxin, as this region is identified as the core chain in UniProt and is present in the mature protein. Other parts, such as the pro-peptide, are removed during post-translational modifications and are not present in the mature protein.

EVQLVQSGAEVKKPGASVKISCKTSGYTFTEYTIH
WVKQASGKGLEWIGNINPNNGGTTYNQKFEDRA
TLTVDKSTSTAYMELSSLRSEDTAVYYCAAGWNF
DYWGQGTTVTVSSGSTSGGGSGGGSGGGGSSDIV
MTQSPSSLSASVGDRVTITCKASQDVGTAVDWYQ
QKPGKAPKLLIYWASTRHTGVPDRFTGSGSGTDF
TLTISSLQPEDFADYFCQQYNSYPLTFGGGTKLEIK
GSTSGGGSGGGSGGGGSSSAALAGTIIEGASLGFQI
LDKVLGELGKVSRKIAVGVDNESGGSWTALNAY
FRSGTTDVILPEFVPNQKALLYSGRKDTGPVATGA
VAAFAYYMSNGHTLGVMFSVPFDYNFYSNWWD
VKVYSGKRRADQGMYEDMYYGNPYRGDNGWH
QKNLGYGLRMKGIMTSAGEAILQIRISR

Each color corresponds to a specific part of the immunotoxin sequence, matching the colors used in Fig. 1a, which provides a schematic representation of the different sections of the designed immunotoxin.

Secondary structure evaluation

The prediction of the secondary structure of a novel-designed immunotoxin, resulting from the SOPMA web server, revealed that the combination of the toxin with the antibody (VL-VH from monoclonal antibody J591) and a flexible linker did not affect their secondary structure.

Figure 1. The schematic representation of the different sections of the designed immunotoxin (A); secondary structure prediction of the novel designed immunotoxin using SOPMA web server (B).

The secondary structure of the chimeric protein comprised 15.86% alpha helix, 28.97% extended strand, 42.99% random coil, and 12.18% beta turn. Fig. 1b illustrates the secondary structure prediction results obtained from the SOPMA web server. The default output width for the secondary structure figure is 70, which means that each line contains 70 amino acids (Singh et al., 2016).

Analysis of physicochemical properties and solubility

Using the ProtParam server, the physicochemical properties of the recombinant constructs were evaluated. The analysis indicated a molecular weight of 46,116.14 Da, with a theoretical pI of 7.79 and a net charge of +1. The aliphatic index was found to be 63.45, while the grand average of hydropathicity (GRAVY) measured -0.341. The instability index (II) was calculated at 32.72, suggesting that the scFv + DSH2a construct is a stable protein (indices below 40 are considered stable). Based on the results obtained from the SOLpro web server, the chimeric protein has a solubility probability of 0.624062.

Building a 3D model, refinement and validation of the modeled structure

After predicting the 3D structure with the Galaxy WEB server, the model was refined by minimizing energy through Galaxy Refine. The model exhibiting the highest percentage of residues in the favored region of the Ramachandran plot was chosen for further analysis. The quality of the selected model was evaluated both before and after the refinement process using PROCHECK **(Fig. 2)**. Table 1 displays the comparison of residues in the Ramachandran plot for the unrefined and refined models, based on PROCHECK data. According to the ProSA-web analysis, the refined model achieved a Z-score of -8.25, which was considered acceptable (Fig. 3A).

Table 1. Amino acid positions of the model in the Ramachandran plot before and after refinement

Servers	Ramachandran plot positions	Before refinement	After refinement
PROCHECK	Residues in the most favored regions	319 (90.1%)	336 (94.9%)
	Residues in additional allowed regions	31 (8.8%)	15 (4.2%)
	Residues in generously allowed regions	1 (0.3%)	0 (0.0%)
	Residues in disallowed regions	3 (0.8%)	3 (0.8%)

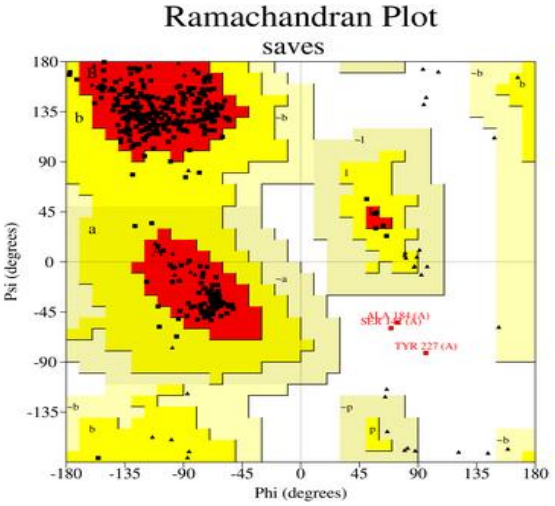


Figure 2. Ramachandran plot on the PROCHECK website for the refined model. Each red dot on the plot represents an amino acid with non-allowed phi and psi angles (Ghorbanpour et al., 2019).

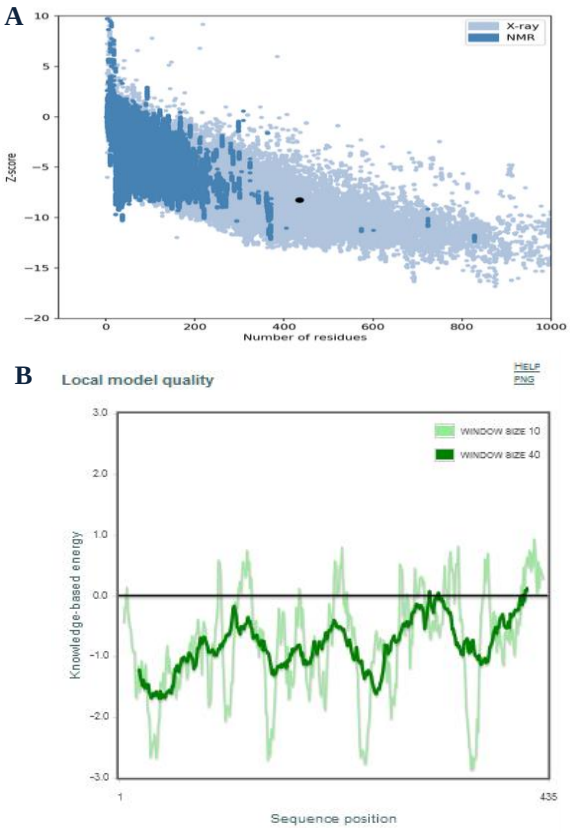


Figure 3. Z-score plot on the ProSA website for the refined model (Z-score: -8.25) (A). The Z-score indicates the overall quality of the model in relation to experimentally determined structures, which are derived from X-ray crystallography (light blue) or NMR spectroscopy (dark blue), based on the lengths of protein chains in the PDB database (Ghorbanpour et al., 2019). Local model quality (B); this plot, which was taken from the ProSA-web, evaluates the protein locally and in sections. It examines energy levels in light green with a resolution of 10 amino acids and in dark green with a resolution of 40 amino acids.

The Z-score provides insight into the overall quality of the model by measuring how the total energy of the structure deviates from the energy distribution derived from random combinations. This Z-score, when compared to those of experimentally determined proteins, helps assess the model's quality. A Z-score that falls outside the typical range for native proteins suggests the presence of structural inaccuracies (Wiederstein and Sippl, 2007). The local model quality was shown in the Fig. 3B plot, which was taken from the ProSA-web, and evaluated the protein locally and in sections. It examined energy levels in light green with a resolution of 10 amino acids and in dark green with a resolution of 40 amino acids.

Allergenicity prediction

Based on the results from the AllerTOP v. 2.0 web server, which assessed the allergenic potential of the chimeric protein, the designed immunotoxin could be considered non-allergenic.

Protein–protein docking analysis

After editing, energy minimization, and preparing the ligand and receptor, 3D models of the interaction between the PSMA receptor (PDB ID: 2xeg) and the immunotoxin were created using the Cluspro web server. The model with the lowest energy score (-858 kcal/mol) was selected as the best prediction for ligand-receptor binding and used for molecular dynamics simulations, as shown in Fig. 4A.

The ClusPro 2.0 server utilizes PIPER, a Fast Fourier Transform (FFT) correlation method for protein docking based on pairwise interaction potentials. The dissociation constant (K_d) and binding affinity of the PSMA-toxin complex were predicted using the PRODIGY web server. The 3D structure complex was visualized with PyMOL v2.3.4 software. Finally, interactions between PSMA and the designed immunotoxin were analyzed and visualized using LIGPLOT (ligand-protein interaction diagrams, https://www.ebi.ac.uk/thornton-srv/software/LigPlus/) (Fig. 4B**)** and PDBsum (Fig. 5A and 5B**)**

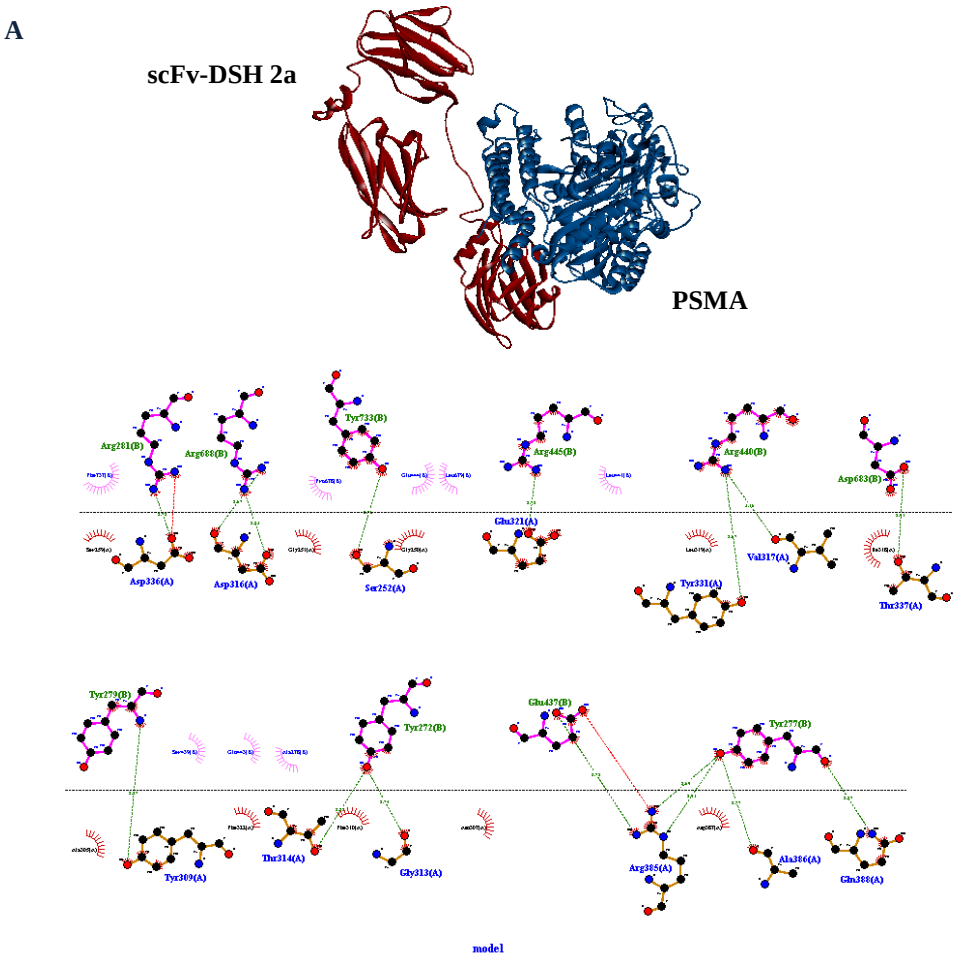
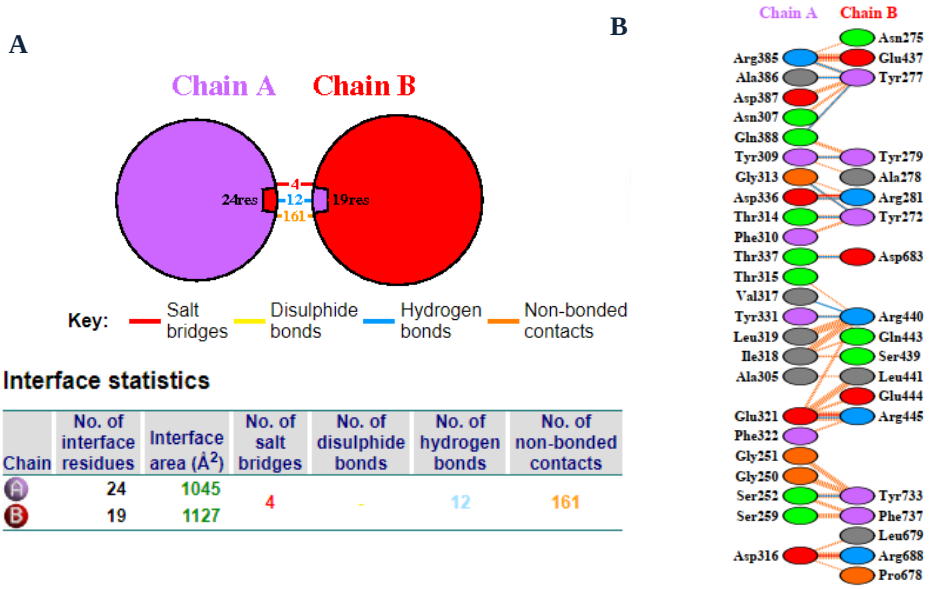


Figure 4. Molecular protein-protein docking (scFv-DSH2a and PSMA) using the Cluspro web server (A); Analyzing docking results with LIGPLOT (B).



Chain	No. of interface residues	Interface area (Å^2)	No. of salt bridges	No. of disulphide bonds	No. of hydrogen bonds	No. of non-bonded contacts
A	24	1045	4	-	12	161
B	19	1127				

Figure 5. Analyzing docking results with PDBsum (A); Evaluation of docking results with PDBsum (B).

Molecular dynamics (MD) simulation study

System temperature analysis

By analyzing the temperature of the system during the simulation, it was observed that the temperature remained relatively stable with minimal fluctuations, indicating that the system has reached a steady temperature suitable for the simulation. According to the graph, the average system temperature is 300 Kelvin, with the heat capacity varying between approximately 308 to 312 Kelvin. The temperature shows convergence, confirming that the simulation was conducted correctly (Fig. 6A).

Volume analysis

During a simulation, monitoring properties like the system's volume is crucial to ensure the accuracy of the analysis. Analyzing the changes in system volume throughout the simulation indicated that this parameter converges smoothly to its equilibrium value. For the protein-ligand complex system, the average volume was approximately 3323, fluctuating between 3292 and 3327 (Fig. 6B).

Potential energy analysis

In the potential energy graph, a decrease in energy was observed during the initial moments of the simulation. Afterward, the energy reached a plateau with minor fluctuations, averaging around -4,222,857.582 kJ/mol. The total energy remained quite stable throughout the simulation, indicating that the simulation was conducted correctly (Fig. 6C).

Total energy analysis

By examining the total energy graph, we observed how the energy values for the protein-ligand complex converge around -3,378,321.813 kJ/mol. If these values had not converged, it would indicate that the simulation had not yet reached energy equilibrium, requiring further continuation before analysis. However, our results demonstrated that the system had achieved equilibrium in this regard (Fig. 6D).

Root mean square deviation (RMSD) analysis

Once the accuracy of the simulation was confirmed, we analyzed the root mean square deviation (RMSD) to evaluate the structural stability of the protein-ligand complex. This analysis involved comparing the deviations of backbone atoms of the ligand protein (A) and receptor (B) from the initial conformation to their final positions. The RMSD indicated the stability of the molecule's conformation during the simulation, with smaller deviations reflecting greater stability.

Using the gmx rms tool in GROMACS, we calculated RMSD values once for the protein backbone and once for the ligand. As shown in the corresponding figure, the RMSD for the ligand protein was lower compared to the receptor protein. RMSD values for both structures gradually decreased over time, eventually reaching relative equilibrium with minimal fluctuations from approximately 60 nanoseconds until the end of the simulation. The average RMSD value was 0.66 for the receptor protein and 0.2 for the ligand protein (Fig. 7A).

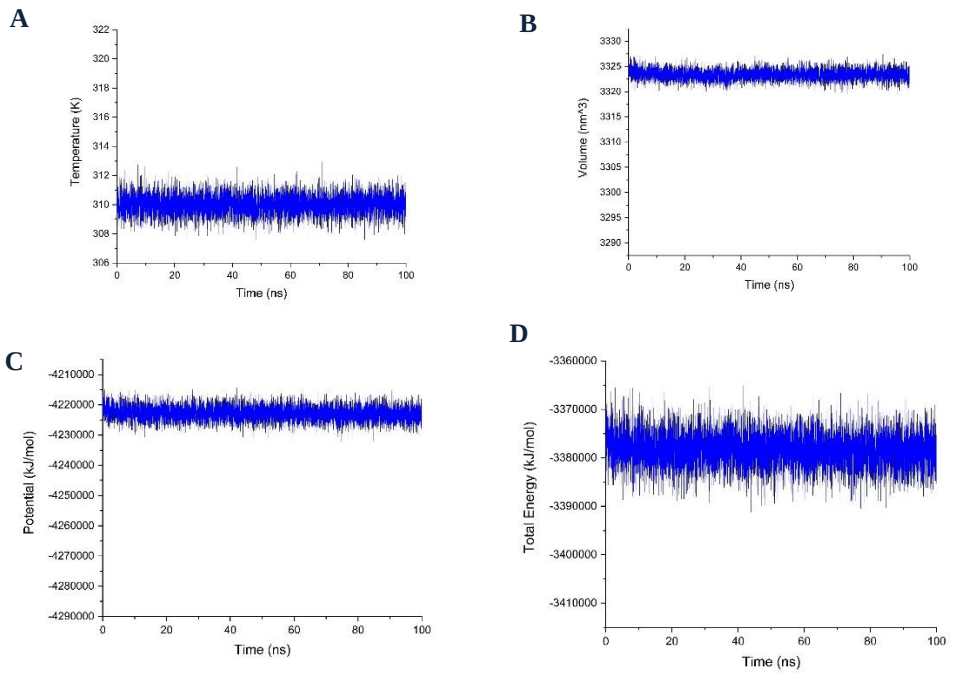


Figure 6. System temperature analysis (A); volume analysis (B); potential energy analysis (C); total energy analysis (D) of immunotoxin and PSMA interaction.

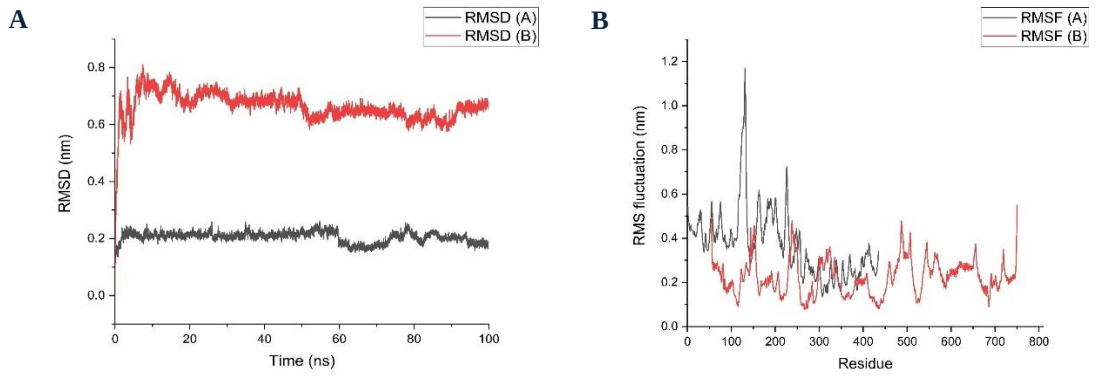


Figure 7. Root mean square deviation (RMSD) analysis (A); root mean square fluctuation (RMSF) analysis (B) using the GROMACS software package.

Root mean square fluctuation (RMSF) analysis

RMSF quantifies the fluctuations in the position of each atom or residue from its average location over the course of the simulation. This analysis offered insights into the flexibility of various regions within the protein, with higher RMSF values signifying greater flexibility during the simulation.

RMSF was calculated using the gmx rmsf tool in GROMACS. The resulting graph showed changes in RMSF values plotted against residue numbers, highlighting which amino acids contributed most to the movement of the molecule and were thus more flexible compared to other regions.

For both the receptor and ligand proteins, RMSF values were calculated for all residues. Peaks in the RMSF plots indicate regions with the greatest fluctuations throughout the simulation. Typically, higher fluctuations are observed in the N-terminal, C-terminal regions, as well as in loops and coils of the protein, due to their inherent structural flexibility. The average RMSF during the simulation was 0.222 nm for the receptor protein and 0.37 nm for the ligand protein (Fig. 7B).

Radius of gyration analysis

Alongside the previous analyses, the radius of gyration (R_g) of the simulated complex was evaluated to gain insight into its compactness and flexibility. The radius of gyration provides an indication of the molecular shape at each time point during the simulation. It is compared with the hydrodynamic radius and calculated using gmx gyrate. A higher radius of gyration is associated with increased system instability, while a lower R_g indicates a more compact complex. A protein that folds stably is likely to maintain a relatively constant R_g value. Conversely, if a protein unfolds, its R_g will increase over time.

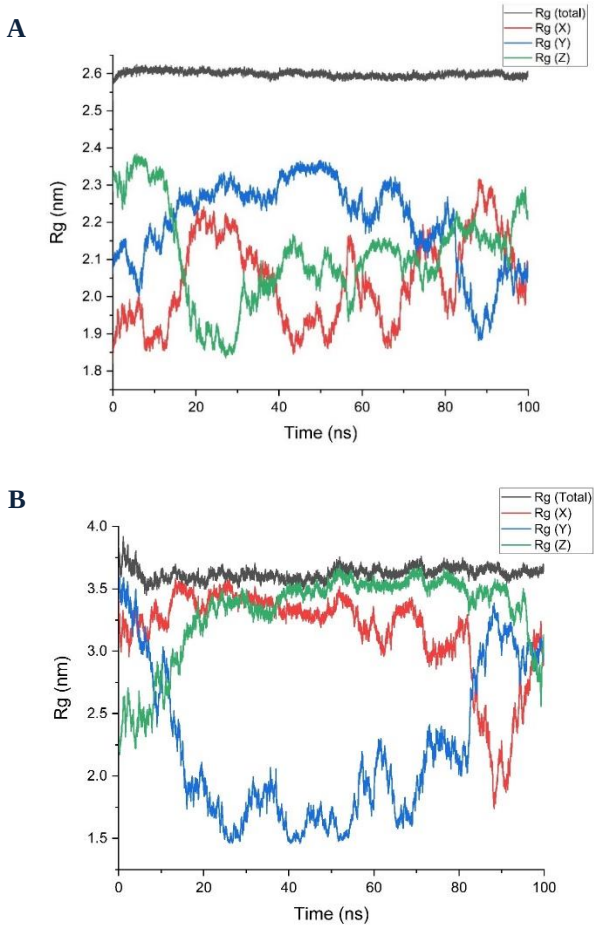


Figure 8. Radius of gyration of the receptor protein (A); radius of gyration of the ligand (B) using the GROMACS software package.

The radius of gyration for both the protein and immunotoxin ligand was evaluated during the simulation. The average R_g value for the receptor protein throughout the simulation was 2.60 nm, indicating a nearly stable conformation (Fig. 8A). For the ligand protein, the average R_g value was 3.62 nm, which showed a slight decreasing trend (Fig. 8B).

Solvent accessible surface area (SASA)

SASA, or solvent accessible surface area, refers to the portion of a biomolecule's surface that is exposed to the solvent, typically measured in square angstroms (Å²). The SASA value of a protein is associated with its folding and stability; proteins with higher SASA values are generally more flexible and less stable, while those with lower SASA values are typically more compact and stable. Consequently, SASA offers important insights into the structural changes happening at the molecular level during simulations.

In this study, SASA was analyzed for our protein complex using the gmx sasa module of the GROMACS software package. Higher SASA values indicated greater exposure of amino acid residues to surrounding water molecules and potential structural unfolding or rearrangement. Conversely, lower SASA values suggested a more compact and less solvent-exposed conformation, indicating a native-like structure. The SASA plot showed two distinct chains, A and B, represented in black and red, respectively, over a time period measured in nanoseconds (ns), Chain B exhibited higher SASA values, ranging from approximately 280 to 300 nm², while chain A had lower SASA values, fluctuating around 220 to 240 nm². Notably, SASA decreased over time, suggesting structural compaction.

Fluctuations in SASA throughout the simulation were expected due to the dynamic nature of the system. However, the overall trend indicated stable behavior following the initial equilibration period, with chain B maintaining its folded state for most of the simulation. It is also important to note that some SASA fluctuations along the trajectory suggested minor structural changes in the protein (Fig. 9).

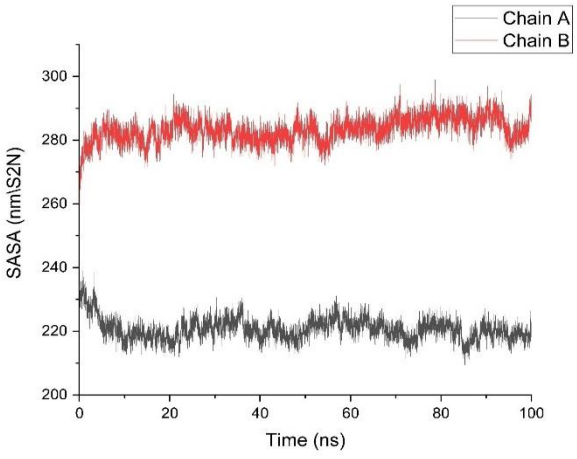


Figure 9. Solvent accessible surface area (SASA) of immunotoxin protein complex using the gmx SASA module of the GROMACS software package.

Hydrogen bond analysis

Protein-protein complexes are stabilized by a variety of interactions, including hydrogen bonds, hydrophobic interactions, and electrostatic interactions, among others. Hydrogen bonds, in particular, are highly specific interactions that are vital for stabilizing the complex. These bonds not only enhance the structural stability of the protein but also significantly influence the binding of the ligand to the receptor, thereby affecting drug specificity.

Consequently, the number of hydrogen bonds formed between the receptor protein and the ligand was determined using the gmx hbond tool within the GROMACS software. Hydrogen bonds were analyzed to understand the dynamic equilibrium of the complex. The hydrogen bonds were formed within a distance of 0.35 nanometers. It was observed that a network of hydrogen bonds existed between the components, varying between 3 and 32 bonds. These bonds were formed and broken due to fluctuations in angles and distances throughout the simulation.

Discussion

Prostate cancer continues to be one of the primary causes of cancer-related deaths among men globally (Strand SH). Depending on the stage of the disease, patients are typically treated with hormone therapy, surgery, and chemotherapy. Despite advancements in diagnostic and therapeutic methods, mortality rates continue to rise (Dohm et al., 2021). Most prostate cancer deaths are due to advanced disease that can metastasize to surrounding tissues via blood or lymphatic spread (Muneer et al., 2019). PSMA is a membrane protein found on prostate epithelial cells that is significantly upregulated in prostate cancer. It was one of the first prostate cancer biomarkers to be successfully cloned (Adamaki and Zoumpourlis, 2021). Since 1993, PSMA has been the subject of extensive clinical research as an imaging and therapeutic agent. Given that ligands are fundamentally internalized by PSMA, PSMA is considered a promising target for advanced prostate cancer treatment (O'Dwyer et al., 2021). PSMA is a non-secreted cell surface membrane protein, making it a suitable target for monoclonal antibody therapy.

Single-chain antibody fragments are chimeric proteins constructed from the VH and VL chains of an antibody linked together by a flexible amino acid linker. Due to their small size, they are valuable as diagnostic and therapeutic alternatives. Compared to mAbs or Fab fragments, scFvs improve tissue penetration, pharmacokinetics, and immunogenicity. The J591 scFv specifically recognizes the extracellular domain of PSMA and has been studied for localized treatment and staging of prostate cancer (Psimadas et al., 2018).

Immunotoxins are a type of therapeutic molecule that combines an antibody with toxin molecules to significantly enhance the ability of antibodies to kill cancer cells. Immunotoxin molecules have great potential in cancer treatment, with several having already been approved for clinical trials (Psimadas et al., 2018).

Similar strategies have been successfully applied in breast cancer research. For instance, Samavarchi Tehrani et al. (2021) designed and evaluated scFv-RTX-A as a novel immunotoxin targeting HER2-positive breast cancer cells. Their results demonstrated that scFv+RTX-A was a non-allergenic and stable chimeric protein with high binding affinity to HER2 receptors, suggesting its potential as a therapeutic candidate for HER2-positive breast cancer patients (SamavarchiTehrani et al., 2021).

Likewise, Movahedpour et al. (2022) employed an *in silico* strategy to design an efficient immunotoxin against HER2-positive breast cancer resistant to Herceptin. Using various bioinformatics tools, they evaluated the structure and composition of the immunotoxin and analyzed its binding affinity through docking and molecular dynamics simulations. Their findings confirmed the structural integrity and stability of the designed immunotoxin, as well as its effective binding to HER2 receptors (Movahedpour et al., 2022).

Recombinant protein technology now allows for the production of proteins with desirable properties (Vasina et al., 2022). In this study, a chimeric protein was designed and tested using various bioinformatics tools. To begin designing an immunotoxin against prostate cancer, the protein sequences of J591 scFv and DSH2a were obtained. The components of the construct were then linked using a flexible linker (GSTSGGGSGGGSGGGGSS). The secondary structure of the designed immunotoxin was evaluated using the SOPMA server. The analysis compared the secondary structure of the sequence before and after the addition of the toxin via the linker, revealing no significant changes that would impede receptor binding and recognition. The alpha helix content was measured at 15.86%, which is below the 20% threshold and unlikely to cause expression issues. The ProtParam online server assessed the physicochemical properties of the immunotoxin, including molecular weight (MW), aliphatic index, and instability index. The instability index (II) was calculated to be 32.72, indicating that the immunotoxin was a stable protein, as values below 40 are deemed stable. The grand average of hydropathicity (GRAVY) was found to be -0.341, with this negative value signifying hydrophilicity. This suggests that the protein

does not excessively favor hydrophobic interactions or exhibit extreme hydrophilicity, both of which could influence target binding. According to SOLpro results, the chimeric protein was predicted to have a solubility probability of 0.624062.

AllerTOP was used to evaluate the potential allergenicity of the designed immunotoxin. The results indicated that the designed structure could be considered a non-allergenic protein that does not elicit inflammatory responses in the human body.

The GalaxyWEB server was utilized to predict the 3D structures of the designed immunotoxin. The most promising model was refined using the GalaxyRefine server. Among the refined models, the one with the greatest proportion of allowed Ramachandran regions and a higher GDT-HA score—indicating structural similarity to the reference structure, with scores ranging from 0 to 1 (higher values reflecting better similarity)—was chosen. The 3D models were further analyzed and validated using ProSA-web and PROCHECK, confirming the appropriateness of the protein structure.

Among the available 3D structures for PSMA in PDB, an appropriate structure was selected and downloaded. After editing and energy minimization, it was used for docking and analyzing interactions with the designed immunotoxin. Cluspro was employed to examine the interaction between the designed immunotoxin, acting as a ligand, and PSMA, serving as the receptor. From the molecular docking analysis, the model with the lowest energy score was identified as the best prediction for ligand-receptor binding and was selected for molecular dynamics (MD) simulations.

Generally, MD simulations offer an efficient and rapid method for assessing protein-protein interactions and the stability of complexes. In this study, GROMACS 2018 was utilized to evaluate the stability of the designed toxin-PSMA complex. The interactions within this complex were simulated for 100 ns. We assessed the stability of the docked complex by analyzing RMSD, R_g, RMSF, and SASA values. The average RMSD value for the receptor protein was 0.66, and for the ligand protein, it was 0.2. RMSD values for both structures gradually decreased over time and eventually reached a relative equilibrium with minimal fluctuations, indicating complex stability. The average R_g value for the receptor protein during the simulation was 2.60 nm, which was relatively stable. The average R_g value for the ligand protein was 3.62 nm, showing a slight decrease, indicating stable complex formation during the simulation.

RMSF values revealed which amino acids contributed most to molecular movement during the MD simulation and highlighted regions with greater flexibility compared to others. Peaks in the RMSF plot indicated areas with the highest fluctuations throughout the simulation. Most fluctuations were observed in the N-terminal, C-terminal regions, and in loops and helices of the protein, as these areas exhibited high structural flexibility. The average RMSF for the receptor protein was 0.222 nm, and for the ligand, it was 0.37 nm.

The SASA (solvent-accessible surface area) plot showed minimal fluctuations, indicating minor structural changes and relative stability. Hydrogen bonds were analyzed to demonstrate the dynamic equilibrium of the complex. Hydrogen bonds formed within 0.35 nm, revealing a network of hydrogen bonds that contributed to the protein-protein complex stability. Post-MD analysis showed an increase in interactions, suggesting the formation of a stable complex.

Overall, the data and minimal fluctuations indicated that the complex was likely stable throughout the simulation, with the amino acids involved in the receptor-ligand interaction remaining stable over 100 ns.

Taken together, these findings align with previous studies in breast cancer immunotoxin design, reinforcing the potential of scFv-based chimeric proteins as targeted therapies. The stability, solubility, and non-allergenicity of scFv-DSH2a, along with its strong binding to PSMA, support its candidacy as a novel therapeutic agent for prostate cancer.

Conclusion

In this study, we aimed to design an effective immunotoxin for prostate cancer using in silico strategies. We employed various bioinformatics tools and servers to evaluate the composition and structure of the immunotoxin. Additionally, we analyzed its binding affinity to the PSMA receptor through docking studies and molecular dynamics (MD) simulations.

The results indicated that our designed immunotoxin has a correct and stable structure. Binding assessments confirmed that it functions as a non-immunogenic and stable chimeric protein with a high affinity for PSMA receptors on prostate cancer cells, effectively binding to the PSMA receptor. Therefore, the scFv-DSH2a construct could represent a promising candidate for immunotoxin therapies targeting prostate cancer. However, in vitro and in vivo conditions are different, and immunological assays should be performed to confirm the effectiveness of the designed structure.

Further experimental studies are necessary to validate these findings, which will be the focus of our future research. In summary, bioinformatics tools and software are essential for understanding molecular mechanisms and may pave the way for new therapeutic approaches in various cancers, particularly prostate cancer.

Ethical Statement

The authors confirm that this study complies with institutional regulations and ethical guidelines and did not involve human or animal subjects. Furthermore, the manuscript does not violate copyright, third-party rights, or other applicable laws.

Competing Interests

The authors declare that there are no competing financial and non-financial interests, direct or indirect, related to this manuscript.

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Data Availability

Additional data will be available from the corresponding author upon reasonable request.

Authors Contribution

S.M. was responsible for designing the project and leading the research, writing, and editing of the article. N.M. coordinated the research activities and reviewed the final manuscript. All authors read and approved the final manuscript.

Declaration of AI Tools Usage

We gratefully acknowledge the use of artificial intelligence (AI) tools solely for the purpose of linguistic revision and to improve the clarity of the manuscript. The content, analysis, and scientific integrity of the manuscript are the sole responsibility of the authors.

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