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Designing and Simulating the Structure of an Effective Immunotoxin in Breast Cancer

Mona Maleknezhad -Yazdi[1*], Ali Akbar Haddad-Mashadrizeh[2]

[1]Biology Department, University Jihad Unit, University of Science and Arts of Yazd, Iran.
[2]Department of Molecular and Cell Biology, Institute of Biotechnology, and Department of Ferdowsi University of Mashhad, Iran.

**Corresponding author: Mona Maleknezhad -Yazdi, Biology Department, University Jihad Unit, University of Science and Arts of Yazd, Iran. Email: moon.maleknejad@gmail.com.*

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Abstract:

Immunotoxins have been used for cancer treatment. The immunotoxin binds to the surface antigen on the cancer cell, enters inside the cell by endocytosis, and destroys the cancer cell. In addition, the components of this type of drug and assembly based on peptide bonds, and the creation of recombinant protein construction were among the requirements investigated in this study. In this bioinformatics research, membrane antigen structural, and functional properties on the surface of breast cancer cells were investigated and evaluated to target cancer cells. An EGFR antigen with a shorter amino acid length for positive binding and INS, which has 110 amino acids, binding +, and a binding score of 0.99, was selected as the most efficient ligand using the AAASGG 3 (GGGGS) linker, resulting in a six-recombinant structure. Hence, the targeted treatment of cancer through immunotoxin with the confirmation of the patent sequence led to the creation of a recombinant structure, which was analyzed with bioinformatics software. To ensure accurate results in the laboratory, we utilized Escherichia coli strain DH5 as a host during the cloning phase for plasmid DNA replication. This enabled a more precise and reliable replication process, thereby confirming the validity of our computational modeling, and the results of this research led to the modeling and simulation of the engineering structure of Cetuximab ZZpe38 immunotoxin. For future research, gene expression in mammalian cells will be the focus.

INTRODUCTION

Cancer is the second-most common cause of death in developed countries and the third-most common cause of death in developing countries. So more than 25 million people in the world are suffering from cancer, and more than 11 million people get this disease every year. Cancer is a group of diseases that involve an abnormal increase in the number of cells and have the potential to invade and spread to other parts of the body. All cancerous tumors are benign tumors that have spread to other parts of the body. There are more than 100 cancers that affect humans (1-3) Therefore, the growth of cancer cells between normal tissues occurs in two ways: malignant tumors, or benign and cancerous tumors which are known as masses. A benign tumor is generally not cancerous and usually grows slowly. Benign tumors are formed from cells that are comparable to ordinary cells. They only have a series of problems: excessive and very large growth, unpleasant and uncomfortable onset, pressure on other organs of the body, and malignant tumors, which, as a rule, develop faster than benign tumors, spreading into and damaging tissues that may occur anywhere in the body, such as the blood and lymphatic system, in the form of secondary tumors, which spread to other parts of the body called metastasis. The sorts of cancers known in people incorporate cancers determined from epithelial cells, which are the most common cancers among the elderly. Most cancers are created within the breast, lung, pancreas, colon, and prostate (4). Breast cancer is the second-leading cause of cancer death and a major health risk for women. although recent early detection advances have improved overall survival rates. The successful treatment of breast cancer in the last 10–15 years is one of the greatest achievements of medical science, particularly in the field of oncology, and is considered a medical revolution (5) Therefore, breast cancer risk factors are involved in the development of all cancers in about 9% of cases. This type of cancer mainly leads to metastasis and death due

to insignificant clinical symptoms (6) Therefore (6), as with other cancers, it is inevitable to pay special attention to this type of disease in terms of early diagnosis and effective treatment, with Immunotoxins occupying a special place in targeted treatment. These types of therapeutic compounds are hybrid proteins with the ability to attack cancer cells and cause their death, based on the presence of a specific ligand associated with the antigen on the surface of the cancer cells and the toxin moiety in their structure (7) In this context, the development of this type of drug was carried out in the context of breast cancer and other cancers, among which the immunotoxin DAB389IL2 was the first immunotoxin approved by the U.S. Food and Drug Administration (FDA), known as the title Denileukin diftitox (Ontak) ® (DD) (8) And pointed out the ability to treat breast cancer and Ontak with FDA approval (9) This type of cytotoxic drug binds to the specific antigen on the surface of the cancer cell due to its ligand moiety, and then the entire antigen-immunotoxin complex can enter the cell (10) Within the cell, the bond between the ligand moiety and the toxin is cleaved, and cell death is triggered by several pathways depending on the sort of toxin, including inhibition of protein synthesis (10, 11). Various isoforms of antibodies, growth factors, and bacterial and nonbacterial toxins such as diphtheria toxin, Pseudomonas exotoxin, and ricin have been used for the structure of Immunotoxins (12), and their development and optimization are on the agenda of many research centers. In the development of this type of drug, as with many recombinant protein drugs, attention is paid to immunogenicity (13) folding, drug structure (14, 15) stability under physiological conditions, temperature stability over sterilization conditions, specific binding to the target cell, and revealing specific antigens on the surface of cancer cells (16, 17). Cell penetration (18). Post-translational modifications (19) and induction of cell death (20). They have a special meaning and deserve attention. In this regard, there are several reports of practical tests related to the optimization of immunotoxin drugs to reduce the immune response (21). Optimization parts of the toxin and ligand. (22) Permeability and increased stability due to amino acid substitution (23) In addition, antibody and non-antibody ligands such as a monoclonal antibody, Na nobody (Nb), folate, transferring, vitamins, carbohydrates, and peptides have been developed for specific binding of the toxin moiety to the cell (24) Although the development and optimization of Immunotoxins and other recombinant drugs are based on laboratory methods, consideration of cost and time justifies the use of modern computational methods (25) Therefore, it is important to know the different components of common Immunotoxins to optimize or introduce new toxins and specific ligands related to antigens on the surface of cancer cells to develop this type of drugs based on data analysis and computational biology (25, 26) In this study, breast cancer, which is one of the most common cancers in women and has a high mortality rate, was examined, for this purpose, it was examined in this study, and the reason of designing an immunotoxin was the selective treatment of this disease. Thus, Immunotoxins allow targeted delivery of potent toxin molecules directly to cancer cells, minimizing toxicity to normal cells. This improves safety compared to traditional chemotherapy. Commonly used ligands include monoclonal antibodies, growth factors, vitamins, and peptides that specifically bind to antigens overexpressed on tumor cells. Popular toxins are bacterial toxins such as Pseudomonas exotoxin A and diphtheria toxin. They inhibit protein synthesis through ADP-ribosylation or RNA N-glycosidase. After endocytosis of the antigen-immunotoxin complex, the toxin domain moves to the cytosol and reaches its intracellular target. The linker domain is cleaved enzymatically or via low pH in endosomes. Second-generation Immunotoxins combine Fv antibody fragments with toxins for smaller size and better tissue penetration. The third generation has mutations for increased activity and stability. Clinical responses have been seen in blood cancers and some solid tumors. Toxins include vascular leakage syndrome. Challenges include immunogenicity, stability, and manufacturing complexity. Linker engineering and simple methods aim to address these issues. Promising areas of research include retargeting Immunotoxins using dual antibodies, nanoparticles for delivery, oncologic viruses, and combination therapies. Immunotoxins are a complex, targeted cancer therapy. Here are some important points about the challenges of immunotoxin development and the techniques researchers are trying to overcome them. Improved stability: amino acid changes to increase intramolecular bonds, use of more stable linker, decreased immunogenicity: amino acid changes in regions recognized by the immune system, covering immunogen molecules, improved targeting: use of monoclonal antibodies, nanoparticles, viral vectors for delivery, Combination with other drugs: such as checkpoint killers to enhance the immune system response, Preparation methods: attached culture for standardized production, use of advanced biological platforms, There is no perfect solution to the challenges and researchers are looking for continuous optimization According to the progress made and the efforts of researchers in the development of optimization techniques of Immunotoxins, the prospect of their approval and commercialization is as follows: The immunotoxin Denileukin diphtitox has been the only commercialized product so far. An increasing number of clinical trials for Immunotoxins are underway,

indicating the development of this field. The use of advanced biotechnological methods will increase production efficiency. Attracting more investors for the research and development of immunotoxin products is increasing. Therefore, we hope to see more Immunotoxins approved and marketed shortly. However, there are still challenges that require further research. There are several main challenges to commercializing immunotoxin: high costs of research and development; design, production, and clinical studies are all expensive. The complexity of biotechnology—preparation, production, and quality control—requires high expertise. Stability issues: Maintaining biological activity throughout the drug's shelf life is challenging. Resistance to the immune system: Lowering immunogenicity requires a lot of effort. Limited market: Unlike chemical drugs, there is no viable market for biological cancer treatments. Regulatory issues: Strict regulations for biologics have become more challenging today. Therefore, they need strong financial resources and industrial cooperation to overcome these obstacles. Some important strategies to reduce research and development costs in the commercialization of Immunotoxins: are leveraging worldwide research networks to increase efficiency and reduce costs; funding from various sources such as venture capitalists, government grants, and industry; cooperating with industrial partners to carry out development and commercialization stages; sharing costs with other companies in joint research projects; using the existing infrastructure in universities and research centers; optimizing production processes and quality control; and using new bioprocessing methods such as stem cells. International cooperation plays an important role in this field.

MATERIALS AND METHODS

The reason for this inquiry was to plan smart immunotoxins to target cancer cells, which to begin with examined the layer antigens on the surface of breast cancer cells from the database www.ncbi.nlm.nih.gov/gen (Figure 1). In this manner, its basic and useful characteristics were observed and examined, and the next step was chosen within the assessment of membrane-bound antigens on the surface of breast cancer cells: EGFR antigen with a length of 3489920–3611495 BP and chromosomal position 4q25. 26 exons and the identification number 1950. Therefore, the three-dimensional structure of the required sequences, including the EGFR antigen against breast cancer, was traced by the https://swissmodel.expasy.org database. In addition, the expression level of antigens related to breast cancer was determined and quantified from the available sources using the Protein Atlas database (PA) (www.proteinatlas.org). For this purpose, by entering the names of selected antigens in the relevant toolbar, the level of antigen expression in cancer cells was determined using this cancer cell database (27). To analyze the topology of antigens in the breast cancer cell membrane, Secondary structure prediction program in the ExPASY database (https://www.expasy.org/) was used, and tracking protein molecules that can bind to specific antigens associated with breast cancer cells was done from the string network web server with the address (http://string-db.org/) (28) In this context, Monoclonal antibodies were used to target the selected antigen. Specificity for the selected antigen was found based on a review of PA sources and databases. Regarding the binding affinity of the ligand to the antigen, we ensured that the ligand had high specificity for the selected antigen and did not bind to other antigens, post-translational modifications, and the size of the ligand were among the factors that were investigated in the selection of the ligand. Determining the three-dimensional structure of target protein sequences, including antigens and components of immunotoxins, by the comparative modeling method based on the Swiss-Model program (29). The RCSB protein structure database was used. In addition, the modeling and assembly of immunotoxin structures were performed using Modeller version 9.15 (30). To this end, we ensured that the ligand had high specificity for the selected antigen and did not bind to other antigens, post-translational modifications, and the size of the ligand were among the factors that were investigated in the selection of the ligand. Then, the structural models

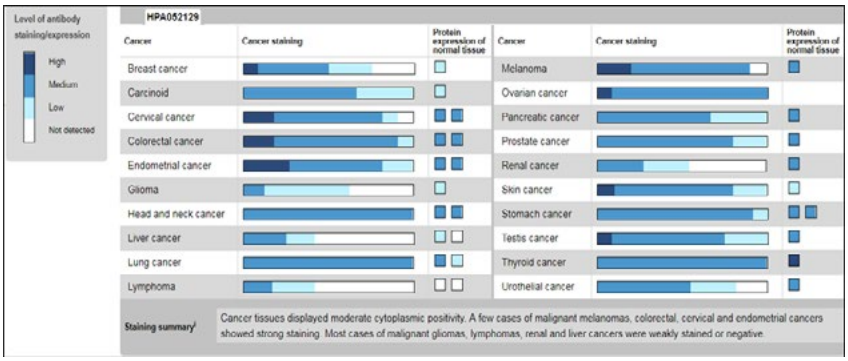


Fig1. Comparison of EGFR antigen expression in different normal and cancer tissues using cancer cell staining (38)

of the components of the immunotoxin structure were transferred to the alignment file, and the correctness of the procedure was verified by re-reading the sequence of each structure in the PyMOL software. Finally, based on the instructions of the program during structure modeling, six structures for each immunotoxin structure were generated and evaluated. The structures were displayed using PyMOL version 1.2 (31). By determining the quality of the modeled structures in the test path by showing the positional score of the amino acids that make up the structure, based on determining the Ramachandran diagram using the RAMPAGE program, ERRAT (32) The most accurate model was checked based on Ramachandran diagram and ERRAT score Optimization of modeled immunotoxin structures and evaluation of structural stability under quasi-physiological conditions were done with Gromacs 4.5–5 molecular dynamics simulation software in a Linux environment and using the Gromos 43a1 force field. For this purpose, after passing through the nptand nvt stages in 100 picoseconds at a pressure of one atmosphere, the immunotoxin structures were placed in a 0.1 nm box in an aqueous environment using the SPC 216 water model for 20 ns at 300 °C. Kelvin degree (33). Hence, to evaluate the functional capabilities of the immunotoxin structures and the ability of the designed immunotoxin structures after optimization in physiological conditions, from the point of view of stimulating the immune response and the ability to bind to cancer cells, they were tested. For this purpose, the identification of detectable epitopes of the humoral immune system was performed using the SVMTriP web server. Therefore, the binding affinity of the constructs to related antigens was evaluated using the molecular docking method using the HADDOCK2.2 program (34). Cetuximab monoclonal antibody and insulin ligand targeted the EGFR antigen in breast cancer cells. Exotoxin A of Pseudomonas aeruginosa $(GGGG)_3$ was used as the toxic part of the Cetuximab ZZpe38 immunotoxin. This toxin has ADP ribosylation activity and inhibits protein synthesis in the cell, so the antibody and toxin were linked by a flexible GGY AAASGG 3 (GGGGS) linker. To identify and confirm the production of immunotoxin, SDS-PAGE electrophoresis tests were used to determine the molecular weight and confirm the binding of ligand and toxin, and liquid chromatography with high separation power was used to confirm the authenticity and proper quality of the production of immunotoxin. Therefore, the use of these analytical techniques can confirm the correct production of immunotoxin for immunotoxin design and analysis. For designing and modeling, using Auto dock and PyMOL software, antibody, and toxin binding optimization was done, and protein sequence and structure information was extracted from the PDB, UniProt, and GENBANK databases. As a result, the use of these banks helped to make a more optimal design.

RESULTS

According to the research, EGFR antigen expression evaluation detected high expression in breast cancer cells. As shown in the figure below, four cases of cancer tissue studied in this database have moderate expression, and only one case has low expression of this antigen on the surface of breast cancer cells. While the average expression of said antigen is low in the corresponding normal tissue cells, the expression of the EGFR antigen is high in breast cancer tissue. Therefore, searching for the structural model of selected target antigens on the surface of breast cancer cells with high expression in the database and determining the relevant model based on computational methods led to the discovery of the three-dimensional structure of EGFR antigens with the desired structure (Figure 2). assessment of the quality of the 3D structure of the EGFR antigen using Ramachandran. EGFR, with structure rich in alpha helices and beta sheets, was in the Ramachandran map with 98% favorable quality, so that only 1.1% of its amino acids were allowed outside the environment (Figure 3).

From determining the topological features of the antigen, the position of each amino acid was obtained in three extracellular, intermembrane, and intracellular positions based on the fact that most of the amino acids

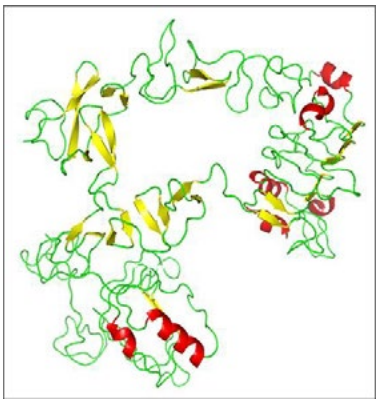


Fig 2. 3D structure of the EGFR antigen

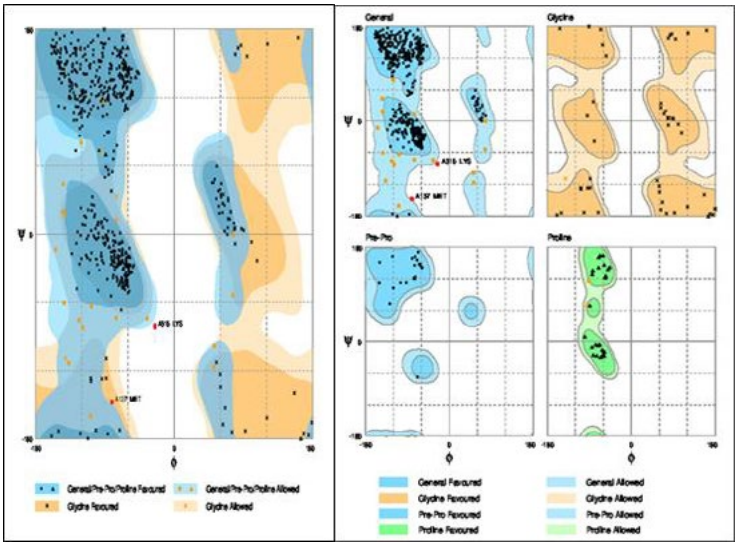


Fig3. Assessment of the quality of the 3D structure of the EGFR antigen using Ramachandran

of this antigen are located outside the cell. that said antigen was a membrane antigen that was recognized by the target proteins on the cell surface (Table1). All amino acids are outside the membrane and are part of the membrane that can be ignored (Figure 4). Then, to determine the epitope position of the EGFR antigen after binding from the IEDB site (Figure 5, 6). The epitope regions of the EGFR antigen were determined to identify the CDRs of the antibody (Table2), determine the molecular interactions of the specific EGFR antigen, and monitor the molecules that can bind to the EGFR surface antigen (Figure7).

Table 1. Study of the position of amino acids on the membrane

Peptide prediction	positions of amino acids
1-600	Extracellular
601-623	transmembrane
624-1091	inside the cell

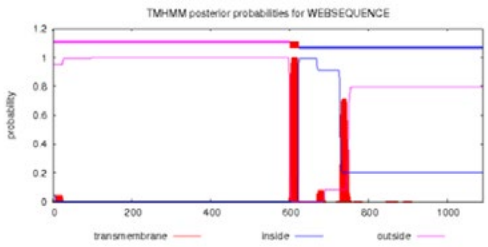


Fig 4. Topological position of the EGFR antigen

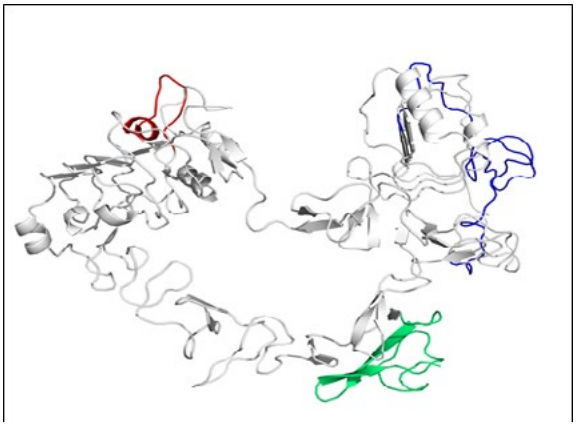


Fig 5. determination of the position of the epitope regions on the antigen

Table 2. EGFR antigen index epitopes included in the IEDB database

Score	sequence	aa Number	Row
0.824	GENNTLVWKYADAGHV CHLCHPNCTYGCTGPGLEGCPT	593-556	1
0.773	LSNYDANKTGLKELPMRNLQGQKC DPSCPNGSCWGAGEENCQKLTKIICAQQCS	175-122	2
0.768	LPVAFRGDSFTHTPPL	342-327	3

Fig 6. Structural features of the second EGFR antigen

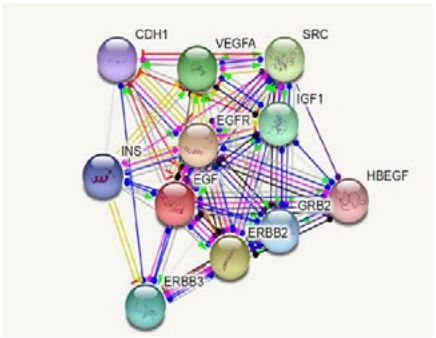


Fig7. Molecules that can bind to the EGFR antigen

This led to the discovery of 10 molecules that interact with the EGFR binding domain and have different functions. Among the ligands that interact with the selected antigen, the ligand that has a shorter amino acid length for positive binding and a higher score is selected as the best ligand. The ligand INS, which has 110 amino acids, a binding +, and a binding score of 0.99, was selected as the best ligand (Table3). The antigen is better selected based on its binding affinity to different proteins to select the binding region for designing a smart immunotoxin. Creating mutations and optimizing immunotoxin design requires a lot of time and money. For this purpose, in this project, the Cetuximab antibody and, as a result, the optimal immunotoxin ZZpe38 Cetuximab were used for drug design. The determination of the three-dimensional structure of the connecting part of the mother structure of the connection of the selected immunotoxin to the target antigen was based on a domain called Cetuximab. Therefore, structural modeling was done for this purpose. The results of determining the structure of the selective domain showed a structure with beta sheets (Figure 8). To evaluate the quality of the structural model of the antibody after assembling the domain and checking the structural quality of the Cetuximab antibody in terms of amino acid position, it showed that 99% of the amino acids were in the correct place. By examining the ERRAT and the crystallographic structure of the protein, the correct placement of the amino acids in the correct position was confirmed, and the diagram was produced with 100% accuracy and a quality factor of 79.503 (Figure 9). To determine the position of the Cetuximab antibody in the mother immunotoxin, where the heavy and light chains of the antibody can bind to the epitope regions of the target antigen (Figure 10). The para-topic regions of this antibody were determined. The para-topic regions of the immunotoxin include six CDRs, three of which were in the heavy chain and three of which were in the light chain of this antibody (Figure 11). Further,

Table 3. Structural and functional features of proteins that can bind to EGFR antigens

Score	linkage	ID	length BP	Gene	Function	length aa	protein	Row
0.999	+	100197	1950	EGF	human epidermal growth factors	1207	EGFR	1
0.995	+	60748	2064	ERBB2	Epidermal plant protein receptor gene	1255	ERBB2	2
0.994	+	6714	60748	SRC	a gene similar to the V-SC gene of sarcoma	536	SRC	3
0.993	-	7422	16279	VEGFA	Vascular endothelial growth factor	412	VEGF	4
0.993	+	7157	12988	IGF1	Insulin-like growth factor1	195	IGF1	5
0.992	+	2065	23483	ERBB3	EGFR epidermal growth factor receptor gene from tyrosine kinase receptors	1342	ERBB3	6
0.991	+	2885	87634	GRB2	The protein encoded by the gene involves The epidermal growth factor receptor	217	GRB2	7
0.990	+	3630	1431	INS	Insulin lowers the blood glucose concentration	110	INS	8
0.989	-	9999	98253	CDH1	adhesion-dependent cell cadherin's are calcium	882	CDH1	9
0.989	+	18361	3761	HBEGF	Growth factor-like EGF	208	HBEGF	10

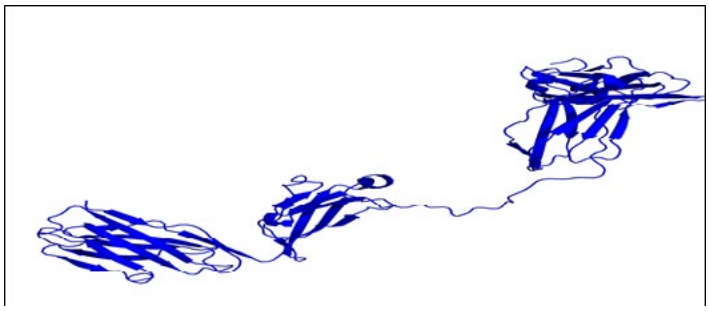


Fig 8.a. 3D structure of the heavy chain of cetuximab-VH,

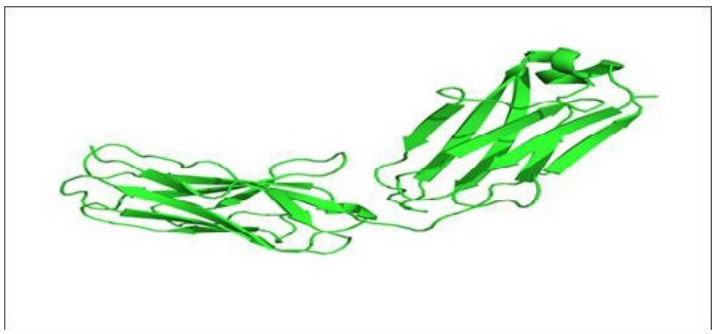


Fig 8. b. VL-Cetuximab light chain structure

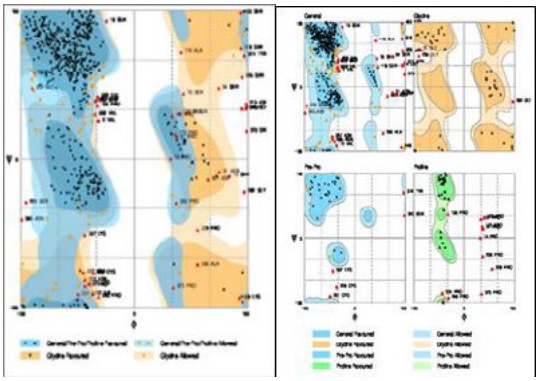


Fig 9. Determination of the quality of the three-dimensional structure of the antibody cetuximab using Ramachandran

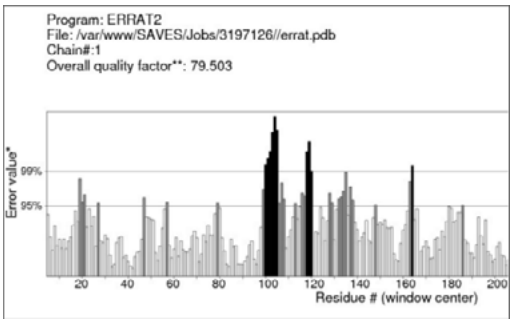


Fig 10. determination of the quality of the 3D structure of the cetuximab antibody using ERRAT software

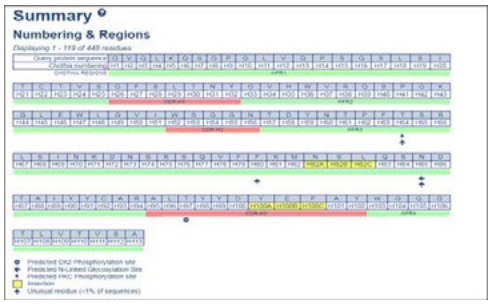


Fig 11.a. Paratopic regions in the antibody heavy chain,

Fig 11.b. Paratopic regions in the light chain of the antibody

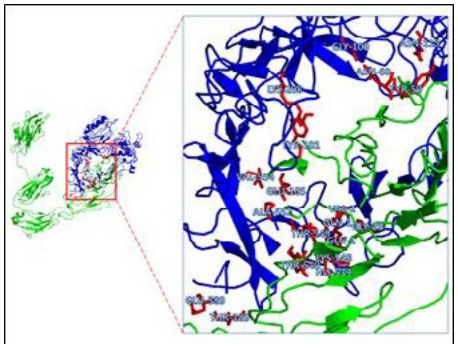


Fig 12. Evaluation of antibody and antigen binding

Table 4. Evaluation of antibody-antigen binding

HADDOCK Score:	-133.3
RMSD	23.9
Z-Score	-1.8

the evaluation of the binding of the antibody to the selected antigen led to the detection of binding with the optimal energy level in the relevant position (Figure 12; Table 4).

To monitor bacterial exotoxin A derived from Pseudomonas in terms of structure, host distribution, and possible function, the ability to induce cell death with multiple potentials and the most efficient selection were studied accordingly. To investigate the structural features of the exotoxin A domain, whose protein sequence is available in the NCBI database with accession number P11439, it is 638 amino acids and 1917 base pairs long, it is derived from the bacterium Pseudomonas aeruginosa, and the results of protein sequence control show the presence of 4 domains including the domain Concanavalin-like lectin glucanase A at position 27 to 276, exotoxin binding domain at position 27 to 275 and exotoxin A domain, intermediate domain at position 277 to 409 and exotoxin A domain, catalytic domain at position 413 to 638 showed (Figure 13,14) From the comparison of the three-dimensional structure and amino acid sequence of the primary toxin A of Pseudomonas aeruginosa with the part used in the structure of Cetuximab ZZpe38 immunotoxin (amino acids 252 to 608) (Figure 15), it seemed that the subunit in the structure of the immunotoxin lacks the IA domain of this toxin because this domain It has the task of identifying the eukaryotic target cell in the pathogenic process of Pseudomonas aeruginosa bacteria. The purpose of using poison in the mentioned immunotoxin structure was to use other toxic domains to enter the eukaryotic target cell and perform the cytotoxic function of its catalytic domain. Therefore, monitoring the sequence of the immunotoxin CituximabZZpe38 led to the identification of the components of the immunotoxin,

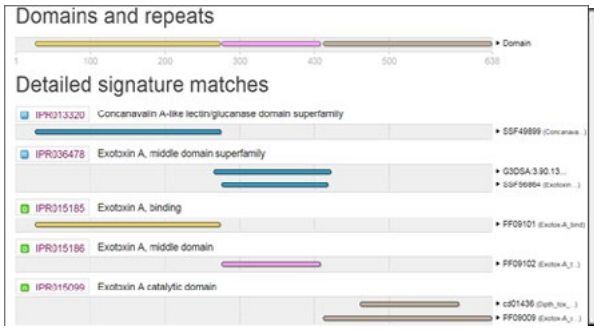


Fig 13. Structural features of the second exotoxin

Fig 14. amino acid sequence and components of cetuximab ZZpe38 immunotoxin

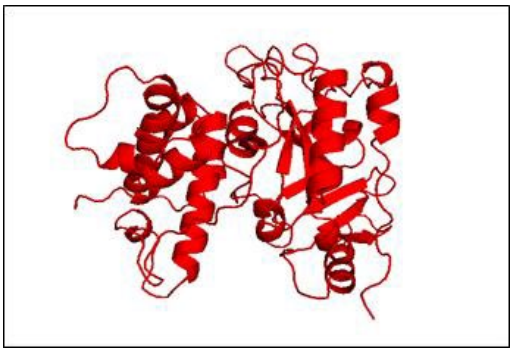


Fig 15. 3D structure of the functional part of the selective toxin

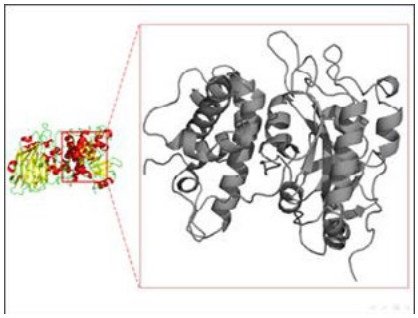


Fig16. Comparison of the complete toxin (native toxin) and the toxin part used in the immunotoxin structure (toxin subunit)

which include the antibody light chain, parts of the antibody linker, the antibody heavy chain, the antibody binding linker to the toxin, and the toxin subunit (Figure 16). In detection, the secondary structures of Cetuximab ZZpe38 immunotoxin were determined in terms of the type and number of B_sheet, helix, and coiled-coil structures that make up Cetuximab ZZpe38 immunotoxin subunits. Examining the results showed that the dominant structure in the immunotoxin antibody subunit is B_sheet. In other words, the only secondary structure in the sheet antibody subunit (a total of 33 structures) is located in this section. The rest of the predicted structure of the antibody sequence can be considered a double-coil structure (Figure 17). This is the case when the toxin subunit has approximately equal amounts of two secondary structures in common. To determine the physical and chemical properties of the immunotoxin CetuximabZZpe38, the parameters obtained from the calculation of the physicochemical properties are: the number of amino acids, the molecular weight of the protein, the isoelectric point of the protein, the composition of amino acids (the percentage of each amino acid in the total composition of the protein), the total number of amino acids with charge negative, the total number of positively charged amino acids, the combination of elements in the protein structure, the chemical formula of the protein in terms of elements, the total number of atoms, molar absorption coefficient, extinction coefficient, half-life, instability index, aliphatic index, and hydrophobicity scale were used (Table 5). From the assembly of immunotoxin domains with an effect on breast cancer, the binding of the Pseudomonas toxin domain to the selected ligand using the linker AAASGG 3 (GGGGS) in a structure that led to the creation of 6 recombinant models with different quality and structure, the best of which, according to the qualitative evaluation, simulations were subjected to pseudo-physiological

Fig 17. Secondary structures of the cetuximabZZpe38 immunotoxin segment

Table 5. amino acid composition of PE38 cetuximab immunotoxin

trilateral	monomial	amino acid number	percentage of	monomial	The amino acid in the structure trilateral	immunotoxin	Percentage of structure constituents
Ala	A	94	% 8	LYs	K	59	5.1%
Arg	R	49	4.2%	Me	M	6	0.5%
Asn	N	56	4.8%	Phe	F	38	3.3%
Asp	D	53	4.5%	Pro	P	79	6.8%
Cys	C	18	1.5%	Ser	S	116	9.9%
Cys	Q	64	5.5%	Thr	T	70	6.0%
Glu	E	71	6.1%	Trp	W	17	1.5%
Gly	G	95	8.1%	Tyr	Y	41	3.5%
His	H	21	1.8%	Pyl	O	77	6.6%
LIe	I	36	3.1%	Sec	U	0	0.0.%

conditions (Table6) (Figure 18). which has the best structural quality. In this regard, the quality of this structure was confirmed by the RMSF and RMSD charts (Figure 19).

The aim of investigating the characteristics of the linear immunotoxin CetuximabZZpe38 in the use of peptide drugs was to determine the lack of immune response in the body and the stability of the drug after injection into the host body. Therefore, the immunogenicity of the drug was checked using SVM Trip software. The results of the immunogenicity evaluation of the selected immunotoxin led to the

Table 6. The position of amino acids after domain assembly

Score	Un allow position aa	allow position aa Appropriate	Structural Model	Row
%98	55	160	951	1
%94	69	92	975	2
%92	84	91	961	3
%91	101	103	933	4
%90	104	103	928	5
%90	101	77	958	6

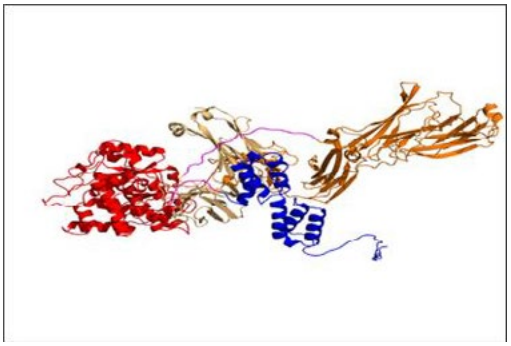
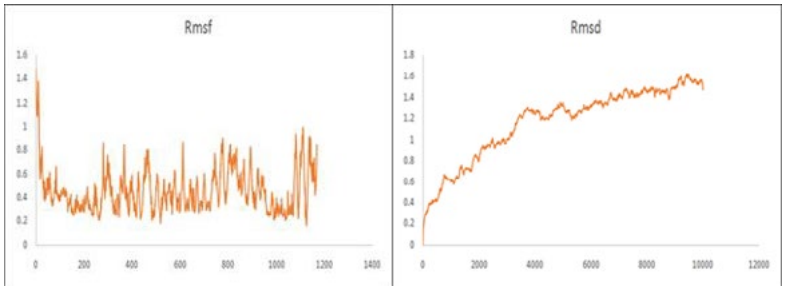


Fig 18. The structural model resulting from the domain composition



a **b**

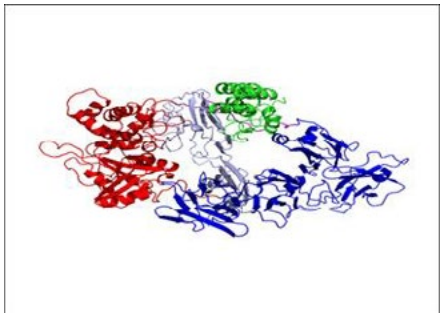


Fig 19:a) RMSF map of the designed structure under quasi-real conditions; b) RMSD map of the designed structure under quasi-real conditions; c) Cartoon model of the best-designed immunotoxin structure with effect on breast cancer from the point of view of structural stability under quasi-physiological conditions

discovery of 10 epitope regions recognizable by the human immune system in different positions of this protein sequence, of which 6 higher immunogenic positions and their spatial arrangement positions were shown (Table7, Figure 20,21). The results of the antigenic evaluation of the designed structure led to the identification of 28 epitope regions, which can be identified after modeling and simulating the immunotoxin CetuximabZZpe38 in our environment and evaluating its binding to the EGFR antigen by MHC-I (cellular immunity) of T cells (Table 8). The mentioned immunotoxin was synthesized. To amplify and validate the synthesized immunotoxin, the following steps were performed in vitro: culture and sensitize bacteria; confirm the absence of resistance genes; and use the E. coli DH5 strain for plasmid DNA replication. Therefore, before starting the laboratory process, it is necessary to confirm the absence of a foreign plasmid in this bacterium. For this purpose, linear cultivation of this strain was done in the environment with and without antibiotics, which showed that the bacteria grow very well in the environment without antibiotics, while they do not grow at all in the environment with antibiotics and have no resistance gene. Therefore, to transfer the plasmid to the bacteria, the sensitization steps must be performed first, so the bacteria have the

Table 7. Immunogenicity assessment and representation of epitope sequences in the designed structure

Rank	location	Epitope	Score	Recommend
1	186-205	QRLVALYLAARLSWNQVDQV	1,000	*
2	655-674	ALTSGVHTFPAVLQSSGLYS	0.989	*
3	762-781	DPEVKFNWYVDGVEVHNAKT	0.987	*
4	428-447	GRLETILGWPLAERTVVIPS	0.984	*
5	872-891	EWESNGQPENNYKTTPPVLD	0.982	*
6	348-367	YIAGDPALAYGYAQDQEPDA	0.982	*
7	783-802	PREEQYNSTYRVVSVLTVLH	0.981	
8	1069–1088	VFIFPPSDEQLKSGTASVVC	0.981	
9	404-423	EVERLIGHPLPLRLDAITGP	0980	
10	218–237	DLGEAIREQPEQARLALTLA	0.980	

The epitopes recommended are labeled by the star

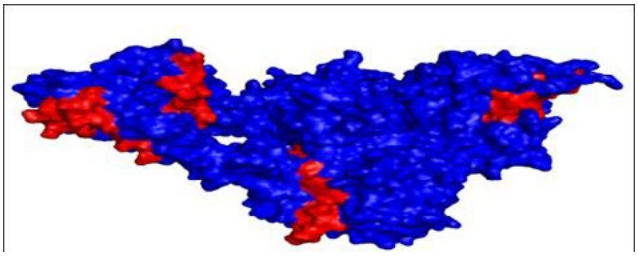


Fig 20. Verification of the location of the immunogenic points of the designed structure (the immunogenic points are shown in red)

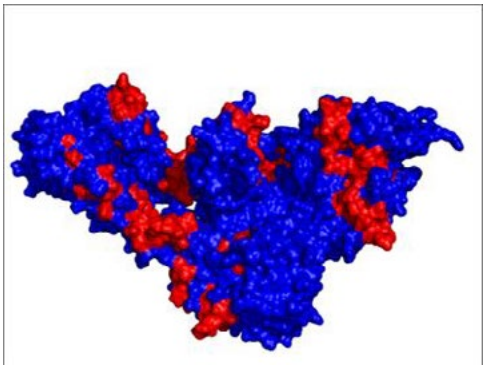


Fig 21. Examination of the location of the cellular immunogenic points of the designed structure (the immunogenic points are shown

conditions to accept the foreign plasmid. After this step, the quality of sensitive bacteria was determined in two environments with and without ampicillin. As seen, the sensitive bacteria grew in the environment without antibiotics and were free from any growth in the environment containing antibiotics.

DISCUSSION

Breast cancer is known to be highly resistant to treatment methods, especially chemotherapy, which has not been efficient due to the resistance of breast cells to systemic treatment with anti-cancer agents. Therefore, there is a need for new treatment options, and immunotherapy approaches that target breast-related antigens have been presented as promising solutions. Normal and transformed cancer cells at the molecular level have made it possible to identify them specifically. And these treatment methods, in addition to their higher efficiency, have side effects. Using low amounts of microbial toxins to stimulate the immune system of cancer patients is a method that replaces chemotherapy and radiotherapy (35) Antibody therapy has become an important component in the management of malignant diseases because it can block tumor growth factors or their receptors, stimulate an immunological attack on the tumor, and be used to deliver carriers such as radioisotopes, cytotoxic drugs, or toxins. Immunotoxins, a new class of antitumor agents including tumor-selective ligands, generally monoclonal antibodies (mAb) attached to highly toxic protein molecules, have the advantage of the precise specificity of antibodies compared to the delivery of selected target drugs and the ability of toxins to eliminate target cells. Toxins have been changed and modified by genetic engineering to remove the natural domains attached to the tissue. Analysis of the amino acid sequence of the specific region for immunogenicity and the signal transduction mechanisms involved in the interaction of immunotoxins with tumor cells will provide a clue for the development of more effective immunotoxins (15). EGFR antigen expression level and its expression range in cancer tissues showed that this antigen is present in malignant tissues of the cervix, skin, stomach, kidney endometrium, glioma, cervix, ovary, prostate, thyroid, kidney, and all cancers. has it. cells. It was an excellent and moderate expression. Regarding EGFR antigen expression in breast cells, it is important to mention that out of 12 cases of cancer tissue examined in this database, 4 cases have moderate expression, and only one case has low expression of this antigen on the surface of cancer cells. It was the chest. At the same time, the average expression of this antigen in the corresponding normal tissue cells was low, whereas its expression of the EGFR antigen in breast cancer tissue was high. Therefore, binding domains for ligands or monoclonal antibodies were utilized as targets for antibody-based immunotherapy in breast cancer patients (36). In this study, the structural and functional characteristics of Cetuximab ZZpe38 immunotoxin were analyzed in the computational section. The results clearly show that in the structure of the existing immunotoxin, according to all common immunotoxin structures, two main parts of antibody and toxin and three sub-parts of a signal peptide, total guide peptide, sequence (His-tag), linker, and KDEL motif can be seen (37). On the other hand, the linker used in this research is AAASGG (GGGGS)3. In general, the linkers used in the fusion of proteins are divided into three categories: flexible, hard, and Cuttable. In the meantime, the linker used in this research was of a flexible type, which has characteristics such as increased stability and favorable folding, so the results of the assembly led to the achievement of 10 models with favorable structural and functional qualities about the immunotoxin structure. The assembly of the components of the drug in this research was done using the homology modeling method and the Modeler software. This antibody is a type of monoclonal antibody against the EGFR antigen. In the toxin subunit of the immunotoxin, there is a part of the sequence including amino acids (252–608) of the complete toxin sequence of exotoxin A of Pseudomonas aeruginosa bacteria that is responsible for transgenic transfer and induction of cytotoxicity of the toxin in the target cell. Also, by identifying the functional capabilities of Cetuximab ZZpe38 immunotoxin, the functional role of its components was determined. The investigation of the linear immunogenicity of the Cetuximab ZZpe38 immunotoxin sequence led to the identification of six epitope positions in the terminal part of its sequence. By looking more closely at the sequence of this part, it is found that, as expected, all six epitope sequences are located in the catalytic domain of the toxin part, which has the property of stimulating the immune system by affecting the target eukaryotic cells. Quality that most of the amino acids used in the structure of the immunotoxin, about 94%, are in the desired or allowed areas in terms of φ and ψ. On the other hand, the results of the ERRAT chart show an overall quality factor value of 73.35. The total of these data indicates the stability of the structure and acceptable quality for the desired immunotoxin. In the next step, Cetuximab ZZpe38 immunotoxin was simulated. The results of the Ramachandran map evaluation of the simulated structure in physiological conditions equal to the environment and the real solvent have proper stability. In other words, of the total amino acids in the structure, about 98% are placed in suitable positions, while the study of the ERRAT diagram of the simulated structure provides a value of 79.913% for the overall quality factor, which itself is proof that the stability of the structure is increased in

the simulated conditions. Comparative studies were conducted between the 3D computer model of the primary immunotoxin and the final simulated structure in the virtual modeling environment. Changes were observed in the orientation of secondary structures and folding of the Cetuximab ZZpe38 immunotoxin protein molecule. The most important of these changes include the folding of more B sheet parts (signal peptide and linkers) towards the center of the macromolecule, the antiparallel orientation of the beta sheets forming the overall structure of the antibody subunit, and the more precise arrangement of the helix structures of the toxin subunit. According to the total findings as well as the data obtained from the evaluation of the structure after simulation in the real environment and solvent, it can be concluded that the said structure has reached acceptable stability. The structure and function of EGFR antigens were analyzed. Monitoring the relationship between antigen structure and function reminds us that the Cetuximab ZZpe38 immunotoxin binding region is located in domain 1 of the N-terminal region, based on the relevant patent. The study of EGFR antigen expression in different cancers shows that in most of these cancers, including breast cancer, the expression level in cancerous tissues is much higher than in healthy tissues. Revealing the epitopes of the antigen, out of the total of 27 epitopes identified for this antigen, 11 epitope sequences are located in domain 1, which confirms our other results in this regard. Finally, the evaluation of the binding affinity of the designed drug after simulation with the selected antigen led to the revelation of the binding affinity with optimal energy levels in the right position. In the laboratory phase of the work to practically verify the results of the computational section, after the culture and susceptibility of the host bacteria E. coli DH5, the growth of normal and susceptible bacteria in LB culture media without antibiotics and the lack of growth of normal and susceptible bacteria in LB culture media with ampicillin were confirmed. Next, the transformation of a plasmid carrying pET-22b was done, and the transgenes were cultivated linearly. Then plasmid DNA was purified by the mini-preparation method. Finally, the movement pattern of the plasmid was checked by electrophoresis on the agarose gel. The movement pattern of the pET-22b enzyme digestion product transformed with the BamH1 enzyme compared to the transformed plasmid and the parent plasmid was displayed near the 1 kbp marker. A comparison of the movement patterns of the primary plasmid, marker, and transgenic plasmid confirmed the desired gene clone in the plasmid in general. The results of this research led to the modeling, simulation, and assimilation of the anti-EGFR immunotoxin called Cetuximab ZZpe38, which can specifically induce the death of breast cancer cells. Its structural and functional capabilities were confirmed in virtual conditions, as was its expression level. was evaluated. Also, the simulated immunotoxin was evaluated for folding changes, structural and functional changes, binding affinity, and immunogenicity. Immunotoxins often referred to as “targeted therapies,” are a combination of proteins consisting of a toxic moiety that is targeted to a specific deletion of target cells. The focus on portion is by and large a monoclonal counteracting agent or hereditarily designed counteracting agent parts. Antibody-based biologics are one of the best-known treatment strategies in cancer therapy. After modeling and simulating the desired immunotoxin in an aqueous medium and evaluating its binding affinity to the EGFR antigen, the immunotoxin was synthesized. To amplify and affirm the synthesized immunotoxin, the following steps were performed in vitro: The bacterial strain E. coli DH5 was utilized to increase plasmid DNA. Hence, since we are just beginning the exploratory steps, it is vital to affirm the absence of any outside plasmids in this bacterium. For this purpose, the linear culture of this strain was performed in an environment with and without antibiotics, which showed that the bacteria in the environment without antibiotics had very good growth, while in the environment with antibiotics, there was no growth, so it lacked the gene for resistance. Thus, new methods based on genes, cells, hormones, and bacteria have made it possible to understand the difference between normal and deformed cancer cells at the molecular level. At that point, after deciding and selecting the finest structure, its blend, cloning, and endorsement were performed in the laboratory. The aim of the targeted treatment of the immunotoxin pathway through sequencing and creation of a hybrid engineering structure or recombinant protein was registered in the patent bank, which was analyzed and modeled with computational software and placed in a vector to confirm it in the laboratory. And the gene synthesis order was issued. In this research, the purpose of expression in E. coli DH5 bacteria was the survival of mammalian cells, and in future research, expression in mammalian cells to destroy cancer cells is on the agenda. The designed immunotoxin showed selective cytotoxicity against the target cancer cells. Electrophoresis and mass spectrometry confirmed the antibody-toxin combination. This product has potential as a targeted cancer therapy for HER2-overexpressing cancers. Clinical trials are needed to demonstrate the safety and efficacy of the treatment in humans. Large-scale production can be achieved cost-effectively through fixed processing methods. The market size for HER2+ breast cancer alone is significant, indicating its commercial viability. Intellectual property protection, such as patents, will be pursued to capture market share. Further preclinical studies are needed to optimize

formulation, stability, and toxicity profiles. A business plan for funding clinical translation and partnership opportunities will be evaluated. With ongoing development, this first-in-class therapy has promising prospects for commercialization and clinical impact. As a result of this research, immunotoxin can be introduced as a potentially superior treatment compared to current treatments with the advantages of safety, efficacy, and lower cost, and even for licensing after phase 2 or joint development with a large pharmaceutical partner to maximize market potential and access. rapid commercialization to patients.

CONCLUSION

Monoclonal antibodies show promise for targeted cancer therapies. However, current production methods face limitations, such as inefficient fermentation processes and high manufacturing costs. To address these challenges, we aimed to develop a recombinant immunotoxin for superior targeting of tumor cells. First, a three-dimensional structural model of the primary immunotoxin was generated in silico using computer modeling and simulation tools. Multiple conformations were analyzed to select the optimal antigen-binding configuration based on structural energetics. This computer-generated model provided a framework for subsequent experimental validation. Escherichia coli DH5α competent cells were selected as the prokaryotic host system. These cells were transformed with the pET-22b (+) expression vector, which contains an IPTG-inducible T7 RNA polymerase promoter for controlled transgene expression. Transformed DH5α cells were cultured in Lysogeny Broth (LB) medium at 37°C and grown overnight with agitation. Bacterial growth was assessed using absorbance measurements and confirmed through the presence/absence of growth on LB agar plates with and without 100 µg/mL ampicillin. Plasmid DNA was then isolated from cultured cells using a commercially available kit following the manufacturer's instructions. Isolated plasmids were visualized via agarose gel electrophoresis alongside a 1kb DNA ladder molecular weight marker to verify the expected plasmid size. Finally, restriction enzyme digestion of isolated plasmids using BamH1 endonuclease followed by gel electrophoresis confirmed transgene insertion into the multiple cloning site of pET-22b (+). The comparison of the movement pattern of the primary plasmid, the marker, and the transformed plasmid confirmed that the clone of the target gene in the field of targeted cancer treatment in a smart way, such as immunotoxin with the method of obtaining a patent from the calculation of the sequence path in the patent is not reliable, but its use is confirmed by modern computational methods. The results provide a foundation for future expression studies. key next steps will be transforming the vector into an inducible bacterial or mammalian cell line. Optimizing expression conditions and testing solubility, folding, and antigen binding of the purified protein will evaluate its potential as an immunotoxin. With further characterization, this immunotoxin could be developed as a targeted therapeutic pending in vivo efficacy studies. In conclusion, we have demonstrated a workflow to computationally design and clone a candidate immunotoxin at the molecular level. Validation of the cloning workflow established technical feasibility and Laid the foundation for optimization of expression and functional assessment. This provides a framework for the rational development of recombinant immunotoxins towards advancing targeted cancer immunotherapy

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Suggestions for further research

1.Changing the linkers to investigate the effect of folding.

2.Changing the toxin and ligand parts to increase the efficiency of the immunotoxin and increase its half-life Mentioning downstream applications requiring exploration, such as expression level, folding, antigen binding.

Author Contributions

All authors have read and agreed to the published version of the manuscript

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