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Computational design of a chimeric toxin against Claudin-4-expressing cancer cells: molecular modeling, docking and molecular dynamics simulation analysis

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Abstract

Cancer is one of the main reasons of mortality all over the world. Over the time, the major ways for cancer-therapy were based on radiotherapy, chemotherapy and surgery. These methods are not specific enough for that purpose, therefore, new ideas for design of new drugs with higher specificity are considered. Chimeric protein toxins are hybrid proteins consisting of a targeting portion and a toxic one which specifically bind and kill the target cancer cells. The main purpose of this study was designing a recombinant chimeric toxin with biding capability to one of the most key receptors namely claudin-4 which is over-expressed in almost all cancer cells. To design it, we utilized the last 30 C-terminal amino acids of *Clostridium perfringens* enterotoxin (CPE) as a binding module for claudin-4 and the toxic module which is the A-domain of Shiga toxin from *Shigella dysenteriae*. Using molecular modeling and docking methods, appropriate binding affinity of the recombinant chimeric toxin to its specific receptor was demonstrated. In the next step, the stability of this interaction was investigated by molecular dynamics simulation. Although partial instability was detected at some time points, however, sufficient stable situation of hydrogens bonds and high binding affinity between the chimeric toxin and receptor were observed in the *in silico* studies which in turn suggested that this complex could be formed successfully.

Introduction

Cancer is the second reason of mortality all over the world.[1] Main reasons of cancer are mutation and dysfunction of genes that control cell cycle which eventually lead to development of a mass of tumor cells.[2] One of the drug types that can be designed and developed for cancer treatment by the help of computational tools, are chimeric protein molecules like immunotoxins and chimeric protein toxins. As the name suggests, immunotoxins are formed of two linked portions: "immune" part which refers to an antibody molecule – intact or just a fragment of it – and the "toxin" part which refers to a protein toxin molecule.[3] Theoretically, studies have shown potential and applications of immunotoxins for elimination of different types of unwanted cells, however, best results were observed in cancer cells. However, antibody moiety of immunotoxins can be replaced by other high affinity protein in particular bacterial toxin for a specific receptor on a target cell which is called chimeric or fusion toxin.[4]

Change of gene expression pattern is one of the main characteristics of cancer cells. Expression of them - like cell adhesion molecules – can be reduced. Many of these molecules are involved in cell-cell and cell adhesion to the basement membrane or extracellular matrix. Such changes are involved in the metastasis of cancer cells.[5] One of these proteins is claudin protein family which is transmembrane protein and found in the tight junction between epithelial and endothelial cells.[6] The expression patterns of different members of the claudin family change during the cancer process. The expression of some members of the claudin family increases in various cancers in humans- like claudin-4 that shows overexpression in many cancers like: Breast carcinoma, ovarian surface epithelial carcinomas, biliary tract carcinoma, prostate carcinoma.[7,8] Expression of certain types of claudin proteins in different tumors can make it a good target in designing chimeric toxin (CT) against cancer, in particular, claudin-4, which is over-expressed in many types of cancer. An interesting example is the effect of *Clostridium perfringens* enterotoxin (CPE)

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on claudin proteins. The receptor for this toxin is located on the extracellular domain (ECL2) of claudin (specially claudin-3 and 4 which are mentioned as receptor for CPE).[9,10] A significant point regarding this binding is experiments confirming that the last 30 amino acids from the c-terminus of this toxin (C-CPE) play a major role in its binding to the receptor.[11,12] It can be inferred that these 30 amino acids could play a role in the design of a recombinant CT protein. In other words, by binding these 30 amino acids to a potent toxin, a recombinant CT similar to an immunotoxin can be designed against claudin-4 expressing cancer cells. For this purpose, there is a wide range of toxins with different origins, however, in our study, Shiga toxin (toxin of the *Shigella dysenteriae*) was used to design the new CT. Actually, there are some studies demonstrating killing effects of CPE-based immunotoxins on cancer cell- for example immunotoxin or recombinant toxin protein obtained from fusing *Pseudomonas aeruginosa* exotoxin A (ETA) to the CLDN4‑binding domain of CPE.[10]

Shiga toxins (Stx) belong to the AB toxin family. The toxin consists of a subunit A with a biological and enzymatic role and five subunits of B which are pentamers involved in the binding of this toxin to its specific receptors on the cell surface.[13] The mechanism of action of Stx is that after holotoxin endocytosis, a part of subunit A (StxA) called A1 causes 60S subunit of eukaryotic ribosome failure which leads to ribosome defect and inhibition of protein synthesis and hence death of the target cell.[14,15] Endocytosis of such recombinant chimeric toxin is challenging since StxA only can be effective once internalized into the cells via endocytosis. In other words, potential of the claudin receptor in triggering endocytosis upon interaction with immunotoxin (targeting or CPE portion) is a key issue. However, there are some evidences proving endocytosis of the claudin receptor and the CPE complex upon binding.[16,17]

Computational biology or bioinformatics is a science which refers to employment of computer or computational methods for analyses of biological data, prediction of molecular structures, modeling and simulations.[18] Nowadays, importance of bioinformatics is undeniable in different aspects of biological studies and has become increasingly important in drug discovery and design processes.[19]

Therefore, in this study we aimed at designing a new recombinant CT composed of the last 30 amino acids at the c-terminal of the CPE and the StxA subunit of the Shiga toxin. To do so, it was modeled by bioinformatics tools and after refinement and optimization of final model, interactions of recombinant CT model and claudin-4 were analyzed using molecular dynamics simulation and molecular docking.

Materials and Methods

Sequence retrieval. The amino acid sequences of both proteins, the last 30 residues of CPE and Stx subunit A of Shiga toxin, were retrieved from uniport. The computational analysis order was as follows: modeling, refinements of models, molecular docking, and finally simulation of interactions between recombinant chimeric toxin (30 last residues of CPE and StxA) and claudin-4 by molecular dynamic simulation.

3D models, refinement and validation. For down-stream analysis, we had to make a reliable model (in PDB format) of recombinant chimeric toxin sequence. To do so, homology modeling method by three Softwares named MODELLER (version 10.4; University of California at San Francisco, San Francisco, USA), I-Tasser (https://zhanglab.dcmb.med.umich.edu/I-TASSER/) and Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) was applied. Then, the modeled structures were refined and validated to choose the best one. For this purpose, various online servers including Galaxy Refine (https://galaxy.seoklab.org/cgi-bin/submit.cgi? type=REFINE), MolProbity (http://molprobity.biochem. duke.edu/index.php), SAVES (https://saves.mbi.ucla.edu) and ProSA (https://prosa.services.came.sbg.ac.at/prosa.php) were utilized and Ramachandran plot were drawn for the models. To do so, back bone of modelled chimeric toxin was considered fixed and side chains flexibility was rebuild and repacked and followed by short molecular dynamics simulation.[20] GalaxyRefine made five refined model for all three models that was obtained from MODELER, I-Tasser and Phyre2, and finally best refined models of these three was chosen for validation. In validation, some servers are used to assess parameters like quality of bonds, dihedral angles, existence of clashes and matching protein sequence to predicted models. The servers used in this research were: MolProbity to obtain clash score and molProbity score, SAVES (The three servers used were: PROCHECK for drawing Ramachandran plot, ERRAT to assess general quality of models and Verify3D to match first structure to third structure) and ProSA to compare general quality of predicted models with structured models in the databases. When using MODELLER, structured-proteins in databases as template for prediction models should be used. Therefore, 1R4Q (StxA code) and 3AM2 (CPE code) (from RCSB database) were used as templates in the modelling by MODELLER.

Molecular docking. After refinement and validation of the models, the best model was selected for docking run. Molecular docking is a computational tool that models the binding of a particular molecule as a ligand to another molecule as a receptor, and most importantly, it predicts the binding affinity of the ligand and receptor and their most possible binding modes. In other words, considering the limitations that the ligand bears, the geometric state of

the ligand during the connection and the physical and chemical interactions of this connection are predicted. Finally, by storing and comparing docking data, molecules attached to a specific receptor can be distinguished from other molecules that do not bind to that receptor at the same binding site.[21] In this regard, molecular docking was performed using ClusPro and HADDOCK web servers. After confirming the ligands' ability to bind to the receptor, haddock server was used to check the docking more closely and obtain correct docking pose. Haddock uses template-based method for docking and obtained results of docking are clustered based on the number of members and members of each cluster are sorted based on Haddock score. Haddock score is a value affected by energies like electrostatic and Van der Walls.[22,23]

Molecular dynamic simulation. After molecular docking investigations, the interaction of chimeric toxin and claudin-4 was evaluated by molecular dynamics (MD) simulation method. MD studies simulate thousands of atoms involved in bonding between biomolecules using algorithms and, most importantly, over time on nanosecond (nsec) scales.[24] In order to evaluate the stability of the chimeric toxin complex and its receptor under physiological conditions, molecular dynamics simulation was performed by GROMACS program (version 5.0.1; Royal Institute of Technology and Uppsala University, Uppsala, Sweden). It was performed in a 1.00 nm diameter box using Gromos43a1 force field. The simulations were performed using periodic boundary conditions (PBC) and Ewald particle mesh method (PME). To perform MD, sodium and chlorine ions were initially used to neutralize and balance the charge. Then, the energy minimization step was performed for 500 picosecond (psec). Subsequently, the system was prepared for the final stage of MD in two equilibration steps. The final simulation was performed for 100 nsec using the LINCS algorithm at a constant temperature of 300 K and with a time interval of 2.00 femtosecond. After performing molecular dynamics, RMSF (root-mean-square-fluctuation) and RMSD (root-mean-square-deviation) diagrams were evaluated and the stability of the complex and its conformational changes in physiological conditions were investigated. The number of hydrogen bonds at the time of binding to the receptor and the alterations in the chimeric protein toxin secondary structure during the simulation time were also evaluated.

Results

Amino acid sequence of StxA and CPE. The sequences of each module of chimeric toxin which were retrieved from protein databases are as follow: KEFTLDFS TAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDSGTGDNLFA VDVRGIDPEEGRFNNLRLIVERNNLYVTGFVNRTNNVFYRF ADFSHVTFPGTTAVTLSGDSSYTTLQRVAGISRTGMQINRHS LTTSYLDLMSHSGTSLTQSVARAMLRFVTVTAEALRFRQIQR GFRTTLDDLSGRSYVMTAEDVDLTLNWGRLSSVLPDYHGQ DSVRVGRISFGSINAILGSVALILNCHHHASRVARMASDEFPS MCPADGRVRGITHNKILWDSSTLGAILMRRTISSsldagqyvlv mkanssysgnypysilfqkf. Upper-case letters belong to StxA and bold lower-case letters belong to CPE.

Homology modeling. For homology modeling three programs were applied namely MODELER, I-Tasser and Phyre2. 1,000 models were made by MODELLER and the best one was selected based on DOPE score (lowest DOPE score is optimum). Also five models were obtained from I-Tasser and 20 models were obtained from Phyre2 followed by selection of the best ones. After modeling, in order to enhance the quality of a predicted protein models, the refinement was applied. Based on the data obtained from softwares (Table 1), servers and also the results of Ramachandran plot (data not shown), the refined and validated model were obtained by Phyre2 software selected for docking steps and molecular dynamics (Fig. 1).

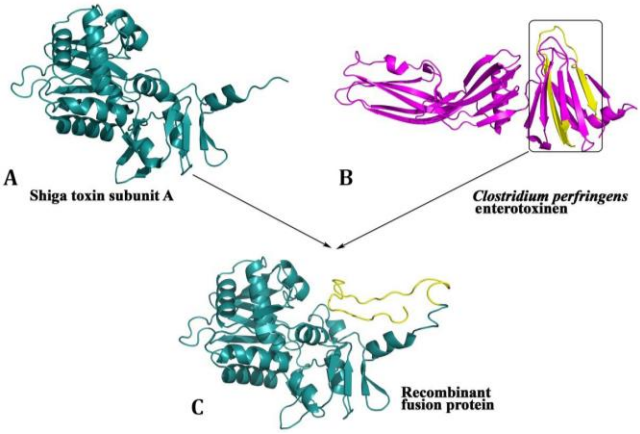

Fig. 1. Final model of chimeric protein toxin **(C)** from Shiga toxin subunit A **(A)**, and CPE of *Clostridium perfringens* enterotoxin **(B)**. Yellow region is 30 last residues of CPE that has been switched from β-strand in β-sheet secondary structure of CPE to a loop in chimeric toxin.

Table 1. Comparison of models from three programs.

Protein	Ramachandran plot quality (%)				MolProbity clashscore	Verify 3D (%)	ERRAT (%)	MolProbity score*
	Most favored	Additionally allowed	Generously allowed	Disallowed				
I-Tasser	89.30	8.30	0.90	2.40	7.42	87.31	95.43	1.73
MODELLER	93.40	5.50	0.00	1.00	5.61	83.90	91.13	1.40
Phyre2	93.80	5.50	0.30	0.30	4.41	83.9	92.39	1.41

* MolProbity score combines the clashscore, rotamer, and Ramachandran evaluations into a single score, normalized to be on the same scale as X-ray resolution.

Molecular docking. In molecular docking studies three complexes were evaluated: Chimeric toxin/claudin-4, Shiga toxin/claudin-4 (as negative control) and CPE in complex with claudin-4 (PDB ID: 7KP4; as positive control). According to the docking results of ClusPro server, the docking score for the reference structure (positive control) was –2,816.70 kcal mol^{-1} while for the negative control which did not have the correct docking pose as expected, it was –1,573.10 kcal mol^{-1}. Therefore, scores close to negative control indicated that the ligand and receptor did not interact properly in our experiment, and complexes with binding scores similar or close to the reference structure were probably well formed and had the proper interactions. In addition to the docking score, complexes were selected based on the docking pose. Interestingly, the docking score for the chimeric toxin was even significantly higher than the reference (–3,958.40). This implied the high quality of the predicted model with an emphasis on the fact that it was very likely that the chimeric toxin had a high ability to interact and bind to the receptor (Fig. 2). The results of the HADDOCK server were given for checking the ΔG to PRODIGY server. The ΔG obtained from PRODIGY server was –13.30 (kcal mol^{-1}) for 7KP4 and –14.30 (kcal mol^{-1}) for chimeric toxin-claudin-4 complex. The results of both molecular docking showed that the chimeric toxin-receptor complexes had excellent temperature stability even better than positive control.

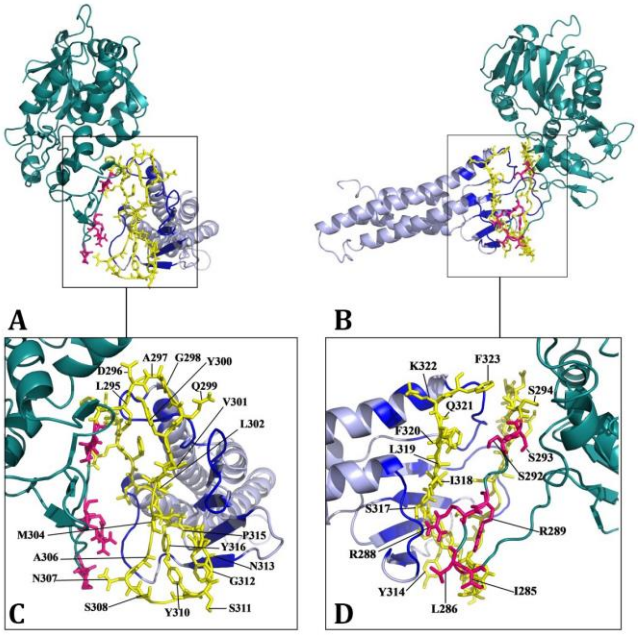

Fig. 2. Docking pose and interacting residues in the chimeric toxin-claudin-4 complex. Residues involved in the interaction are labeled and shown in stick. 30 residues of CPE are represented in yellow, StxA portion is marked turquoise in color and claudin-4 is shown in blue. Residues which interacted with receptor illustrated in Magenta, but belong to N-terminal (StxA portion). **A** and **C** are front view, **B** and **D** are lateral view.

RMSD analysis. One hundred nanoseconds MD simulation was performed for the chimeric toxin-caudin-4 complex. Conformational changes in simulation times were evaluated by RMSD calculations that could be used to measure the stability of the complex. In fact, the less conformational change in proteins over time occurred, the smoother the RMSD graph became. The RMSD plot showed that the complex had undergone many changes during the simulation time. It ranged from about 0.40 to just over 1.00 nm, indicating relatively variable conformational changes at complex. The most drastic conformational changes were observed at the beginning of the simulation and around the first 7,500 psec, which showed that the complex with these changes was far from its original state to be able to reach its stable form. Over the simulation, the relative stability was seen for a long time, during which small changes in RMSD indicated the stability of the complex. This stage of relative stability could be observed between 7,500 and 67,500 times. Near the end of the simulation, the fluctuations in RMSD diagram were increased again so that at 82,500 psec, another increase in the RMSD number was observed indicating a re-conformation change of the complex (Fig. 3A).

RMSF analysis. In order to examine the fluctuations of each residue in more detail, RMSF analysis was conducted. This analysis showed the spatial changes of alpha carbon of each amino acid over time and was a measure of the flexibility of the residues. In fact, the larger the RMSF number for a residue, meant the more conformational change of the residue and greater displacement in space. Residues located in areas without a regular second structure, such as long loops, would have a higher RMSF value. Based on the RMSF diagram prepared for the recombinant chimeric toxin, residues of the C-terminal portion which included the StxA end portion and the CPE portion showed more fluctuations than the rest of the protein and had a higher RMSF. The CPE section is marked with a yellow box and contains residues 294 to 323. As previously shown in the chimeric toxin, this part was composed of long loops that were inherently free to operate and could be noticeable in the displacement space. In fact, since these end loops were not surrounded by special structures, they had a lot of flexibility that could slow down the interaction with the receptor. Despite the fact that this was the position of the connected areas, even in the complex state, the residues were fluctuated a lot, which in turn reduced the stability of the complex (Fig. 3B).

Evaluation of hydrogen bonds. Evaluation of the number of hydrogen bonds showed that there was an average of 27 hydrogen bonds between the recombinant chimeric toxin and its receptor. The number of hydrogen bonds was fluctuated between 14 and 40 during the simulation time. In general, after the start of the

simulation, the number of hydrogen bonds was stable and slightly increased, which indicated that the complex conditions were favorable in terms of the number of hydrogen bonds (Fig. 3C).

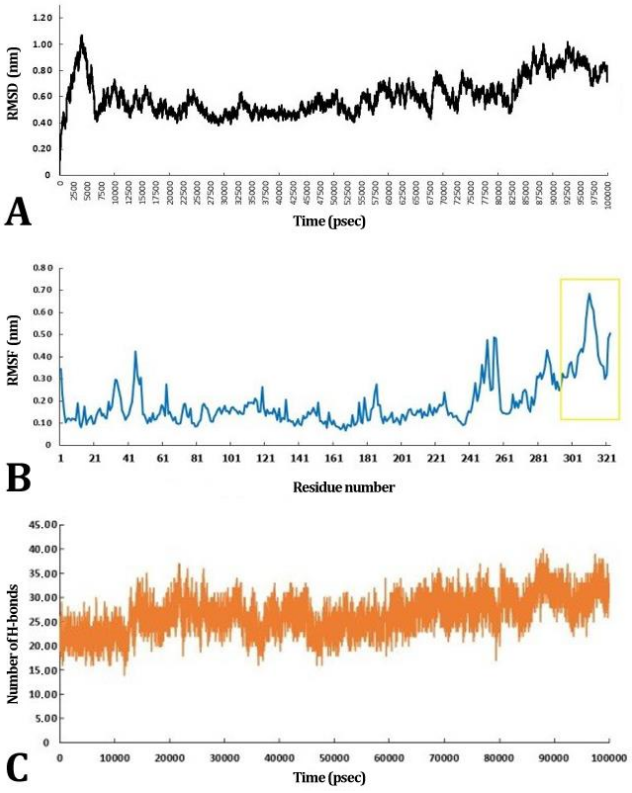

Fig. 3. A) RMSD plot. The RMSD plot shows that the complex has undergone many changes during the simulation time. RMSD ranges from about 0.40 to just over 1.00 nm, indicating relatively variable environmental changes at complex conformation. **B)** RMSF plot. This analysis shows the spatial changes of alpha carbon of each amino acid over time and is a measure of the flexibility of the residues. In fact, the larger RMSF number for a residue means the more conformational change of the residue and greater displacement in space. **C)** Hydrogen bonds plots.

Secondary structures of chimeric toxin. Generally, the secondary protein structures were more stable in the N-terminal half of the protein (approximately residues 1 to 165). Due to the three-dimensional shape of the protein, these regions had a large number of α-helix and β-sheet, which with proper compaction formed a stable nucleus in the protein. Conversely, the C-terminal half of the protein showed noticeable structural fluctuations with changes in helixes to turns and beta sheets to coils. The most important area for us was the CPE part, main structure between the coil and the bend, which were the second irregular structures, therefore, it was clear that this area was not taken on a regular structure during the simulation. This observation confirmed the high RMSF in this area as seen in the previous interpretation (Fig. 4).

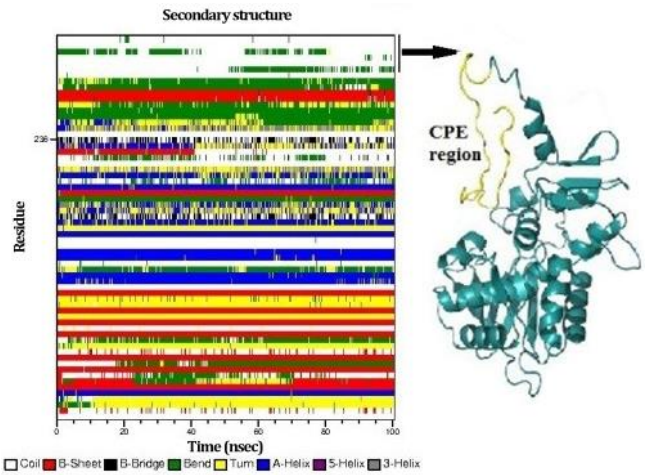

Fig. 4. Secondary structure of recombinant chimeric toxic during the simulation time.

Discussion

In general, the design of chimeric toxins targeting antigens whose expression increases during the process of cancer is a powerful strategy to fight cancer. One of the most important successful factors in such an approach is the correct selection of target molecules as chimeric toxins receptors. The claudin family has unique characteristics for this purpose which most probably the important feature is the higher expression of proteins of this family in a very high number of cancers.[6,7] In other words, in 9 out of the 12 types of cancers its over-expression was evident. This has a direct effect on the specificity of such treatment. Another interesting feature is that claudin family proteins often appear on the apical surfaces of cancer cells. In healthy cells, however, these proteins are found only in the intercellular spaces between adjacent epithelial cells. This means that even if the amount of proteins in this family does not increase significantly during the cancerous process, their location is such that they are still available for the targeting. One issue that may be considered as one of the disadvantages of selecting claudin proteins as chimeric toxins receptors, is its low antigenicity. In other words, there are few antibodies either native or synthetic that can bind to the extracellular loops of claudin proteins.[12,25] Therefore, the options that they can be selected as targeting molecules are very limited and are probably limited to the C-terminal of the CPE.

As stated in the result section, according to docking data, it could be presumed that the chimeric toxin had a very high affinity for binding to its specific receptor. One possible explanation could be the alteration of the binding region of chimeric toxin - the last 30 amino acids of CPE - from the beta strand to a loop. This change in structure leads to more flexibility in the structure of the interaction interface. Therefore, this area becomes more suitable for connecting to the receptor and effective connection occurs.

However, it also raises issues. The loop structure is a more unstable structure than the beta strand. However, this may cause problems in further steps of bioinformatics calculations in particular molecular dynamics simulation. Extending the time of the molecular dynamic simulation may make the results clearer. To prevent this situation, it is suggested that it is better to select the entire C-terminal of the CPE (from amino acids 194 to 319) rather than the last 30 amino acids.

Another important point is that the designed chimeric protein must not only bind to its specific receptor on the surface of cancer cells, however, must also be able to somehow transported into the cell due the fact that the enzymatic or toxic component of the toxin has to reach the target cell ribosomes and then kill the cell by inhibiting protein synthesis. This contradiction did not pose a problem in the process of the current research. It is worthwhile to note that tight junctions despite the high precise organizations are dynamic and undergo structural changes.[26] These structural alterations or remodeling lead to changes of two adjacent cells connection. This is a relatively common response at the time cells encountering some situation such as pathological, bacterial toxins, pathogens, and precursor cytokines conditions. In other words, it can be said that after exposure to bacterial toxins, their endocytosis will be mostly likely. Despite the possible proposed mechanism, the precise process of endocytosis has not been yet clearly elucidated. However, two suggested endocytosis mechanisms are clathrin-dependent endocytosis in some cell lines, or micro-pinocytosis which in case of claudin-4 is supposed to be clathrin-dependent endocytosis.[27,28]

In comparison between monoclonal antibodies in cancer therapy and chimeric toxins it should be mentioned that even though antibodies have shown great success and high rate in various cancer treatment, but, they also impose adverse effects such as hypertension, kidney damage, production cost, weakness, headache, nausea, vomiting and diarrhea.[29] It seems research and pharmaceutical companies show high interest for optimized and engineered small protein drugs with more specific, less toxic and more cost-effective properties. Based on the computational results and the affinity and stability of the designed chimeric toxin for claudin-4 receptor we suggest experimental assessments such as cellular activity and mechanism of action and the possibility of its endocytosis and entry into the cell.

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Conflict of interest

No conflicting interests and no funding in connection with this paper are applicable.

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