Research in Pharmaceutical Sciences, August 2022; 17(4): 428-444
Received: 22-12-2021
Peer Reviewed: 10-01-2022
Revised: 01-02-2022
Accepted: 16-03-2022
Published: 14-07-2022

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Original Article

Design of two immunotoxins based rovalpituzumab antibody against DLL3 receptor; a promising potential opportunity

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Abstract

Background and purpose: The lack of a new effective treatment for small cell lung cancer (SCLC) is an unresolved problem. Due to the new identification of delta-like ligand 3 (DLL3) and its high expression in SCLC patients, the use of DLL3 in target therapy can be effective. The use of bacterial toxins belonging to the ADP-ribosyl transferase toxins family and human enzymes to remove cancerous cells has been effective in the structure of immunotoxins. In this study, single-chain fragment variable of rovalpituzumab antibody fused to granzyme B (Rova-GrB) and PltA of typhoid toxin (Rova-Typh) as immunotoxins were designed, and bioinformatics analysis was done.

Experimental approach: *In silico* analysis including the physicochemical properties, evaluation of the secondary and tertiary structure, refinement and validation of 3D models, and docking were performed. Immunotoxin genes were cloned and expressed in the *Escherichia coli* BL21 (DE3) host, purified, subsequently confirmed by western blotting and their secondary structure was evaluated by the circular dichroism method.

Findings/Results: The bioinformatics analysis showed that Rova-GrB and Rova-Typh had hydrophilic properties, their codon optimization parameters were standard, validation parameters were improved after immunotoxin refinement, and docking analysis showed that the binding domain of immunotoxins could bind the N-terminal region of DLL3. immunotoxins had high expression and after purification under denaturing condition by Ni-NTA column, the immunotoxins were dialyzed against PBS buffer.

Conclusion and implications: The immunotoxins had the right structure and can be produced in a prokaryotic host. The recombinant immunotoxins against DLL3 can be promising therapeutic agents for SCLC cancer.

Keywords: DLL3; *In silico* design; Rovalpituzumab; SCLC.

INTRODUCTION

Lung cancer has the most mortality rate according to the Globocan 2018 and 2020 report (1). To date, conventional small cell lung cancer (SCLC) treatments such as surgery, chemotherapy, and radiotherapy have had moderate therapeutic effects, but most SCLC patients relapse and rarely survive more than two years. Drug resistance and high genetic variation in SCLC patients are the main factors in the lack of approved treatment (2). Despite common cancer treatments, recurrence of SCLC is a challenge and no one has yet been able to prevent a recurrence of SCLC (3).

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Access this article online

Website: http://rps.mui.ac.ir

DOI: 10.4103/1735-5362.350243

Considering the disadvantages of conventional therapies, complementary therapies such as molecular targeting therapy, anti-angiogenesis therapy, immunotherapy, apoptosis regulation, signal transduction therapy, and nucleic acid-based therapy have attracted the interest of researchers (4,5).

Immunotoxin therapy is an anticancer drug that kills cancer cells. Immunotoxins are therapeutic agents that contain a targeting segment present on cancer cells and a cytotoxic agent like bacterial, fungal, and plant toxins or cytotoxic enzymes (6). The most important feature of an immunotoxin is that it can specifically kill cancer cells while normal cells remain intact. Immunotoxins can bind to the cancerous cells, internalized, and kill them by the toxic active segment (7,8). Till now, the denileukin diftitox, tagraxofusp, and moxetumomab pasudotox have been approved by the US Food and Drug Administration (9).

To design an effective immunotoxin, instead of using the whole antibody or toxin, the smallest binding, and catalytic domains as much as possible should be used. In other words, the parts that do not play a role in binding and catalytic activity can be removed (10). In the design of an immunotoxin, the selection of specific biomarkers is a key factor to differentiate cancer cells from normal cells (11).

Delta-like ligand 3 (DLL3) is known as a new biomarker in SCLC, which is expressed 80% in SCLC while not seen in normal lung cells (12). DLL3 is an inhibitory ligand belonging to the notch signaling pathway. DLL3 inhibits the Notch signaling pathway in SCLC and other neuroendocrine tumors, which in turn inhibits apoptosis and promotes tumor growth, migration, and invasion (13).

After DLL3 binding to the Notch receptor, the Notch intracellular domain enters the nucleus and binds to the CSL complex (C promoter-binding factor 1/suppressor of hairless, and longevity-assurance gene-1), acts as the transcription factor for downstream genes including the hairy and enhancer of split (HES) family genes and the HES related with a YRPW motif (HEY) family genes. Notch signaling is inhibited by DLL3 at the cell surface, Golgi apparatus, and in the nucleus of tumor cells. Notch activates skip2, HES1, pre-Tα, cyclin D, and cyclin-dependent kinases to increase tumor cell proliferation and protect tumor cells from apoptosis (14-16).

Rovalpituzumab tesirine (Rova-T) is the first antibody-drug conjugate to be used specifically to bind to DLL3 in patients with SCLC and shows antitumor effects in recurrent SCLC and is well-tolerated (17). The bispecific T cell engager immuno-oncology therapy (BiTE) includes anti-DLL3 and CD3 antibodies. BiTE cause T cell activation, cytokine production, and shifts T cells to remove DLL3-expressing cells (18). AMG 119 (another example of targeting DLL3) contains a cytotoxic T cell that, by making genetic changes, causes the expression of a transmembrane chimeric antigen receptor on its surface. Subsequently, AMG 119 can bind to DLL3 and inhibit the growth of tumor cells (19).

In the cytotoxic part of immunotoxins, usually bacterial and plant toxins are used. Exotoxin A from *Pseudomonas aeruginosa* and *Diphtheria toxin* is the most common bacterial toxins in the structure of immunotoxins. These toxins can do the ADP-ribosylation of elongation factor-2 using NAD^+ and inhibit protein synthesis (20).

Among the toxins in the ADP-ribosyl transferase toxins family, typhoid toxin is known as a toxin that has two catalytic components. Typhoid toxin has an A2B5 novel structure, with two catalytic domains (A2) and pentamer domains (B5), which have the role of binding to the receptor. PltB is a pentameric binding domain that has hydrophobic binding to PltA with an ADP-ribosyl transferase activity. The second catalytic subunit of typhoid toxin is CdtB, which has nuclease activity and stops the cell cycle. (21-23). PltA catalyzes the ADP-ribosylation of α subunits of the heterotrimeric Gi/o protein family and inhibits the G proteins from connecting with their cognate G protein-coupled receptors. ADP-ribosylation of the α subunit of heterotrimeric Gi/o proteins (Gαi/o) stops the α subunits into an inactive state (GDP-bound form), subsequently the adenylate cyclase enzyme is not stopped. This modification of Gαi/o proteins causes the increase of cAMP and disrupts cellular signaling pathways (24,25).

Despite the promising results of immunotoxins in killing tumor cells, due to the immunogenicity of the toxic domain and the non-specific toxicity, that causes vascular leak syndrome, the use of immunotoxins has been challenged. Recently, in order to solve this problem, the use of an endogenous proteins of humans, like RNase and granzyme has been considered. There are 5 types of granzyme in humans, of which granzyme B is the most potent. The granzyme B is a serine protease (32 kDa) is produced by cytotoxic T lymphocytes and natural killer cells. Granzyme cleaves several substrates like procaspases, caspase, and Bid protein substrates, which leads to the induction of apoptosis (26). In this study, we designed two immunotoxins against DLL3. Bioinformatics analysis, expression, purification, and structural analysis will also be examined. The first immunotoxin contains a single-chain variable fragment rovalpituzumab antibody (scFv-Rova) that binds to the granzyme B which is named Rova-GrB. The second immunotoxin including the scFv-Rova that connected to the PltA of typhoid toxin which is called Rova-Typh. The aim of the research was to design and produce two immunotoxins, one based on bacterial toxins and the other based on human enzymes. After evaluation of immunotoxins in terms of Physico-chemical properties, stability of mRNA structure, the secondary and tertiary structure, solubility rate, the number of disordered regions, the interaction between ligand and receptor, the convenience of expression and purification of immunotoxins, one of these two immunotoxins was selected to continue cytotoxicity assay and apoptosis.

MATERIALS AND METHODS

Bioinformatics structural analysis

Immunotoxins design

Each immunotoxin consists of three parts, binding, translocation, and a catalytic domain. In the binding domain, the amino acid sequence of an scFv of rovalpituzumab antibody was extracted from the US 9770518B1 patent (27). The $(G_4S)_3$ linker is placed between the light and heavy chains of the antibody. Also, the ASGGPE linker is located between the binding and the translocation domains. The translocation domain contains the amino acids 278 to 429 of exotoxin A *Pseudomonas aeruginosa* P11439 UniProt accession number (minus amino acids 390-405 of domain Ib). The catalytic domain of the first immunotoxin is human granzyme B P10144 UniProt accession number and the second immunotoxin is a PltA subunit of typhoid toxin A0A286LNU8 UniProt accession number. To purify the immunotoxins, His-taq sequences were used at the 5′ end of structures. The stop codon sequence (*TAA*) was placed at the 3′ end of the structure after the His-taq sequences. The *BamH*I, *Eco*RI, *Hin*dIII, and *Xho*I were used based on the restriction enzymes map in the pET-28a expression vector (Thermo Scientific, USA). The construct of immunotoxins and restriction sites is shown in Fig. 1.

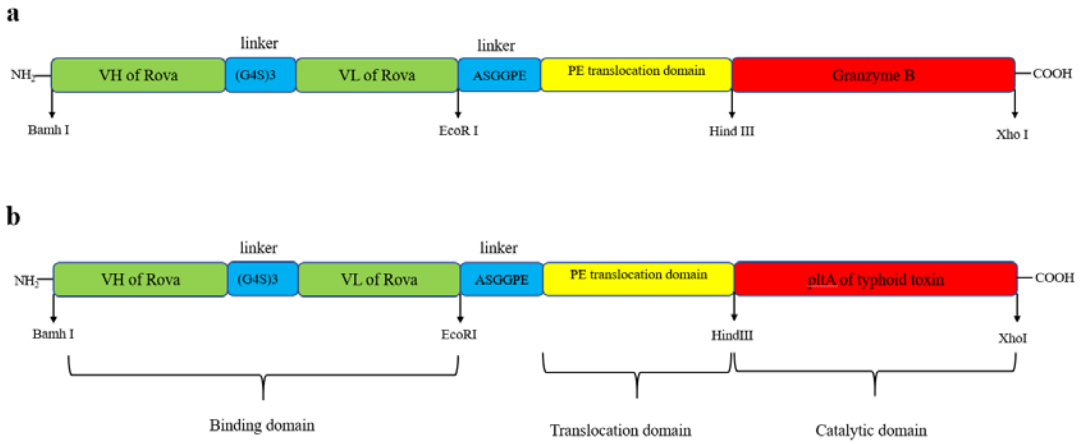


Fig. 1. Schematic representation of immunotoxins named (a) *Rova-GrB* and (b) *Rova-Typh*. Rova, Rovalpituzumab; Typh, typhoid; GrB, granzyme B. Rova, Rovalpituzumab; Typh, typhoid; GrB, granzyme B.

The codon usage of both immunotoxin genes was set according to the codon bias of *Escherichia coli*. The structure's properties such as rare codon analysis, codon used distribution, and GC content was confirmed by GenScript online server (https://www.genscript.com/tools). The immunotoxins construct was analyzed in terms of Physico-chemical properties by Expasy's ProtParam server (http://web.expasy.org/protparam/).

In the end, constructs were synthesized by ShineGene Molecular Biotech (Shanghai, China) into pET28a cloning vector. The parts of the two immunotoxins are listed in Fig. 1.

Secondary and tertiary structure analysis

The GOR IV server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_gor4.html) as an ideal online bioinformatics tool for the prediction of the secondary structure of immunotoxins was used. This server can predict thestructure and function of proteins by creating the best three-dimensional structures. The recombinant immunotoxins including rRova-GrB and rRova-Typh, to obtain the best tertiary structure, were submitted in the I-TASSER online server. To view the results of I-TASSER models, Accelrys Discovery Studio 1.7 software was applied.

Optimization and evaluation of protein structure

The tertiary structures of immunotoxins were refined by 3DRefine server (http://sysbio.rnet.missouri.edu/3Drefine/). Therefore, the immunotoxins PDB file of I-TASSER was uploaded to 3DRefine server for protein structure refinement. Validation analyses of refined immunotoxins were done by PROCHECK server (https://saves.mbi.ucla.edu/). In this sever, the best 3D structure was qualitatively evaluated by Verify3D (https://saves.mbi.ucla.edu/) and ERRAT (https://saves.mbi.ucla.edu/) online servers. The PROCHECK was applied for assessing the stereochemical quality of a given protein structure. The Ramachandran plot data of immunotoxins were determined in the server (https://www.doe-mbi.ucla.edu/services/). In the end, the overall quality score of immunotoxins refined in the previous step was calculated by the ProSA-web server (https://prosa.services.came.sbg.ac.at/prosa.php).

Disordered parts prediction and solubility analysis of immunotoxins

DISOPRED3.server.(http://bioinf.cs.ucl.ac.uk/psipred/?disopred=1) was applied to investigate an intrinsically disordered region by computational methods. Also, protein solubility of recombinant immunotoxins was analyzed by SOLpro (http://scratch.proteomics.ics.uci.edu/) and also Protein-Sol web server (http://protein-sol.manchester.ac.uk).

Docking of immunotoxins against receptor

The Docking of rRova-GrB and rRova-Typh and also scFv of Rovalpituzumab with DLL3 receptor (isoform 1,2) was performed by ZDock online server (https://zdock.umassmed.edu/). The best model of rRova-GrB, rRova-Typh, and DLL3 in PDB file, which was obtained from I-tasser, were submitted to ZDock online server for receptor-ligand binding affinity assessment.

Immunoinformatics analysis

Antigenicity and allergenicity of immunotoxins

Antigenicity of rRova-GrB and rRova-Typh were performed by the VaxiJen V.2 server (http://www.ddg-pharmfac.net/vaxijen), also the allergenicity of immunotoxins was analyzed by Algpred server at (https://webs.iiitd.edu.in/raghava/algpred/submission.html) and AllerTOP v.2.0 server at (http://www.ddg-pharmfac.net/AllerTOP/).

Immune response prediction

C-ImmSim online server at (https://150.146.2.1/C-IMMSIM/index.php) was used to evaluate the immune system response against immunotoxins.

B and T cell epitopes prediction

Continuous and discontinuous B cell epitopes were determined by ABCpred (https://webs.iiitd.edu.in/raghava/abcpred/ABC_submission.html), DiscoTope 2.0 server (https://services.healthtech.dtu.dk/service.php?DiscoTope-2.0), and ElliPro online server (http://tools.iedb.org/ellipro/).

Experimental studies

Confirmation of the recombinant genes in the pET28a vector

Rova-GrB and Rova-Typh constructs in pET28a vector were synthesized by a ShineGene Molecular Biotech Company, China. The immunotoxin constructs were transferred to *E. coli* BL21DE3 competent cell by calcium chloride (20 mM) heat-shock transformation method. Enzymatic digestion reaction (BamH1 #ER0051 and Xho1 #ER0691, Thermo Fisher Scientific, USA) and polymerase chain reaction (PCR) were applied to confirm the genes.

Expression and purification of the recombinant protein

A single colony of bacteria containing recombinant plasmid was inoculated in 5 mL of Luria-Bertani liquid medium (LB) containing 50 µg/mL kanamycin overnight. Then, 1 mL of overnight culture was cultured into 50 mL LB media containing 50 µg/mL kanamycin at 37 °C with a constant agitation of 120 rpm. When an optical density of samples at 600 nm was equal to 0.7, the expression of the protein was started by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated at 37 °C for 2, 4, 6, and 8 h and overnight. Then, the cells were harvested using centrifugation at 4000 rpm for 10 min. The cells were suspended in the native buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH: 8.0) and then sonication was done (75 W for 20 s). After centrifugation at 12,000 rpm, 4 °C for 10 min, the supernatant was stored in order to analyze the presence of recombinant protein in the soluble phase. The cell sediment of the soluble phase was dissolved in denatured buffer (8 M urea, 100 mM NaH2PO4, 100 mM tris, pH 8.0). To determine whether the rRova-GrB and rRova-Typh are in the soluble or insoluble phase, the samples of both phases were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at a constant voltage of 100 V for 1 h. The electrophoresis buffer including tris, glycine, and SDS was prepared at pH 8.3. The recombinant proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) affinity column under native and denature protocols (Ni-NTA Spin kit, Qiagen, Iran; Cat. No. 31314). At last, the purified proteins were examined by 12% SDS-PAGE. The Bradford method was used to determine the concentration of immunotoxins.

Confirmation of recombinants immunotoxins by western blot method

Purified recombinant immunotoxins were loaded on SDS-PAGE, and the contents of SDS gel were transferred to poly(vinylidene fluoride) (PVDF) membrane by transfer buffer (39 mM glycine, 48 mM tris base, 0.037% SDS, and 20% methanol) at a constant voltage of 100 V for 1 h and then, the membrane was blocked with 5% skim milk at 37 °C, 120 rpm, for 2 h. The PVDF membrane was washed twice using a wash buffer TBST (tris-buffered saline + 0.05% Tween® 20). The horseradish peroxidase (HRP) conjugated mouse anti-His tag antibody (Sigma, USA) at 1:2000 dilutions in TBST buffer was poured on PVDF membrane and incubated for 2 h at 37 °C on a rotary shaker (120 rpm). Ultimately, 3, 3′-diaminobenzidine (DAB) solution substance was added as an enzyme-substrate to observe antibody binding.

Determination of secondary structure by circular dichroism method

The ultraviolet circular dichroism (CD) is a useful tool for determining the secondary structure and folding properties of proteins produced by the recombinant method (28). CD method was used to analyze the amount of alpha-helix, beta-sheet, beta-turn, and other conformations in unknown proteins. The purified immunotoxins (0.2 mg/mL) were evaluated by a CD spectrum (J-810-150S, Japan) at 180 to 240 nm at the biochemistry laboratory of Tarbiat Modares University and its data was saved. The standard protein and solvent used in the CD assay are bovine serum albumin and phosphate-buffered saline (PBS), respectively.

RESULTS

Bioinformatics structural analysis

Secondary structure prediction

The comparison between the prediction of the secondary structure of the scFv rovalpituzumab, the PltA of typhoid toxin, and granzyme B in the structure of immunotoxins

and single-mode are mentioned in Table 1. The details of the prediction of secondary structures of immunotoxins and comparing them with the results of the CD method are listed in Table 2. Also, the secondary structure of both linkers ($(G_4S)_3$ and ASGGPE) in immunotoxins was the random coil.

Tertiary structure prediction

Both immunotoxins were analyzed in the I-Tasser server and the results are listed in Table 3 and Fig. 2a and b. C-score or confidence score estimates the predicted structure's quality which is typically in the range of -5 and 2. Template modeling (TM)-score shows a topological similarity of protein structures which is rated from 0 to 1. TM-score > 0.5 shows that frequently, the query and template protein is in the same fold and if the TM-score was less than 0.5, it means that proteins are not in the same fold. A common scale for comparing protein structures is to measure a root-mean-square deviation (RMSD). RMSD is obtained by calculating the distance between pairs of atoms after being at the best position. The ideal RMSD is as small as that for closely homologous proteins (< 3 Å) (29). C and TM scores of the immunotoxins were been in the standard range.

Table 1. Comparison of secondary structures of immunotoxins and their components in a single state and in case of binding to other components.

	scFv in rRova-GrB and rRova-Typh (%)	scFv of Rova (%)	Granzyme B in rRova-GrB (%)	Granzyme B (%)	pltA in rRova-typh (%)	pltA of typhoid toxin (%)
Alpha helix	8.57	5.31	10.97	10.97	23.93	23.93
Extended strand	36.33	39.18	27.0	25.74	19.23	17.95
Random coil	55.10	55.51	62.02	63.29	56.84	58.12

scFv, Single-chain variable fragment; rRova, recombinant rovalpituzumab; Typh, typhoid; GrB, granzyme B.

Table 2. The comparing results of secondary structures of immunotoxins by bioinformatics and CD method.

Protein name	Number of amino acids	Method of analysis	Alpha helix (%)	Extended strand (Beta sheet) (%)	Random coil (%)
rRova-GrB	624	Predicted	17.95	25.64	56.41
		CD	49.2	-	50.8
rRova-Typh	621	Predicted	22.87	23.03	54.11
		CD	54.8	-	45.2

CD, Circular dichroism; rRova, recombinant rovalpituzumab; Typh, typhoid; GrB, granzyme B.

Table 3. Result of I-TASSER analysis of immunotoxins.

Protein name	Length	C-score	Template modeling score	RMSD (Å)
rRova-GrB	624	-2.50	0.42 ± 0.14	14.0 ± 3.9
rRova-Typh	621	-4.20	0.27 ± 0.08	18.8 ± 2.1

rRova, Recombinant rovalpituzumab; Typh, typhoid; GrB, granzyme B; RMSD, root-mean-square deviation.

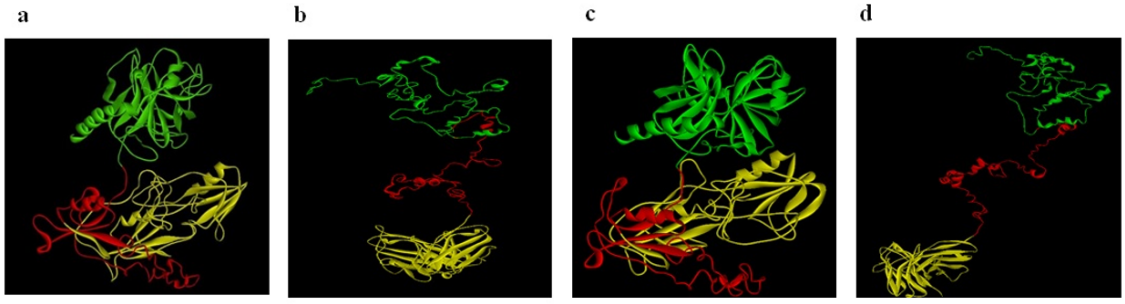


Fig. 2. Predicted initial and refined models of the tertiary structure of immunotoxins. The initial model of (a) rRova-GrB and (b) rRova-Typh by I-TASSER server and refined model of the tertiary structure of (c) rRova-GrB (d) and rRova-Typh were shown. In all figures, the scFv of rovalpituzumab is yellow, the exotoxin A *Pseudomonas aeruginosa* as translocation domain is red, and the GrB and *pltA* of typhoid toxin are green. rRova, Recombinant rovalpituzumab; Typh, typhoid; GrB, granzyme B; scFv, Single-chain variable fragment.

Refinement and validation of tertiary structure

To get the best refinement of the protein structure, 3Drefine web server optimizes a network of hydrogen bonds in the protein structure and combines it with the model with the lowest level of atomic energy. The refinement result is reported as 3Drefine score. 3Drefine score is the potential energy of the refined model based on 3Drefine force field. A lower score usually shows a better quality structure (30). 3Drefine scores of rRova-GrB and rRova-Typh were 43997 and 45623, respectively. The tertiary structure of rRova-GrB and rRova-Typh after refinement is shown in Fig. 2c and d.

After refinement of the immunotoxins, validation was performed by online programs such as ERAT, Verify3D, Procheck, and proSA-web servers. All data before refinement and after refinement are listed in Table 4.

ERRAT server can determine the correct and incorrect parts of the protein structure according to characteristic atomic interaction. The overall quality factor is the percentage of the protein that their protein structure is acceptable. ERRAT analysis of immunotoxins after refinement showed that the higher percentage of amino acids had the correct structure (Fig. 3a and b).

Verify3D server determines what percentage of amino acid sequences are folded into a known 3D structure. In the acceptance state, at least 80% of the amino acids must be scored ≥ 0.2 in the 3D/1D profile. Ninety-one percent of residues of rRova-GrB had averaged 3D/1D score ≥ 0.2 (Fig. 3c and d).

PROCHECK program calculates stereochemical properties such as phi and psi angles in helixes, disulfide bridges, C_α-N-C-C_β, and etc of all amino acids. In the following, the results are compared with the well-known protein structures in the Protein Data Bank. According to the study of 118 structures of resolution of at least 2.0 Å, a proper quality structure of the protein in the Ramachandran plot must have over 90% of amino acids in the most favored regions. As shown in Table 4 and Fig.3e and f the details of amino acids dispersion in the Ramachandran plot were shown. In overall, more than 88% of the amino acids in the structure of immunotoxins are located in the most favoured and additional allowed region.

The ProSA-web server checks for errors in the protein structure by comparing the overall quality score of a recombinant protein with native proteins. The result of the comparison is a Z-score that is shown graphically. In this plot, the structure of native proteins obtained through nuclear magnetic resonance (NMR) and X-ray method is blue and the desired protein is shown in black point. In general, the validation of results showed that structural analysis had improved after refinement. The result of immunotoxins validation by the ERRAT, Verify3D, PROCHECK, and proSA-web programs are shown in Fig. 3g and h.

Table 4. The result of validation analysis of immunotoxins.

	rRova-GrB		**rRova-Typh**	
	Before refinement	**After refinement**	**Before refinement**	**After refinement**
ERAT online program Overall quality factor	67.82	78.57	58.59	79.11
Verify 3D online programs	86.86	91.03	56.36	61.03
Ramachandran plot				
Residues in most favoured regions	293 (56.2%)	335 (64.3%)	266 (50.6%)	340 (64.6%)
Residues in additional allowed	164 (31.5%)	133 (25.5%)	187 (35.6%)	124 (23.6%)
Residues in generously allowed	42 (8.1%)	27 (5.2%)	44 (8.4%)	38 (7.2%)
Residues in disallowed regions	22 (4.2%)	26 (5.0%)	29 (5.5%)	24 (4.6%)
z-score	-4.94	-6.51	-2.05	-4.27

rRova, Recombinant rovalpituzumab; Typh, typhoid; GrB, granzyme B.

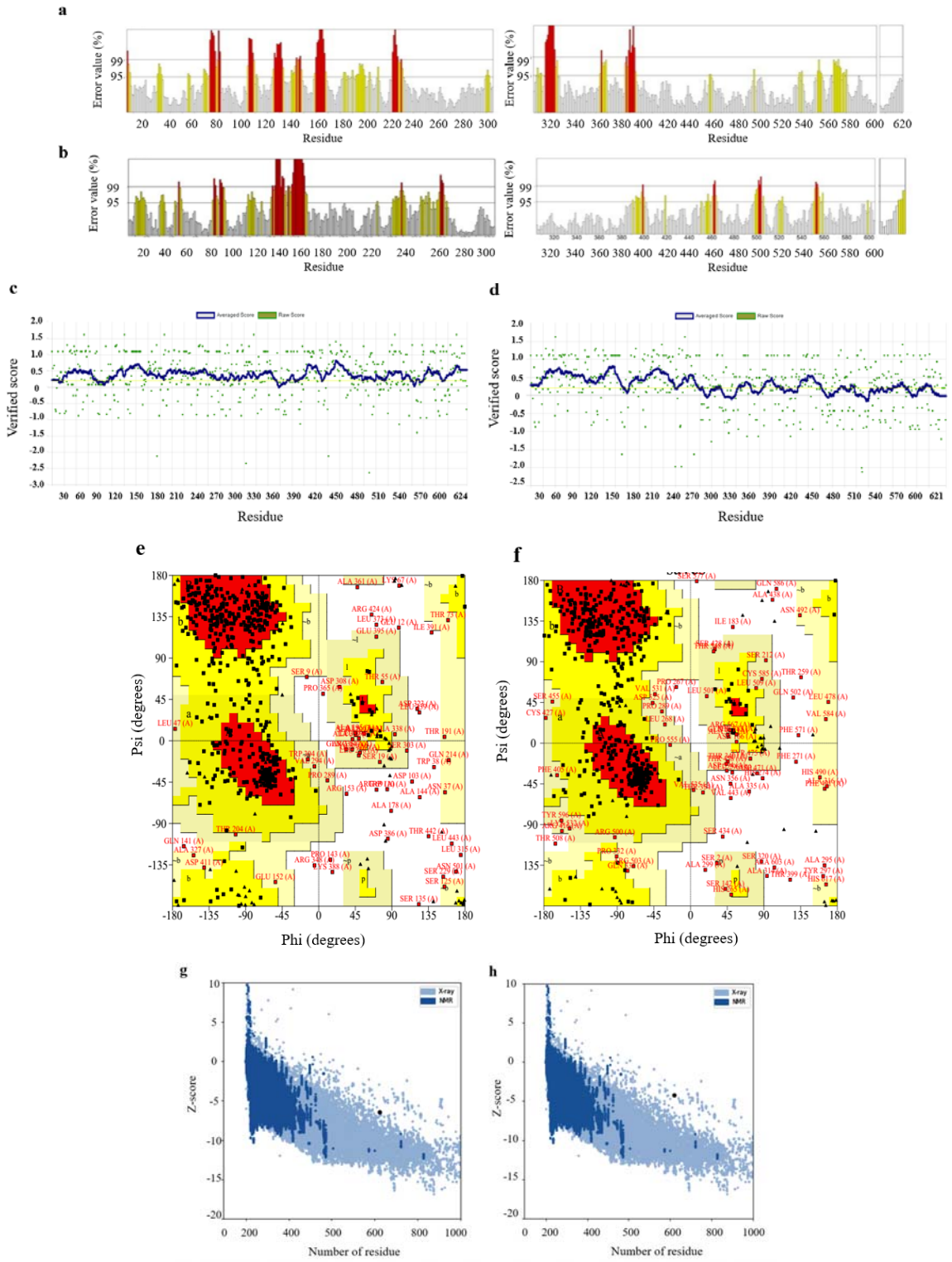


Fig. 3. The result of immunotoxins validation by the ERRAT, Verify 3D, PROCHECK, and proSA-web program. The validation of 3D model of (a) rRova-GrB and (b) rRova-Typh by ERRAT program were shown; the 3D profile of the protein structure of (c) rRova-GrB and (d) rRova-Typh by Verify3D program; the Ramachandran plots of (e) rRova-GrB and (f) rRova-Typh show more than 80% of residues are in the desired region; the z-score of the 3D structure of (g) rRova-GrB and (h) rRova-Typh. rRova, Recombinant rovalpituzumab; Typh, typhoid; GrB, granzyme B.

Determining of intrinsic protein disorder

DISOPRED3 server shows the intrinsic protein disorder regions. The 3D structure of the intrinsic protein disorder regions is not stable under physiological conditions. Also, DISOPRED3 neural networks can predict long disorder regions. The server compares the structure of each window of seven residues in the desired protein with the amino acids profile in the data bank to see if it is an order or disorder (31-32).

The number of the intrinsic protein disorder in the rRova-GrB and rRova-Typh by PSIPRED online server were 47 and 62 amino acids, as disorder regions, respectively. As shown in Fig. 4a and b, the distribution of intrinsically disordered regions is mainly located between the amino acids 120 to 140, 355 to 380, and the carboxyl-terminus of the immunotoxins.

Prediction of solubility

SOLpro web server examines the possibility of a protein to be soluble or insoluble on overexpression in *E. coli* from the primary sequence. In fact, SOLpro can determine whether the protein is solvent or not. The solubility index of rRova-GrB (soluble with probability 0.76) and rRova-Typh (insoluble with probability 0.68) were determined by SOLpro web server.

The use of recombinant proteins in industry and therapy has made it very important to predict protein solubility. The server examines protein for amino acid count, the presence of aromatic side chains, number of negatively charged amino acids, lysine to arginine ratio, number of β-strands, the possibility of producing inclusion body, and then predicts protein solubility. QuerySol is defined as the predicted solubility value obtained based on the average of the laboratory results and is equal to 0.45. If the QuerySol of the target protein is higher than 0.45, it means that the recombinant protein has more solubility than *E. coli* proteins (33). The QuerySol of rRova-GrB and rRova-Typh were 0.229 and 0.182, respectively, therefore the immunotoxins are considered insoluble protein (Fig. 4c).

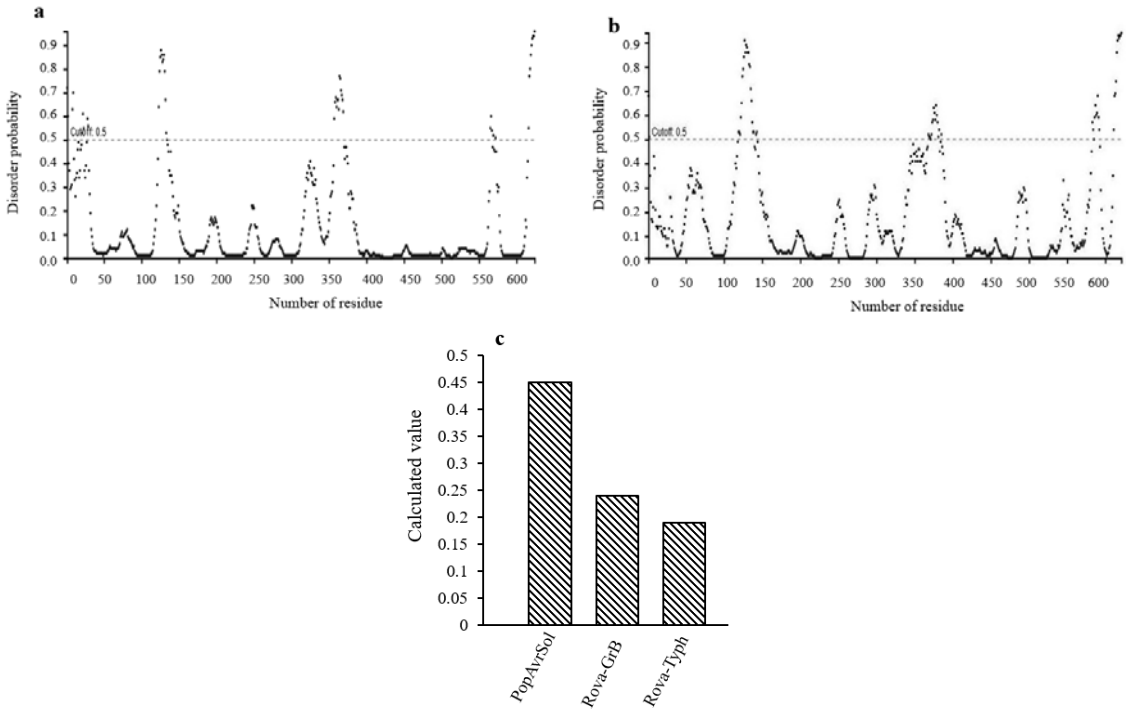


Fig. 4. Prediction of intrinsic disorder profile and solubility of immunotoxins. The result of intrinsic disorder regions of (a) rRova-GrB and (b) rRova-Typh by DISOPRED3 server was shown. (c) The prediction of solubility of rRova-GrB and rRova-Typh by Protein-Sol web server shows that immunotoxins were unsoluble. PopAvrSol, Population average solubility of *E. coli* proteins; rRova, recombinant rovalpituzumab; Typh, typhoid; GrB, granzyme B.

Docking results

The binding of immunotoxins to DLL3 was predicted by the Z-Dock online server. As shown in Fig. 5, the rRova-GrB and rRova-Typh from the scFv of rovalpituzumab region are connected to N-terminal domain of DLL3. The binding of scFv of rovalpituzumab to DLL3 by the Z-Dock online server showed that scFv of rovalpituzumab binds to the EGF-like domain of DLL3.

Immunoinformatics results

Antigenicity and allergenicity

Evaluation of the allergenicity of immunotoxins by Algpred and AllerTOP v.2.0 server showed that they do not have the proven IgE epitopes in their structures, so it seems they are not allergens. The VaxiJen V.2 server that acts based on the alignment approach for antigen prediction, has 70 to 89% accuracy. This server also showed that the Rova-GrB probably is not an antigen while the Rova-Typh has some antigenic regions.

Immune stimulation

The C-ImmSim program is based on machine learning techniques and a mesoscopic scale simulator of the immune system to predict the interaction between pathogens and immune system components. The first response of the immune system to both immunotoxins is IgM. The highest number of IgM and IgG is produced 13 days after the injection of immunotoxins. IgG antibody responses are lower than IgM antibody responses in both immunotoxins (Fig. 6a and, b). The Rova-Tyh seems to produce less antibody response. The intensive decrease in the plasma cell population is observed from day 10 onwards (Fig. 6c and d) and also, T regulatory cells have been significantly activated against immunotoxins (Fig. 6e and f). Among the cytokines and interleukins, interferon-gamma and interleukin-2 had the highest production level against both immunotoxins (Fig. 6g and h).

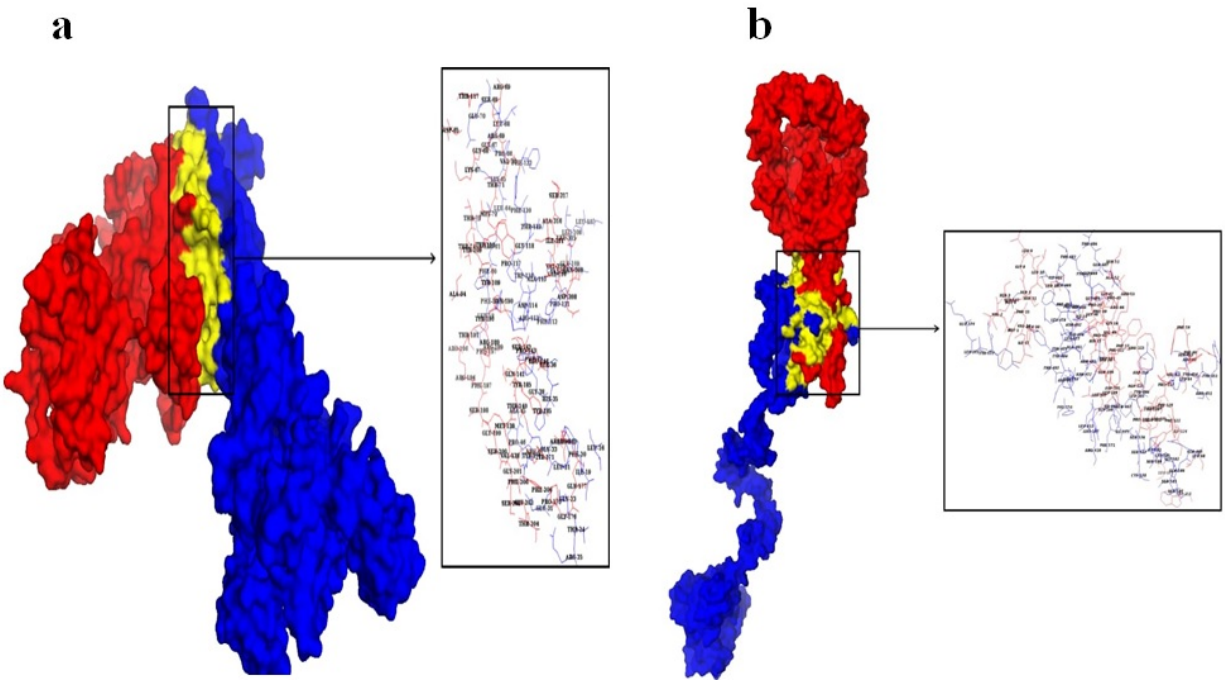
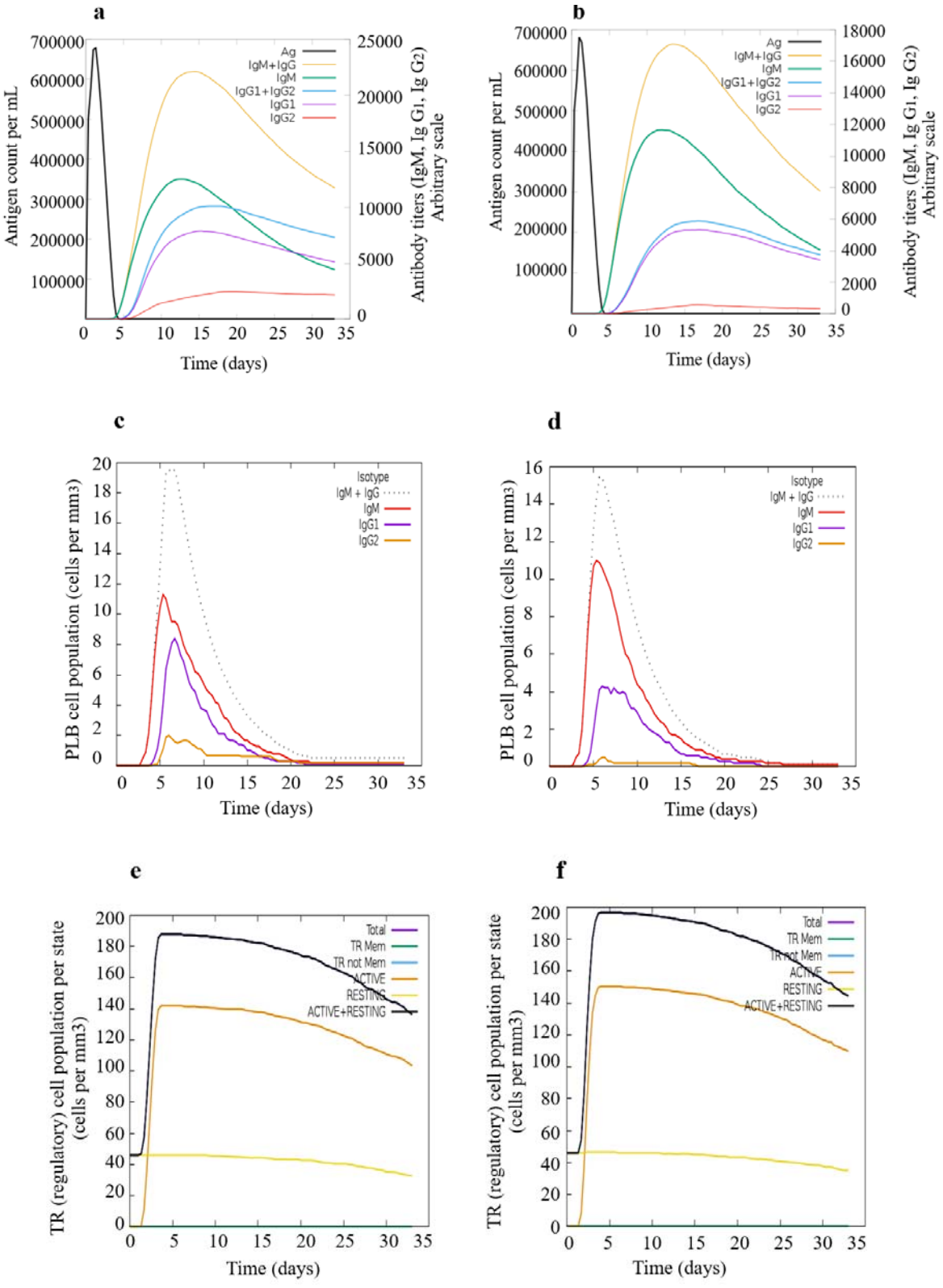


Fig. 5. Molecular docking of DLL3 against immunotoxins. The binding prediction between DLL3 as a receptor and (a) rRova-GrB and (b) rRova-Typhas ligands. In all diagrams, DLL3 is red, rRova-GrB and rRova-Typh are blue, and the interface residues are yellow. DLL3, Delta-like ligand 3; rRova, recombinant rovalpituzumab; Typh, typhoid; GrB, granzyme B.


a
Antigen count per mL
Antibody titers (IgM, Ig G1, Ig G2)
Arbitrary scale
Time (days)
Ag
IgM+IgG
IgM
IgG1+IgG2
IgG1
IgG2
b
Antigen count per mL
Antibody titers (IgM, Ig G1, Ig G2)
Arbitrary scale
Time (days)
Ag
IgM+IgG
IgM
IgG1+IgG2
IgG1
IgG2
c
PLB cell population (cells per mm3)
Time (days)
Isotype
IgM + IgG
IgM
IgG1
IgG2
d
PLB cell population (cells per mm3)
Time (days)
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IgM
IgG1
IgG2
e
TR (regulatory) cell population per state (cells per mm3)
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Total
TR Mem
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ACTIVE
RESTING
ACTIVE+RESTING
f
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ACTIVE+RESTING

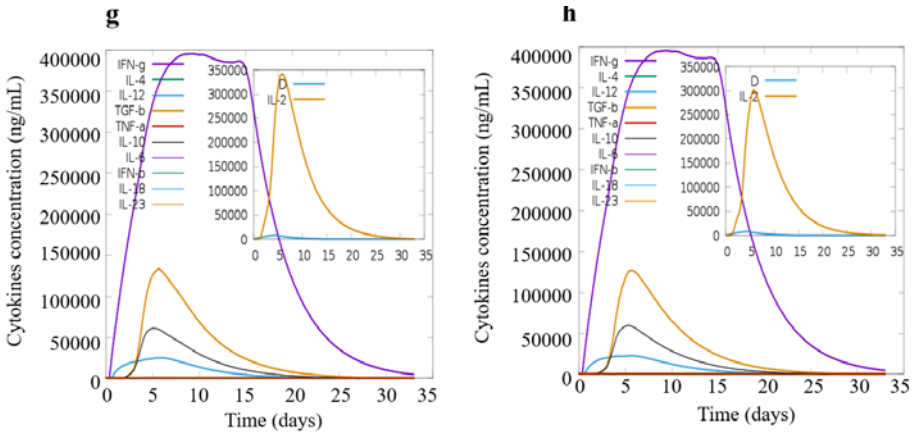

Fig. 6. Results of predicting immune system responses by the C-ImmSim server. The IgM and IgG antibody responses of (a) Rova-GrB and (b) Rova-Typh; the plasma cells population of (c) Rova-GrB and (d) Rova-Typh; the prediction of T regulatory cell amounts of (e) Rova-GrB and (f) Rova-Typh; the increased rate of cytokines and interleukins after injections of (g) Rova-GrB and (h) Rova-Typh. Rova, Rovalpituzumab; Typh, typhoid; GrB, granzyme B.

Table 5. Continuous and discontinuous B cell epitopes of immunotoxins.

	Server	**Rova-GrB**			**Rova-Typh**		
		Sequence	**Start position**	**Score**	**Sequence**	**Start position**	**Score**
Linear epitopes	ABCpred	DDFKGRVTMTTDTSTSTAYM	64	0.93	DDFKGRVTMTTDTSTSTAYM	64	0.93
		GDPEIKKTSFKGDSGGPLVC	559	0.93			
	ElliPro	CHLPLETFTRHRQPRGW	264	0.90	RYIATTSSVNQTYAIARAYYSRSTFKGNLYRYQI	435	0.75
Discontinuous epitopes	DiscoTope 2.0	A:S82, A:D117, A:T119, A:K120, A:P122, A:P123, A:R124, A:P158, A:R170, A:P172, A:Q204, A:Y205, A:418E, A:503E, A:504D, A:506A, A:507R, A:510A.			A: G 1, A: S2, A: P16, A: G17, A: N33, A: Y56, A: T57, A: E59, A: D64, A: S120, A: G121, A: S135, A: I137, A: R273, A: H274, A: Q276, A: P277, A: R278, A: E281, A: Q282, A: Q285, A: A316, A: S317, A: P318, A: D357, A: P378, A: T399, A: P400, A: P401, A: A452, A:Y453, A: S455, A: R456, A: K460, A: G461, A: N462, A: L463, A: R465, A: N473, A: S476, A: P479, A: S480, A: I481, A: T482, A: Y483, A: E485, A: T486, A: Q487, A: G488, A: N550, A: P551, A: P555 TO A: Q565, A: S599, A: Y601 TO A: E621.		
	ElliPro	A:V7, A:Q8, A:S9, A:G10, A:A11, A:E12, A:V13, A:K14, A:K15, A:P16, A:G17, A:A18, A:S19, A:V20, A:K21, A:V22.			A: F421 to A: R465. A: N473, A: Y475 to A: L522. A: Y524, A: D525, A: T528. A: G534 to A: S541. A: L543 to A: E621.		

Rova, Rovalpituzumab; Typh, typhoid; GrB, granzyme B.

B cell epitopes

The ABCpred server indicated that the Rova-GrB has two main B cell epitopes, one in the binding region and the other in the catalytic region of the immunotoxin. The important epitope of the Rova-Typh is in the binding region. The DiscoTope 2.0 server showed that the Rova-GrB and Rova-Typh contain 18 and 9 amino acids, respectively, that are involved in creating the discontinuous B cell epitopes. The presence of a continuous B cell epitope in the translocation domain of Rova-GrB and a continuous B cell epitope in the catalytic domain of the Rova-Typh was determined by the ElliPro server. The B cell epitopes of immunotoxins are shown in Table 5.

Experimental results

Confirmation of Rova-GrB and Rova-Typh genes in pET28a

The constructs genes containing *rova-grb* and *rova-typh* were transferred to the *E. coli* Bl21DE3 and analyzed by PCR and restriction enzymatic digestion (Fig. 7a and b). The Results showed that the *Rova-GrB* gene (1875 bp), *Rova-Typh* gene (1866 bp) had expected bands on the 1% agarose gel.

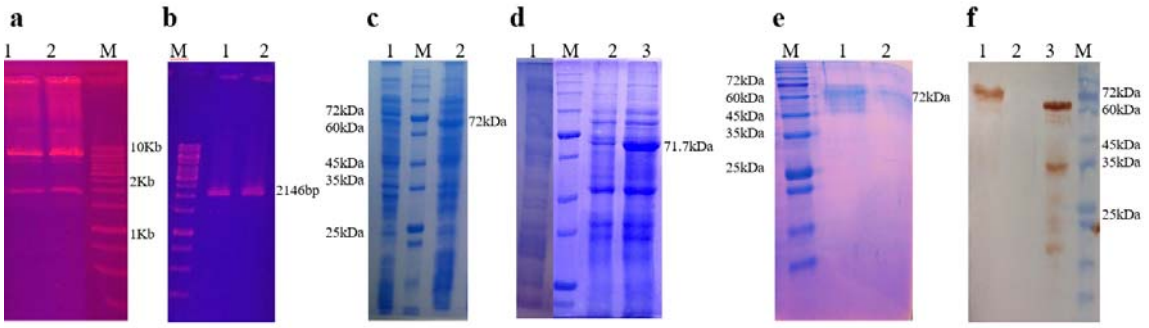

Fig. 7. The Result of enzymatic digestion, PCR, expression, purification and western blot analysis of the rRova-GrB and rRova-Typh. (a) Restriction enzyme analysis: (1) *Rova-GrB* and (2) *rova-typh*; (b) the PCR results: (1) *Rova-Typh*, (2) *Rova-GrB*, (M): 1 Kb DNA ladder, SM0313, Thermo Fisher Scientific); (c) expression of rRova-GrB: (1) before induction by IPTG, (2) induced expression during 16 h; (d) expression of rRova-Typh: (1) *E. coli* BL21 without pET28a and (2) before induction by IPTG, (3) induced expression during 16 h; (e) protein purification of immunotoxins: (1) unpurified protein before column, (2) rRova-GrB purified, (3) rRova-Typh purified; (f) western blotting analysis for rRova-GrB and rRova-Typh: (1) cell extract from 16 h expression of rRova-GrB, (2) non-induced cell, (3) cell extract from 16 h expression of rRova-Typh, (M) protein marker, PM1700, SMOBIO Technology). PCR, Polymerase chain reaction; IPTG, isopropyl β-D-1-thiogalactopyranoside; rRova, recombinant rovalpituzumab; Typh, typhoid; GrB, granzyme B.

Expression, purification and western blot analysis of rRova-GrB and rRova-Typh

The maximum expressions of recombinant immunotoxins were induced by the 1 mM IPTG and 16 h incubated time. The results of the evaluation of protein expression in the native and denature method showed that the protein expression was observed in the insoluble phase. The desired proteins were evaluated on the 12% SDS-PAGE gel (Fig. 7c and d). The rRova-GrB and rRova-Typh were purified based on denaturing method by Ni-NTA column (Fig. 7e). The purified immunotoxins were transferred to the dialysis sac with a 14 kDa cutoff (Sigma Aldrich, USA). The dialysis process against PBS 1X buffer was done overnight by changing the buffer four times. Using the Bradford method, the concentration of rRova-GrB and rRova-Typh were 130 µg/mL and 80 µg/mL, respectively. All purified proteins were stored at -20 °C. To confirm the rRova-GrB and rRova-Typh, western blotting was done by mouse anti-his tag antibody HRP conjugated (Fig. 7f).

CD Spectroscopy

To determine the secondary structure of the immunotoxins, CD analysis was done on the purified protein by Circular Dichroism spectrophotometers. Table 3 shows the comparison of the percentage of the secondary structure of immunotoxins *via* bioinformatics predictions and CD spectrophotometers. The data obtained from the CD spectrophotometers were not compatible with the bioinformatics predictions.

DISCUSSION

For the past three decades, the first line of treatment for SCLC has been the chemotherapy drug-like "etoposide-platinum". Unique features of SCLC including fast growth, primary metastasis, obtained therapeutic resistance, and mutation in the tumor suppressor genes cause failed drug development. In recent years, immunotherapy approaches have raised hopes in clinics (234). Selecting a specific biomarker that has a high expression in cancer cells while its expression is low in normal cells is a fundamental phase in the design of immunotoxins. The overexpression of DLL3 in SCLC versus normal organs, including the lung was identified in 2015. Among the biomarkers of SCLC, DLL3 has shown promising effects. Rova-T is an antibody-drug conjugate that targets DLL3 in preclinical and clinical studies. Due to its side effects, using the Rova-T drug has been stopped; however, DLL3 is an ideal

target for drug development against SCLC. In such a way, anti-DLL3/anti-CD3 bispecific T-cell engager is being evaluated (2,15).

So far, no immunotoxin that targets DLL3 has been designed, and in the present study, the new immunotoxins based on the recombinant humanized scFv of rovalpituzumab antibody against DLL3 were made.

The issue of immunogenicity of immunotoxin components is a limiting factor in its use in the clinic (35). The scFv of rovalpituzumab which has the ability of binding to the DLL3, is humanized antibody. Also, in the catalytic domain of immunotoxins, in addition to using bacterial toxins such as pltA of typhoid toxin, a strong human enzyme such as GrB has been used. Based on the experience of our previous studies, how determining the position of the binding and catalytic domains in the immunotoxin structure (N-terminal or C-terminal) is important and required bioinformatics studies. Bioinformatics analysis is very helpful in obtaining an ideal immunotoxin construct and reducing costs. Rare codon analysis results in the GenScript online program showed the Codon Adaptation Index (CAI), GC content, and Codon Frequency Distribution (CFD) parameters are in the ideal range, so the immunotoxin genes will be expressed strongly. The results of the GenScript were similar to the results of previous work (36-40). The hydrophobicity index of rRova-GrB and rRova-Typh based on ProtParam server showed that the recombinant immunotoxins had hydrophilic properties. The results of the analysis of secondary structures by GORIV server showed that about 55%, 25%, 20% of the secondary structure of immunotoxins were random coil, beta-sheet, and alpha-helix, respectively, while the results of experimental analysis by CD spectrophotometers showed that about half of the secondary structure of immunotoxins were random coil and the other half were alpha helix.

Also, the outcomes of the GORV server showed that the linkage of scFv rovalpituzumab to the translocation domain and catalytic domain (GrB and pltA of typhoid toxin) caused small changes of about 2 to 3% in the extended strand and random coil of the secondary structure, respectively. In general, a comparison of the secondary structure of the components of immunotoxins in the single and fusion protein states showed that the secondary structure of immunotoxins did not change significantly. Moreover, the results of the PredictProtein server showed that most amino acids of immunotoxins were not exposed, so it can be concluded that the immunotoxins expressed in *E. coli* are hydrophobic and insoluble. The results of the bioinformatics analysis were consistent with the results of the experimental analysis. In fact, all recombinant proteins of immunotoxins and their components were dissolved in 8 M urea and purified by Ni-NTA column using the denaturing method. The dialysis method against PBS to solubilize immunotoxins was used stepwise. Like all fusion proteins designed in previous studies, the C-score of immunotoxins and their components analyzed by the I-Tasser server is within the standard range (41-42).

The intrinsic protein disorder region in the rRova-GrB and rRova-Typh showed that most of the disorder regions of immunotoxins were related to the (G4S)3 linker, which is located between the light and heavy variable chains of rovalpituzumab antibody, and the six histidines (6x His tag) residues that are located in the carboxylic terminus of the immunotoxins. Validation analyses of rRova-GrB and rRova-Typh after refinement showed that their structure and folding have improved, which in turn indicated the correct performance of immunotoxins. There are few researches on the immunotoxins that target SCLC.

In the first immunotoxin, we have tried to use GrB, which is a human enzyme, to reduce the immunogenicity of Rova-GrB. Our expectation was that the Rova-GrB has less immunogenicity compared to the Rova-Typh. The results obtained from the VaxiJen V.2 confirmed the reduction of the antigenic property of Rova-GrB.

The main purpose of determining B cell epitopes is to determine the number and location of the B cell epitopes (43). If a severe immune response is observed in the practical phase, the immunogenicity of immunotoxins can be reduced by more detailed bioinformatics analyses and alterations in the B-cell epitope sequence. According to the immune system

responses to Rova-GrB and Rova-Typh, the use of immunotoxins in lung cancer cell line and tumor models and comparison of experimental results with bioinformatics results is required.

The first models of the immunotoxins against SCLC were developed from the chemical conjugation of antibodies to toxins. Wawrzynczak and co-workers developed the first immunotoxins against the SCLC cell line that include a mouse monoclonal antibody (SWA11 and SWA20) that attaches to ricin and abrin toxins. The immunotoxins in the animal model cause a delay in the growth of tumor cells (44-45). Concurrent use of three immunotoxins, MOC-31-PE + NrLulO-PE + MLuCl-PE, in SCLC cell line has been tested. This method removes SCLC cells quickly and effectively from bone marrow after high-dose chemotherapy (46). In 2002, the N901-blocked ricin immunotoxin was tested on nine patients and the results were unsatisfactory. They concluded that designing newer constructs with less immunogenicity could have favorable therapeutic effects (47).

In 2012, the combined use of HB21-PE40 as immunotoxins and ABT-263 (an anticancer drug, which is a Bcl-2 inhibitor) effectively killed the SCLC cells, while immunotoxin and drug alone did not have inhibitory effects (48). Recently, a cell membrane glycoprotein called glypican-3 (GPC3) has been identified that is involved in cell growth and proliferation. An immunotoxin including an anti-GPC3 antibody and *Pseudomonas aeruginosa* exotoxin A 38 (PE38) has cytotoxic effects on liver cancer and SCLC cell lines that have high expression of GPC3 (49).

CONCLUSION

The results showed that bioinformatics analysis is an essential step in designing an effective immunotoxin. Our bioinformatics studies showed that the designed immunotoxins had the right structure and can be produced in a prokaryotic host. The designed and expressed immunotoxins are expected to have beneficial therapeutic effects on SCLC, which will require further experimental analysis.

Acknowledgments

This paper was extracted from a Ph.D. thesis submitted by Mohammad Hossein Ataee, which was financially supported by the Applied Microbiology Research Center and Student Research Committee of Baqiyatallah University of Medical Sciences, Tehran, Iran (Grant No. 452/13971124) and Iran National Science Foundation (Grant No. 981002). The technical help of protein expression and purification by university laboratory staff is gratefully acknowledged.

Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by J. Amani, H. Mahmoodzadeh Hosseini, S.A. Mirhosseini, R. Mirnejad, and E. Rezaie. The first draft of the manuscript was written by M.H. Ataee and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394-424. DOI: 10.3322/caac.21492.
2. Taniguchi H, Sen T, Rudin CM. Targeted therapies and biomarkers in small cell lung cancer. Front Oncol. 2020;10:741-747. DOI: 10.3389/fonc.2020.00741.
3. Baize N, Monnet I, Greillier L, Quere G, Kerjouan M, Janicot H, *et al.* Second-line treatments of small-cell lung cancers. Expert Rev Anticancer Ther. 2017;17(11):1033-1043. DOI: 10.1080/14737140.2017.1372198.
4. Ke X, Shen L. Molecular targeted therapy of cancer: the progress and future prospect. Frontiers in Laboratory Medicine. 2017;1(2):69-75. DOI. org/10.1016/j.flm.2017.06.001.
5. Shoari A, Khodabakhsh F, Ahangari Cohan R, Salimian M, Karami E. Anti-angiogenic peptides application in cancer therapy; a review. Res Pharm Sci. 2021;16(6):559-574. DOI: 10.4103/1735-5362.327503

6. Jahanian-Najafabadi A, Bouzari S, Oloomi M, Habibi Roudkenar M, Shokrgozar MA. Assessment of selective toxicity of insect cell expressed recombinant A1-GMCSF protein toward GMCSF receptor bearing tumor cells. ResPharm Sci. 2012;7(3):133-140.
   PMID: 23181091.
7. Phillips GL, editor. Antibody-Drug Conjugates and Immunotoxins: from Pre-Clinical Development to Therapeutic Applications. New York: Springer Science & Business Media; 2013. pp.255-272.
   DOI:10.1007/978-1-4614-5456-4
8. Allahyari H, Heidari S, Ghamgosha M, Saffarian P, Amani J. Immunotoxin: a new tool for cancer therapy. Tumor Biol. 2017;39(2):1-11.
   DOI: 10.1177/1010428317692226.
9. Hamamichi S, Fukuhara T, Hattori N. Immunotoxin screening system: a rapid and direct approach to obtain functional antibodies with internalization capacities. Toxins (Basel). 2020;12(10):658-673.
   DOI: 10.3390/toxins12100658.
10. Onda M, Nagata S, FitzGerald DJ, Beers R, Fisher RJ, Vincent JJ, *et al*. Characterization of the B cell epitopes associated with a truncated form of *Pseudomonas exotoxin* (PE38) used to make immunotoxins for the treatment of cancer patients. J Immunol. 2006;177(12):8822-8834.
    DOI: 10.4049/jimmunol.177.12.8822.
11. Rezaie E, Amani J, Pour AB, Hosseini HM. A new scfv-based recombinant immunotoxin against EPHA2-overexpressing breast cancer cells; high *in vitro* anti-cancer potency. EurJ Pharmacol. 2020;870:1-10.
    DOI: 10.1016/j.ejphar.2020.172912.
12. Saunders LR, Bankovich AJ, Anderson WC, Aujay MA, Bheddah S, Black K, *et al*. DLL3-targeted antibody-drug conjugate eradicates high-grade pulmonary neuroendocrine tumor-initiating cells *in vivo*. Sci Transl Med. 2015;7(302):302ra136,1-28..
    DOI: 10.1126/scitranslmed.aac9459.
13. Furuta M, Kikuchi H, Shoji T, Takashima Y, Kikuchi E, Kikuchi J, *et al.* DLL3 regulates the migration and invasion of small cell lung cancer by modulating Snail. Cancer Sci. 2019;110(5):1599-1608.
    DOI: 10.1111/cas.13997.
14. Wu W-S, Hu C-T, editors. Signal Transduction in Cancer Metastasis. Vol 15. New York Springer Science & Business Media; 2010. pp. 157-174.
    DOI:10.1007/978-90-481-9522-0
15. Leonetti A, Facchinetti F, Minari R, Cortellini A, Rolfo CD, Giovannetti E, *et al.* Notch pathway in small-cell lung cancer: from preclinical evidence to therapeutic challenges. Cell Oncol(Dordr). 2019;42(3):261-273.
    DOI: 10.1007/s13402-019-00441-3.
16. Xiu MX, Liu YM, Kuang Bh. The role of DLLs in cancer: a novel therapeutic target. Onco Targets Ther. 2020;13:3881-3901.
    DOI: 10.2147/OTT.S244860.
17. Rudin CM, Pietanza MC, Bauer TM, Ready N, Morgensztern D, Glisson BS, *et al*. Rovalpituzumab tesirine, a DLL3-targeted antibody-drug conjugate, in recurrent small-cell lung cancer: a first-in-human, first-in-class, open-label, phase 1 study. The Lancet Oncol. 2017;18(1):42-51.
    DOI: 10.1016/S1470-2045(16)30565-4.
18. Owen DH, Giffin MJ, Bailis JM, Smit MAD, Carbone DP, He K. DLL3: an emerging target in small cell lung cancer. J Hematol Oncol. 2019;12(1):1-8.
    DOI: 10.1186/s13045-019-0745-2.
19. Byers LA, Chiappori A, Smit M-AD. Phase 1 study of AMG 119, a chimeric antigen receptor (CAR) T cell therapy targeting DLL3, in patients with relapsed/refractory small cell lung cancer (SCLC). Am J Clin Oncol.; 2019;37-15_suppl.
    DOI: 10.1200/JCO.2019.37.15_suppl.TPS8576.
20. Alouf J, Ladant D, Popoff MR. The comprehensive sourcebook of bacterial protein toxins. 4th ed. Elsevier; 2015. pp. 1151-1182.
    DOI: 10.1016/C2013-0-14258-4.
21. Simon NC, Aktories K, Barbieri JT. Novel bacterial ADP-ribosylating toxins: structure and function. Nat Rev Microbiol. 2014;12(9):599-611.
    DOI: 10.1038/nrmicro3310.
22. Chong A, Lee S, Yang Y-A, Song J. The role of Typhoid toxin in *Salmonella typhi* virulence. Yale J Biol Med. 2017;90(2):283-290.
    PMID: 28656014.
23. Lara-Tejero M, Galán JE. A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein. Science. 2000;290(5490):354-357.
    DOI: 10.1126/science.290.5490.354.
24. Mangmool S, Kurose H. Gi/o protein-dependent and-independent actions of Pertussis toxin (PTX). Toxins(Basel). 2011;3(7):884-899.
    DOI: 10.3390/toxins3070884.
25. Miller R, Wiedmann M. Dynamic duo-the Salmonella cytolethal distending toxin combines ADP-ribosyltransferase and nuclease activities in a novel form of the cytolethal distending toxin. Toxins(Basel). 2016;8(5):121-140.
    DOI: 10.3390/toxins8050121.
26. Mathew M, Verma RS. Humanized immunotoxins: a new generation of immunotoxins for targeted cancer therapy. Cancer Sci. 2009;100(8):1359-1365.
    DOI: 10.1111/j.1349-7006.2009.01192.x.
27 Stull RA, Saunders L, Dylla SJ, Foord O, Liu D, Torgov M, *et al*., inventors. Anti-DLL3 antibody drug conjugates. Patent No. US-9770518-B1. Available on: patents.google.com/patent/US9770518B1.
28. Greenfield NJ. Using circular dichroism spectra to estimate protein secondary structure. Nat Protoc. 2006;1(6):2876-2890.
    DOI: 10.1038/nprot.2006.202.
29. Xu J, Zhang Y. How significant is a protein structure similarity with TM-score = 0.5? Bioinformatics. 2010;26(7):889-895.
    DOI: 10.1093/bioinformatics/btq066.
30. Bhattacharya D, Nowotny J, Cao R, Cheng J. 3D refine: an interactive web server for efficient protein structure refinement. Nucleic Acids Res. 2016;44(W1):W406-W409.
    DOI: 10.1093/nar/gkw336.

31. Jones DT, Cozzetto D. DISOPRED3: precise disordered region predictions with annotated protein-binding activity. Bioinformatics. 2015;31(6): 857-863.
    DOI: 10.1093/bioinformatics/btu744.
32. Kosciolek T, Buchan DW, Jones DT. Predictions of backbone dynamics in intrinsically disordered proteins using *de novo* fragment-based protein structure predictions. Sci Rep. 2017;7(1):6999-7011.
    DOI: 10.1038/s41598-017-07156-1.
33. Hebditch M, Carballo-Amador MA, Charonis S, Curtis R, Warwicker J. Protein-Sol: a web tool for predicting protein solubility from sequence. Bioinformatics. 2017;33(19):3098-3100.
    DOI: 10.1093/bioinformatics/btx345.
34. Saltos A, Shafique M, Chiappori A. Update on the biology, management, and treatment of small cell lung cancer (SCLC). Front Oncol. 2020;10:1074-1087.
    DOI: 10.3389/fonc.2020.01074.
35. Li M, Liu ZS, Liu XL, Hui Q, Lu SY, Qu LL, *et al*. Clinical targeting recombinant immunotoxins for cancer therapy. Onco TargetsTher. 2017;10:3645-3665.
    Doi: 10.2147/OTT.S134584.
36. Gharbavi M, Danafar H, Amani J, Sharafi A. Immuno-informatics analysis and expression of a novel multi-domain antigen as a vaccine candidate against glioblastoma. Int Immunopharmacol. 2021;91:107265-107284.
    DOI: 10.1016/j.intimp.2020.107265.
37. Goleij Z, Mahmoodzadeh Hosseini H, Amin M, Amani J, Behzadi E, Imani Fooladi AA. *In silico* evaluation of two targeted chimeric proteins based on bacterial toxins for breast cancer therapy. Int J Cancer Manag. 2019;12(2):e83315,1-10.
    DOI: 10.5812/ijcm.83315.
38. Keshtvarz M, Salimian J, Yaseri M, Bathaie SZ, Rezaie E, Aliramezani A, *et al.* Bioinformatic prediction and experimental validation of a PE38-based recombinant immunotoxin targeting the Fn14 receptor in cancer cells. Immunotherapy. 2017;9(5):387-400.
    DOI: 10.2217/imt-2017-0008.
39. Moghadam ZM, Halabian R, Sedighian H, Behzadi E, Amani J, Fooladi AAI. Designing and analyzing the structure of DT-STXB fusion protein as an anti-tumor agent: an *in silico* approach. Iran J Pathol. 2019;14(4):305-312.
    DOI. 10.30699/ijp.2019.101200.2004
40. Rezaie E, Pour AB, Amani J, Hosseini HM. Bioinformatics predictions, expression, purification and structural analysis of the PE38KDEL-scfv immunotoxin against EPHA2 receptor. Int J Pept Res Ther. 2020;26(2):979-996.
    DOI. 10.1007/s10989-019-09901-8
41. Mohammadi M, Rezaie E, Sakhteman A, Zarei N. A highly potential cleavable linker for tumor targeting antibody-chemokines. J Biomol Struct Dyn. 2020:1-11.
    DOI: 10.1080/07391102.2020.1841025.
42. Rezaie E, Mohammadi M, Sakhteman A, Bemani P, Ahrari S. Application of molecular dynamics simulations to design a dual-purpose oligopeptide linker sequence for fusion proteins. J Mol Model. 2018;24(11):313-321.
    DOI: 10.1007/s00894-018-3846-x.
43. Shafiee F, Rabbani M, Behdani M, Jahanian-Najafabadi A. Expression and purification of truncated diphtheria toxin, DT386, in *Escherichia coli*: an attempt for production of a new vaccine against diphtheria. Res Pharm Sci. 2016;11(5):428-434.
    DOI: 10.4103/1735-5362.192496.
44. Wawrzynczak E, Derbyshire EJ, Henry RV, Parnell GD, Smith A, Waibel R, *et al*. Selective cytotoxic effects of a ricin A chain immunotoxin made with the monoclonal antibody SWA11 recognising a human small cell lung cancer antigen. Br J Cancer. 1990;62(3):410-414.
    DOI: 10.1038/bjc.1990.308.
45. Wawrzynczak EJ, Zangemeister-Wittke U, Waibel R, Henry RV, Parnell GD, Cumber AJ, *et al.* Molecular and biological properties of an abrin A chain immunotoxin designed for therapy of human small cell lung cancer. Br J Cancer. 1992;66(2):361-366.
    DOI: 10.1038/bjc.1992.271.
46. Myklebust AT, Godal A, Pharo A, Juell S, Fodstad Ø. Eradication of small cell lung cancer cells from human bone marrow with immunotoxins. Cancer Res. 1993;53(16):3784-3788.
    PMID: 8393381.
47. Fidias P, Grossbard M, Lynch Jr TJ. A phase II study of the immunotoxin N901-blocked ricin in small-cell lung cancer. Clin Lung Cancer. 2002;3(3):219-222.
    DOI: 10.3816/clc.2002.n.006.
48. Mattoo AR, Fitzgerald DJ. Combination treatments with ABT-263 and an immunotoxin produce synergistic killing of ABT-263-resistant small cell lung cancer cell lines. Int J Cancer. 2013;132(4):978-987.
    DOI: 10.1002/ijc.27732.
49. Rodakowska E, Walczak‐Drzewiecka A, Borowiec M, Gorzkiewicz M, Grzesik J, Ratajewski M, *et al.* Recombinant immunotoxin targeting GPC3 is cytotoxic to H466 small cell lung cancer cells. Oncol Lett. 2021;21(3):222-233.
    DOI: 10.3892/ol.2021.12483.