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OPEN PEER REVIEW

Design and Characterization of Ricin Based Immunotoxins Against EPHA2 Receptor for Breast Cancer Therapy: An In-Silico Study

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1 Original submission

1 comment · 23 Aug 2024

Referee 1 Reviewer

MAJOR REVISION 23 Aug 2024

Comments:
1- The MD simulation extended to 100 ns.
2- Which force field was suitable for the MD simulation?
3- Which server was used to predict the protein structure? Alpha fold or I-TASSER?
4- The number of disallowed amino acids was high; the author should predict the structure using other software, such as Robetta, and refine it using the galaxy web server.
5- The saves web server was a popular tool for analyzing the predicted structure.
6- The author should repeat the MD simulation of immunotoxin 2; the MD simulation started from 3.8.
7- The author mentioned that immunotoxin 2 was better than immunotoxin 1, but the RMSD of immunotoxin 2 was higher than that of immunotoxin 1. The lower RMSD indicated higher stability.
8- The RG values of the two complexes were similar. How do you interpret these results?
9- The RMSF values of the two complexes are compared with each other.
10- Does the EphA2 protein have an active site? Immunotoxin 1 and immunotoxin 2 interacted with different amino acids. How do you interpret these results?

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Revision 1

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Reviewer 1:

Comments for authors:

1-in the abstract please mention the method in order as they are done in the manuscript. Also, the result in the abstract did not completely indicate the outcome of this study. Please revise it.

Response: the abstract is now revised according to the reviewer instructions.

2-The introduction was not written correctly. The introduction should be written from general to detail. First mention breast cancer, then receptor EPH receptor, and after that mention using different toxins used in cancer treatment and studies which were done previously that use immunotoxins-antibody in treating cancer like this article “A new scFv-based recombinant immunotoxin against EPHA2-overexpressingbreast cancer cells; High in vitro anti-cancer potency” and “Breast cancer targeted/ therapeutic with double and triple fusion Immunotoxins”. After that mention why this study was done and what is its outcome. Also, remove sentence lines 48-49 and mention it in the last paragraph. Moreover, mention why use ricin as a toxin in this work and previous studies.

Response: the introduction is now revised according to the reviewer instructions and the mentioned studies are now cited within the text.

3-In the material and methods please mention the sequence of ricin and ScFv of EphA2 receptor were retrieved from which database and if they have ID code please mention it in the manuscript. Also, please explain how its binding site was found because using the whole EpHA2 protein as an active residue reduced docking accuracy.

Response: the sources for the sequences are now added to the methods section. Although we agree with the reviewer about the benefits of determining the active site, we did not have access to the exact site of interaction between the scFv and the EpHA2 receptor. Thus, to avoid any bias against any site of the receptor we used the whole receptor as the active amino acids. Mostly, do to the complementary structures of the scFv and the receptor, which are selected due to high affinity, the docking guides the ligand to the right active site with high binding affinity.

4-Please mention the RNA fold analysis after the MD simulation in material and method and result. First, we should check the physicochemical and structure of each construct and then the ability to interact with the receptor and check the stability with MD and then we check the ability of expression of each construct in the host. Please see this article “Design of two immunotoxins based rovalpituzumab antibody against DLL3 receptor; a promising potential opportunity”.

Response: the methods and the result sections are now rearranged as the reviewer instructed.

5-Please mention and show the linkers to join both VH and VL and ScFv to ricin with their length. Please see Figure 1 of the article “Design of two immunotoxins based rovalpituzumab antibody against DLL3 receptor; a promising potential opportunity”.

Response: the linker sequence was just used between the VH and VL domains and it is now mentioned and shown in the Figure 1 as the reviewer instructed.

6-Please mention the outcome of the prediction of the mRNA structure. which constructs have a better mRNA structure?

Response: the construct 2 had the better stability scores, which is now mentioned within the results section as the reviewer instructed.

7-In the material and methods, authors mentioned the GORIV was used for secondary structure prediction and alpha fold for tertiary structure while in the result section the I-Tasser webserver was mentioned too. Please correct this part. Also, please mention the secondary structure result and quality of the tertiary structure in the table and add more evaluation web servers such as ERRAT and Verfiy3D in the manuscript. In addition, there’s no need to mention how many models were generated by Alphafold in the Figure 1 caption. Please remove it from the Figure 1 caption and mention it in the text.

Response: The I-Tasser webserver is now deleted as the reviewer most correctly pointed out. We also performed the quality assessment analyses by the ERRAT and Verfiy3D tools and the necessary explanations are added in methods and results sections. About the addition of a Table,

we could not add a new one due to the limitations of the journal for the number of allowed Tables and Figure, but we have tried to make the results clear.

8-The manuscript did not have any table to better indicate and compare the results between the two constructs. So please use tables for indicating the result of Physical-chemical properties, docking, refinement, binding affinity analyses, and 2D plot analysis.

Response: as we explained in the previous section, we agree with the reviewer about the benefits of having Tables. However, due to the limitations of the journal for the number of allowed Tables and Figure, we were not able to add extra Tables or Figures.

9- To compare the MD simulation result of both constructs please show both MD constructs results on one plot instead of two separate plots. Also, in the MD simulation in the text, authors mention which constructs have a more stable complex because as can be construct 2 has more fluctuation and RMSD value which means less stability of this complex compared to construct 1. Please see this article" A Novel Anti CD22 scFv.Bim Fusion Protein Effectively Induces Apoptosis in Malignant B cells and Promotes Cytotoxicity".

Response: The Figure is now revised as the reviewer instructed. Regarding the RMSD values, we believe that the large initial changes in the RMSD value does not show the instability of the complex. It is just an indication that the structure is how different from the initially used structure. Higher RMSD in the beginning does not necessarily mean lower stability. The steady state of the RMSD fluctuations during the MD time is more important for us to evaluate the complexes. Keeping the equilibrated state of the complex is observed for both complexes and reducing RMSD values towards the end of the MD simulation is more evident in complex 2.

10- the antigenicity assessment of constructs just needed to be analyzed in tumor cells and did not require to analysis in bacteria and viruses.

Response: the section is revised as the reviewer instructed.

11- In the first paragraph, lines 283-302 are better to mention in the introduction than the discussion, and in the discussion in the first paragraph just briefly mention briefly about the treatment of immunotoxin and its importance for current work and its novelty. It is the same as the second paragraph it is better to mention it in the introduction.

Response: the first paragraph of the discussion is revised as he reviewer instructed and some replacements were performed within the introduction section.

12-The discussion part should be revised and compare the current work result with previous studies.

Response: the discussion is now revised and we have tried to include previous studies specially the studies pointed by the reviewer in previous sections:

1. ” A Novel Anti CD22 scFv.Bim Fusion Protein Effectively Induces Apoptosis in Malignant B cells and Promotes Cytotoxicity”.
2. Design of two immunotoxins based rovalpituzumab antibody against DLL3 receptor; a promising potential opportunity”.
3. “A new scFv-based recombinant immunotoxin against EPHA2-overexpressingbreast cancer cells; High in vitro anti-cancer potency”.
4. “Breast cancer targeted/ therapeutic with double and triple fusion Immunotoxins”.

Reviewer 2:

Comments:

1- The md simulation extended to 100 ns.

Response: we well agree with the reviewer about the benefits of longer MD simulation time, which could give of a better understanding of the behavior of complexes during time. However, our access to longer computation time is limited and we do not have the resources to perform 100 ns simulations. However, to reconfirm our MD simulation results we used the WebGro server and the results were obtained which confirmed the reliability of the current results.

2- Which force field were suitable for md simulation?

Response: we have used the GROMOS force field. GROMOS is a general-purpose molecular dynamics computer simulation package for the study of biomolecular systems. It also incorporates its own force field covering proteins, nucleotides, sugars etc. and can be applied to chemical and physical systems ranging from glasses and liquid crystals, to polymers and crystals and solutions of biomolecules.

3- Which server used for predicted structure of protein? Alpha fold or I-TASSER?

Response: we only used the Alpha fold 2 prediction tool. The text is revised to only include the alpha fold2.

4- The disallowed amino acids were high, author should be predicted structure with other software such as Robetta and refine with galaxy web server.

Response: we agree with the reviewer that using other tool may increase the quality of the predicted structure. The predictions by other servers were also taken in to account. But their results were not better than the Alpha fold results. According to the CASP studies, currently Alpha fold is the most successful prediction tool for the prediction of protein 3D structures. Thus, we only reported the Alpha fold results for the protein modelling section.

5- The saves web server was a popular tools to analysis predicted structure.

Response: As the reviewer instructed we have used the SAVES server to include the quality assessment results from ERRAT and Verify 3D tools. The methods and result sections are revised accordingly.

6- The Author should be repeated md simulation of immunotoxin 2, the md simulation stated from 3.8.

Response: Regarding the RMSD values, we believe that the large initial changes in the RMSD value does not show the instability of the complex. It is just an indication that the structure is how different from the initially used structure. It seems that owing to the different sequence of the

interacting molecules, a structural movement has accrued at the start of the simulation and the RMSD increased to get better interaction orientation. Higher RMSD in the beginning does not necessarily mean lower stability. The steady state of the RMSD fluctuations during the MD time is more important for us to evaluate the complexes. Keeping the equilibrated state of the complex is observed for both complexes and reducing RMSD values towards the end of the MD simulation is more evident in complex 2.

7- Author mention immunotoxin 2 was better than immunotoxin 1 but the rmsd of immunotoxin2 was higher than immunotoxin 1. The lower rmsd indicated higher stability.

Response: Regarding the RMSD values, we believe that the large initial changes in the RMSD value does not show the instability of the complex. It is just an indication that the structure is how different from the initially used structure. It seems that owing to the different sequence of the interacting molecules, a structural movement has accrued at the start of the simulation and the RMSD increased to get better interaction orientation. Higher RMSD in the beginning does not necessarily mean lower stability. The steady state of the RMSD fluctuations during the MD time is more important for us to evaluate the complexes. Keeping the equilibrated state of the complex is observed for both complexes and reducing RMSD values towards the end of the MD simulation is more evident in complex 2.

8- The RG of two complexes were similar. How do you interpret these results?

Response: as the reviewer pointed out the RG plots seemed similar. Thus, we have redrawn the RG plots and now they can be compare more efficiently. The results show that the complex 2 has lower RG values.

9- The Rmsf of two complexes are compared to each other.

Response: as the reviewer pointed out, it should be noted that the main factors for comparison of the two complex were the RMSD and the RG plots.

10- Does EphA2 protein have an active site?immunotoxin 1 and 2 interacted with different amino acids. How do you interpret these results?

Response: Although we agree with the reviewer about the benefits of determining the active site, we did not have access to the exact site of interaction between the scFv and the EpHA2 receptor. Thus, to avoid any bias against any site of the receptor we used the whole receptor as the active amino acids. Mostly, due to the complementary structures of the scFv and the receptor, which are selected due to high affinity, the docking guides the ligand to the right active site with high binding affinity. Having different amino acids in the interaction surface just shows that the orientation of interaction for the molecules are different. This seem quite normal since the composition the two complexes are different and the amino acids available for interaction are different. It’s important that both designed immunotoxin interact with the extracellular domain of the receptor with high affinity.

2 Revision 1

1 comment · 14 Nov 2024

Referee 1 Reviewer

REJECT 14 Nov 2024

1- The immunotoxins 1 and 2 interacted with different amino acids, so molecular docking wasn't accurate.
2- The MD simulation extended to 100 ns.

3

Revision 2

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Reviewer 1:

1-What does the author mean by the word “sawing” in line 6?

Response: we have meant fusing and linking. To avoid confusion, we replaced it with “fusion”.

2-the name of the molecular dynamics tool is corrected in line 12. It should be “Gromacs”.

Response: we have corrected the misspelled word as the reviewer instructed.

3-As the authors said they have limitations in adding the quality of the predicted model and comparing the physicochemical properties of two constructs in the table in the manuscript, I suggest authors add these tables in a separate file as a supplementary file for this article. Also, as I understand this manuscript has not any table. How do authors have limitations in adding tables in the manuscript?

Response: the author instructions have instructed the number of Tables and Figures all together should not exceed 5 and we already included 5 Figures. Thus, we were not able to include any new Figure or Table. As the reviewer instructed we prepared a supplementary file for the suggested Table.

4- The sentence in line 181” EphA2- C- ricin (construct 1), and EphA2- N- ricin fusion genes (construct 2)” is confusing please revise it to “from N terminal to C terminal subunit A ricin-EphA2 (build 1) and EphA2- subunit A ricin (construct 2) fusion genes.

Response: the sentence is now revised as the reviewer instructed.

5-Please correct the word “contract” in line 204. Also, please check the whole manuscript grammatically.

Response: the misspelled word is now corrected and the whole study is now revised for English writing.

6—The ERRAT and Verify 3D values show a moderate-quality model, not high quality. Please correct it at line 207.

Response: the correction is made as the reviewer instructed.

7-Please make a table for docking results and binding affinity assessment in the supplementary file.

Response: As the reviewer instructed we prepared a supplementary file for the suggested Table.

8- Why the authors used PDB code 5nka which belongs to Ephrin A2 in complex with compound 2g instead of using 1MQB as a free form of Ephrin A2.

Also, which domain of Ephrin A2 was used for docking? As the Ephrin A2 complex with monoclonal antibody existed in the PDB bank (3SKJ) why authors did not use this binding site for docking?

Response: the structure of the 5nka (2017) was far more recent than the 1MQB (2002) and more importantly the resolution of the 5nka was equal to 1.38 Å which was far better than the resolution of the 1MQB (2.30 Å). Moreover, the accompanying compound of the 5nka was a small molecule which could not drastically change the native coordinates of the ephA2.

About using the binding site of the 3SKJ, it should be noted that the targeting moiety of the designed immunotoxins were obtained from an antibody reported in a patent without structural data. We were not sure about the epitope which is targeted by this scFv. The antibodies in the patent and 3SKJ may target totally different epitopes in different regions of the target protein. Therefore, we did not use the binding site of the 3SKJ.

9- If authors used Prodigy for assessing binding affinity please mention the KD of immunotoxin 1 and 2 in the manuscript.

Response: the KD is now added to the results and the related supplementary Table.

10- According to the RMSD plot, immunotoxin1 had around 4 nm (a high RMSD value) which is far from the RMSD value of 1 for immunotoxin 2. This means these two immunotoxins had different folding and immunotoxin 2 had better protein structure stability. Also please mention the unit of RMSD, RG, and RMSF in the y-axis and the revised interval of time of the x-axis to better represent of plot. Please see this article:

Response: as the reviewer instructed the Figure is revised with rescaling of the axes and addition of the units for RMSD, RG, and RMSF.

11-Please add “, respectively” at the end of sentence in the line 287.

Response: the sentence is revised as the reviewer pointed out.

12- Please add the energy plot of predicting mRNA structure in the manuscript as a supplementary file.

Response: the energy plots of the mRNA are now added as supplementary file.

13- As the design immunotoxins are probable antigens for the immune system they could easily be removed from the body or it can trigger the immune system. How can authors interpret it?

Response: Thank you for your valuable comment. We acknowledge that the design of immunotoxins, particularly those incorporating foreign protein moieties, could indeed elicit an immune response and lead to their clearance from the body. The immunogenicity of immunotoxins is a well-recognized challenge, and several factors must be considered when evaluating their potential to provoke immune responses. In this regard, we have just used a scFv to include the smallest part possible of the antibody. We also used the sequence of the antibody form a patent which includes the humanized sequences of the antibody. Although we have taken these precautions, we believe that the inclusion of immunosuppressive agents or engineered alterations to the immunotoxin structure (e.g., polyethylene glycol (PEG) PEGylation or glycosylation modifications) in the future in vivo studies can help to evade recognition by the immune system and extend the half-life of the immunotoxin in circulation. the use of polyethylene glycol (PEG) or other conjugates would increase molecular size and reduce renal filtration, thus improves pharmacokinetics and prolongs systemic circulation

14-Please mention the allergen city and antigenicity result of two design constructs in the table in the supplementary file.

Response: a new supplementary Table is now added as the reviewer instructed.

15-the order of mentioned material and method, result, and discussion should be the same. Currently, the discussion order is not the same as the material method, result, and discussion.

Response: the order of discussion is now revised to be in line with methods and results sections and the section about the mRNA structure repositioned.

16- In discussion please do not repeat the result. Instead, compare the results of two designs of immunotoxin and conclude which one had better quality and stability to be a drug candidate and mention it in conclusion.

Response: the revised discussion contains the comparison of our study with several similar studies and two immunotoxins are compared. The conclusion is now revised as the reviewer instructed.

Reviewer 2:

1- The immunotoxin 1 and 2 interacted with different amino acids so molecular docking weren’t accurate.

Response: As the reviewer most correctly pointed out the immunotoxins interacted with different amino acids, which indicates they have different orientation in the interaction with the EphA2 receptor. However, we do not believe this is equal with the inaccurate docking. Since the sequences of two constructs different, different structures are expected for each contracts. Given these circumstances, different orientation of interaction and consequently different interacting

amino acids were already anticipated. The interacting amino acids reveals that both Constructs interact with the same region of the EphA2 receptor with different orientations.

2- The md simulation extended to 100 ns.

Response: Thank you for your insightful comment. We fully appreciate the importance of conducting long-duration molecular dynamics (MD) simulations to gain more comprehensive insights into the system's behavior over time. However, as you have noted, extending the simulation length requires significant computational resources, which, unfortunately, are currently beyond the scope of our available infrastructure. However, to reconfirm our MD simulation results we used the WebGro server and the results were obtained which confirmed the reliability of the current results.

Reviewer 3:

In my opinion, the manuscript now is suitable for publish in IJPR.

Response: we thank the reviewer for his/her positive feedback.

Reviewer 4:

1- In Background “The ricin and EphA2-containing immunotoxins will be generated by attachment of the N or C terminal of subunit A of ricin to antibody fragments (single chain variable fragment or scFv) against the EphA2 receptor. In this way, we can specifically target the cancer cell antigens and solve the specificity issue in old-fashioned chemotherapy methods” please edit to “Therefore, we were generated the ricin and EphA2-containing immunotoxins will be generated by attachment of the N or C terminal of subunit A of ricin to antibody fragments …”.

Response: the revision is now made within the text as the reviewer instructed.

2- In 2.1. Design of recombinant immunotoxins. Please provide a detailed explanation of how these constructs were prepared. What software was used to design these structures, and how were the amino acid sequences assembled?
Also,
“Two immunotoxins are constructed by the fusion of the subunit A of ricin (retrieved from UniProt: P02879) with scFv against the EphA2 receptor (the sequence of the scFv was retrieved form 1F12 mAb of the Patent number US20090304721, which was designed using three repeats of G4S linker in our previous study”
please edit to “the first, structure of the subunit A of ricin (retrieved from UniProt: P02879) and then the structure of scFv retrieved form 1F12 mAb of the Patent number US20090304721,…”
Also,
“We used SnapGene Viewer software (5.3.1) to analyze the correct construction of vectors. “For analyzing the correct construction of vectors by SnapGene, what parameter was used to analyze?

Response: the sentence pointed out by the reviewer is now revised for more clarity. However, we do not use the structures of the ricin and scFv (as the reviewer mentioned), we used their sequences. Thus, we do not use the exact sentence of the reviewer. Regarding the use of SnapGene, we just used this software to make the sequence of immunotoxins and SnapGene was used like a text editor and no specific analyses were done by SnapGene.

3- In 2.2. The secondary and tertiary structure prediction and quality assessment. In sentence "and AlphaFold2 server (https://neurosnap.ai/) were used to predict the secondary and tertiary protein structure, respectively" . This sentence is unclear, Please write the name of protein.

Response: As the reviewer most correctly pointed out the sentence is now corrected.

4- In 2.3. Physical-chemical properties. In sentence "ProtParam (https://web.expasy.org/protparam/) is used to compute different physical and chemical parameters." What protein characteristics do the authors want to investigate? Please write the name of the target protein.

Response: the name of the proteins is now added. It is explained that the properties such as molecular weight, theoretical PI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index, and grand average of hydropathicity are examined.

5- In 2.4. Protein-receptor docking. Please write a reference for "HADDOCK Server v.24".

Response: a reference is added for HADDOCK as the reviewer instructed.

6- In 2.4. Protein-receptor docking. "In this regard, the structure of the EphA2 as the target receptor was obtained from the RCSB Protein Data Bank (RCSB PDB) at" please delete RCSB.

Response: the correction is made according to the reviewer instruction.

7- In 2.4. Protein-receptor docking. Please write a reference for "PDBEditor software.".

Response: a reference is added for PDBEditor as the reviewer instructed.

8- In 2.4. Protein-receptor docking. In sentence "To do the docking analyses, the scFv regions of the designed immunotoxins and the whole EphA2 protein were set as the active amino acids of the interaction." Why has been selected whole EphA2? Why has not been selected active site?

Response: Although we agree with the reviewer about the benefits of determining the active site, we did not have access to the exact site of interaction between the scFv and the EpHA2 receptor. It should be noted that the scFv was obtained from a patent and its target epitope in not reported to be used as the active site. Thus, to avoid any bias against any site of the receptor we used the whole receptor as the active amino acids. Mostly, due to the complementary structures of the

scFv and the receptor, the docking would guide the ligand to the right active site with high binding affinity.

9- In 2.5. Docking refinement and Binding affinity calculation. In sentence " using FireDock at http://bioinfo3d.cs.tau.ac.il/FireDock/." Please change to "Edited,using FireDock (http://bioinfo3d.cs.tau.ac.il/FireDock/)."

Response: the revision is now made within the text as the reviewer instructed.

10- In 2.6. 2D interaction diagrams. please it changes to "Visual presentations".

Response: the revision is now made within the text as the reviewer instructed.

11- In 2.7. Molecular Dynamics (MD) simulation. MDs for 50-nanosecond is very short. It should be 100-200 ns. Also, you should use the last version of Gromacs for run MDs. For writing the steps of Gromacs, it be suggested to use some articles such as:
https://www.tandfonline.com/doi/full/10.1080/07391102.2020.1824816
https://www.tandfonline.com/doi/abs/10.1080/07391102.2019.1588784
https://www.sciencedirect.com/science/article/abs/pii/S1093326319306618
https://onlinelibrary.wiley.com/doi/abs/10.1002/prot.26448

Response: Thank you for your insightful comment. We fully appreciate the importance of conducting long-duration molecular dynamics (MD) simulations to gain more comprehensive insights into the system's behavior over time. However, as you have noted, extending the simulation length requires significant computational resources, which, unfortunately, are currently beyond the scope of our available infrastructure. However, to reconfirm our MD simulation results we used the WebGro server and the results were obtained which confirmed the reliability of the current results.

12- In 2.8. The mRNA structure prediction. What is the purpose of predicting mRNA structure, and why is this evaluation important?
Also,
In the mFold web server what parameters have been used?

Response: predicting mRNA secondary structure is a critical step in optimizing the design of an immunotoxin. It provides insights into translation efficiency, mRNA stability, regulatory elements, and potential pitfalls in expression, all of which are essential for ensuring the success of immunotoxin-based therapeutic strategies. It should also be noted that all default values were used to make the prediction by mFold server.

13- In 2.9. Allergenicity and antigenicity. What is the purpose of Allergenicity and antigenicity, and why is this evaluation important?

Also,
In the AlgPred server and VaxiJen server what parameters have been used?

Response: Evaluating allergenicity and antigenicity is crucial in immunotoxin design to ensure both safety and efficacy. Allergenicity assessment helps identify potential allergic reactions to the protein, reducing the risk of hypersensitivity or anaphylaxis in patients. Antigenicity evaluation, on the other hand, determines the likelihood of the immunotoxin triggering an immune response that could lead to the production of neutralizing antibodies, which may reduce its therapeutic effectiveness. Both evaluations are vital for minimizing adverse immune reactions, optimizing the immunotoxin's performance, and ensuring successful clinical use without compromising patient safety or treatment outcomes. It should also be noted that all default values were used to make the prediction by AlgPred server and VaxiJen server.

14- In 3.1. Design and generation of recombinant immunotoxins. This section is unclear. Please, the results of steps explained completely. Also,
To evaluation the validation of generated constructs, you should utilize bioinformatic approaches. The phrase " further we used western blotting to detect their expression levels (data not shown since they will be used for another paper) " is not acceptable.

Response: this section is now revised according to the reviewer instructions.

15- In 3.2. The prediction of secondary and tertiary structure of immunotoxins and validity assessment. "GORIV predicted there are alpha helix …" Please change to "GOR IV serve".
Also,
"The ERRAT results showed that the contruct 1 and 2 have the Overall Quality Factor of 86.5731 and 89.4212, respectively." Please "contruc" edit to "constructs".
Also,
"VERIFY 3D results showed that 73.84% of the residues have averaged 3D-1D score of >= 0.1 and 73.84% of the residues in the construct 2 have averaged 3D-1D score of >= 0.1. These results could be construed as high quality of the predicted 3D structures." Please edit to
"VERIFY 3D results showed that construct 1 includes 73.84% of the residues have averaged 3D-1D score of >= 0.1 and construct 2 have 73.84% of the residues in the averaged 3D-1D score of >= 0.1"
Also,
The sentence "These results could be construed as high quality of the predicted 3D structures." Is unclear please it edits.

Response: all of the revisions suggested by the reviewer are now exerted within the text.

16- In 3.3. Physical-chemical properties of immunotoxins. Please, tables prepare to present the results of the physical-chemical properties of the designed immunotoxins, and the results compare. You can refer to these articles for further assistance:

https://pubmed.ncbi.nlm.nih.gov/38403216/

https://jaem.qom.ac.ir/article_2553.html

also,
“Its extinction coefficient is 77615 M-1 cm-1.” What is the extinction coefficient, and what results does this analysis yield?

Response: due to the limitation of the journal we cannot include more than five Figure/Table. Thus new table could not be included. However, we have added these data in supplementary files.

Regarding the extinction coefficient of a protein, its a measure of how strongly it absorbs light at a specific wavelength, typically around 280 nm, and is expressed in units of $M^{-1} \cdot cm^{-1}$. It is crucial for determining the concentration of a protein solution using the Beer-Lambert law, which relates absorbance to concentration and path length. The coefficient also provides insight into the protein's amino acid composition, especially aromatic residues like tryptophan, tyrosine, and phenylalanine. Additionally, comparing the measured extinction coefficient with expected values can help assess protein purity and structural changes, offering valuable information about the protein's folding, stability, and homogeneity.

17- In 3.4. Protein-receptor docking of immunotoxins. The sentences of this part are unclear. Please edit.

Response: the section is now revised as the reviewer instructed.

18- In 3.5. Interaction refinement and binding affinity analyses. “The best complexes between the 3D structure of the immunotoxin and EphA2 3D were refined by the FireDock server. The refined complexes were used to calculate the binding affinity between the structure of immunotoxin and EphA2.” Please, these sentences correct in terms of Grammarly.

Response: The Grammarly software was used to analyze the section and necessary edition are made as the reviewer instructed.

19- In 3.6. 2D protein interaction plot. The results of the evaluation of the interaction between the designed immunotoxins and the EphA2 receptor have not been well explained. Please more explain.

Response: more explanation is added to the section according to the reviewer instructions.

20- In 3.6. 2D protein interaction plot. In figure 4. Which software was used to prepare these figures? These figures do not appear to be outputs from the ligplot software. Were they created using another software?

Response: these results are made by the Ligplot software. We just changed the presentation adjustments of the software to make the output result more clear due to high number of interactions between the interacting molecules. Although due to the exerted adjustments the Figure seems different from usual Ligplot Figures, no other software was used.

21- In 3.7. MD simulation. The results of the MD simulation have not been explained clearly. Please rewrite this section with more detail.
response: the whole section is now revised as the reviewer instructed.

22- In figure 5. The quality and resolution of figures are low. Please, the caption of figure 5 edits.

Response: Figure 5 and its caption are now revised according to the reviewer's instructions.

23. In 3.8. The prediction of the mRNA structure. These results have not been clearly explained. Please provide more detailed explanations of the findings.

Response: the whole section is now rewritten to make it more clear

24- In 3.9. Allergenicity and antigenicity of immunotoxins. These results have not been clearly explained. Please provide more detailed explanations of the findings.

Response: the whole section is now rewritten to make it more clear

25- In 4. Discussion. "EphA2 is displayed on the cell surface, which makes it an ideal target for immunotoxins. To design the immunotoxin, we selected ricin as the toxin part, since this protein toxin has been already used to generate other immunotoxins. It has a small size, which is important for cell internalization. Although ricin is made up of two A and B chains, only the A chain is enough to kill the cancer cells. The killing mechanism is related to the inhibition of the translation process inside the cells (22). To design an immunotoxin different factors should be taken into account (17).". These sentences can be improved for better clarity.

Response: some edition is now made to make the section clearer as the reviewer instructed.

26- In 4. Discussion. You write these sentence "It is very useful to use bioinformatics tools to evaluate the immunotoxin design and construction before experimental settings" while in the sentence write "Thus, the purpose of this study is not to find the differences between the two immunotoxins but mainly to use bioinformatics tools to test the feasibility of their construction and their use as immunotoxins to treat breast cancer". you designed two immunotoxins by using bioinformatic tools; based on the results of bioinformatic analysis. You should compare results of two designed immunotoxin and then select best immunotoxin.

Response: as the reviewer most correctly pointed out the necessary changes are made within the text.

27- In 4. Discussion. “The stability of mRNA expressed from two different constructs was tested by mFold software seems good enough. Based on the thermodynamic properties, the mRNA expressed from immunotoxin 2 is more stable to a higher extent. For the best model, for immunotoxin 1, the ΔG is -613.69 kcal/mole, while for immunotoxin 2, the ΔG is -624.88 kcal/mole.” Is there a threshold for ΔG to evaluate mRNA stability? What is the basis for your conclusion that the mRNA expressed from immunotoxin 2 is more stable to a higher extent than immunotoxin 1?
“The obtained results in our study resembles the results of the in silico section in various studies. Ataee et al., have performed a bioinformatics study on the single-chain fragment variable of rovalpituzumab antibody fused to granzyme B (Rova-GrB) and PltA of typhoid toxin (Rova-Typh) as immunotoxins. Their docking analysis demonstrated that the binding domain of immunotoxins could bind the N-terminal region of delta-like ligand 3 (DLL3). ……. ……
In light of the results of these studies, it could be anticipated that the designed ITs in our study could exert similar effects in the elimination of suitable cell lines and consequently act as amenable anti-cancer agents”
In the all studies, designed immunotoxins evaluated in vitro. While in this study only immunotoxin designed. Therefor how can you discus and compare the results of yourself with other studies. You only other studies report, there are not show any discussion. Also,

“EPHA2” edit to EphA2.

Also,

“Recombinant DNA technology and a new recombinant IT was produced.” “IT “What is abbreviation?

Response: To address the reviewer’s comment, it is important to note that while there is no universally defined absolute threshold for ΔG to evaluate mRNA stability, a more negative ΔG generally correlates with greater stability in the secondary structure of mRNA. Studies have shown that a lower (more negative) Gibbs free energy (ΔG) typically reflects stronger and more stable base-pairing, resulting in a less likely tendency for the mRNA to undergo degradation or denaturation. In our study, the ΔG value for immunotoxin 1 is -613.69 kcal/mol, while for immunotoxin 2, it is -624.88 kcal/mol, indicating that the mRNA expressed from immunotoxin 2 has a more thermodynamically favorable structure. This difference in ΔG suggests that the mRNA from immunotoxin 2 is likely to be more stable than that from immunotoxin 1, as it has a more negative ΔG, which aligns with previously reported correlations between ΔG values and mRNA stability (1). Therefore, our conclusion that the mRNA from immunotoxin 2 is more stable is based on the lower (more negative) ΔG value, reflecting enhanced structural stability.

Regarding the issue of comparing our results with other studies with experimental analyses, we agree with the reviewer that such comparison without experimental part is not suitable. However, it should be noted that our comparison was made based on the fact that most of these studies contain bioinformatics sections which were confirmed by empirical sections. Our purpose was to point out that since their corresponding bioinformatics predictions were successfully confirmed by experimental section, our bioinformatics predictions could also be confirmed by experimental studies.

EPHA2 is now edited to EphA2as the reviewer instructed.

IT is now omitted and replaced with immunotoxin.

28- In Conclusions. “In this in silico study, we investigated how bioinformatics analyses by different tools or web servers can be used to assess the design and construction of two immunotoxins, EphA2- C- ricin and EphA2- N- ricin immunotoxins.” This sentence is unclear. Do you designed a software or tools? you used from designed a software or tools by other researchers. Therefore, you con not claim “we investigated how bioinformatics analyses by different tools or web servers can be used to assess the design and construction.”

Response: the sentence is revised as the reviewer most correctly pointed out.

1. Mathews DH, Sabina J, Zuker M, Turner DH. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. Journal of molecular biology. 1999;288(5):911-40.

3 Revision 2

0 comments

No public comments in this round.