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In silico Design and Evaluation of Novel Cell Targeting Melittin-Interleukin-24 Fusion Protein: A Potential Drug Candidate Against Breast Cancer

(Reka Bentuk *in silico* dan Penilaian Penyasaran Sel Baru Protein Gabungan Melitin-Interleukin-24: Calon Dadah Berpotensi Melawan Kanser Payudara)

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ABSTRACT

Fusion proteins are designed to achieve new functionality or improved properties synergistically by incorporating multiple protein domains into one complex. The fusion of two genes to translate a recombinant protein for cancer treatment can enhance the bioactivity of drug and can introduce novel drug candidate with wide range of applications in pharmaceuticals and biotechnology. Interleukin-24 (IL-24) is a novel cancer growth-suppressing and apoptosis inducing cytokine while melittin is a natural honeybee derived cationic polypeptide having anti-tumor activity against breast cancer cells. The current study was aimed to perform *in silico* design and analyses of a melittin-IL-24 fusion protein against breast cancer. The amino acid sequences of the IL-24 and melittin peptide were used to design the fusion protein via a rigid linker. Using the online softwares we predicted the secondary and tertiary structures along with physicochemical properties of the designed fusion protein. The validation and quality of the fusion protein was confirmed by Rampage and ERRAT2. The top ranked structure from I-TASSER showed 18.1KD molecular weight by ProtParam, quality factor of 94.152 by ERRAT and a valid structure by Ramachandran plot with 88.5% residues in favoured region. The docking and simulation studies were performed using ClusPro and Desmond software. The quality, validity, interaction analysis and stability of the fusion protein depicted a functional molecule. The *in silico* analysis finding and expression predicted value of 0.86 in *E. coli* on SOLUPROT tool suggest that the melittin- IL-24 fusion protein can lead to develop a potent therapeutic drug against breast cancer.

Keywords: Breast cancer; fusion protein; interleukin 24; melittin; molecular docking

ABSTRAK

Protein gabungan telah direka bentuk untuk mencapai fungsi baharu atau sifat yang dipertingkatkan secara sinergi dengan menggabungkan berbilang domain protein ke dalam satu kompleks. Gabungan dua gen untuk menterjemah protein rekombinan untuk rawatan kanser boleh meningkatkan bioaktiviti dadah dan boleh memperkenalkan calon dadah baharu dengan pelbagai pengaplikasian dalam farmaseutikal dan bioteknologi. Interleukin-24 (IL-24) ialah sitokin penekan pertumbuhan kanser baharu dan apoptosis manakala melitin ialah polipeptida kation perolehan lebah madu semula jadi yang mempunyai aktiviti anti-tumor terhadap sel kanser payudara. Kajian semasa ini bertujuan untuk menjalankan reka bentuk *in silico* dan analisis protein gabungan melitin-IL-24 terhadap kanser payudara. Jujukan

asid amino IL-24 dan peptida melitin digunakan untuk mereka bentuk protein gabungan melalui penghubung tegar. Dengan menggunakan perisian atas talian, kami meramalkan struktur sekunder dan tertiari bersama-sama dengan sifat fizikokimia reka bentuk protein gabungan. Pengesahan dan kualiti protein gabungan telah disahkan oleh Rampage dan ERRAT2. Struktur kedudukan teratas daripada I-TASSER menunjukkan berat molekul 18.1KD oleh ProtParam, faktor kualiti 94.152 oleh ERRAT dan struktur yang sahih oleh plot Ramachandran dengan 88.5% residu di kawasan yang digemari. Kajian dok dan simulasi dilakukan menggunakan perisian ClusPro dan Desmond. Kualiti, kesahan, analisis interaksi dan kestabilan protein gabungan menggambarkan molekul fungsian. Penemuan analisis *in silico* dan pengekspresan nilai ramalan 0.86 dalam *E. coli* pada perkakas SOLUPROT menunjukkan bahawa protein gabungan melitin-IL-24 boleh membawa kepada pembangunan ubat terapeutik poten terhadap kanser payudara.

Kata kunci: Dok molekul; interleukin 24; kanser payudara; melitin; protein gabungan

Introduction

The cancer describes hundreds of malignancies affecting the cells and tissues of different organs characterized by proliferating abnormal cells in uncontrolled manner (Ghavimi et al. 2020). The World Health Organization (WHO) has warned about the mortality rate to reach up to 80% in the next 10 years (Keshtvarz et al. 2021). Breast cancer is ranked second among all other types of cancer after lung cancer. The principle heterogeneous development of breast cancer mechanism is still uncertain genetically and histopathologically (Sahna, Cakir & Tunali-Akbay 2022).

The clinical strategies employed for breast cancer treatment include chemotherapy, surgery, biotherapy, and radiotherapy. However, these available therapies are seemed to be less efficient due to serious side effects and poor prognosis (Gajski & Garaj-Vrhovac 2013). Additionally, the conventional anti-cancer therapeutic drugs have limitations with deleterious effect on healthy body cells and develop drug resistance (Chabner & Roberts 2005). Few drugs may also lead to secondary tumor development although the resting and slow growing cells are not affected (Naumov et al. 2003). The continuous growing prevalence of breast cancer demands the development of new drug candidates with less side effects, targeted delivery and enhanced anti-cancer activity.

Cytokines possess a promising regulatory role in immune system and have extensively been used as potent anti-tumor agents. Among all cytokines, interleukin-24, interleukin-15 and interleukin-2 exhibit the most efficient anti-tumor activity (Balimane & Chong 2005). But the requirement of high dose administration has limited the choice of interleukins to boost immune system. One solution is adopting the targeted drug delivery method in cancer treatment. The targeted approach not only selectively kills the cancerous cells but also prevents the chemo resistance mechanism and increases the drug efficiency (Smith et al. 2000).

The use of natural peptides as anti-tumor agent has the advantage of enhanced specificity and less toxicity (Sato et al. 2006). The strategy of fusing homing peptide with a killer peptide in cancer pharmacotherapy has the advantage of high selectivity, good solubility, targeted delivery and less toxicity which suggests the use of peptides as a suitable candidate for drug designing (Ghavimi et al. 2020). Recombinant DNA technology has made possible to fuse the gene of a homing peptide with a killing peptide gene to take advantage of dual function of single polypeptide chain i.e., targeting and killing tumor cells. Additionally the synergic therapeutic effect of this recombinant protein is increased up to 10-20 fold (Chen et al. 2005).

Among interleukin-10 family members, interleukin-24 (IL-24) plays an important role in inflammation, autoimmune diseases and action against infections (Cruz et al. 2016). IL-24 generates the signal for apoptosis after its specific interaction with a heterodimer receptors molecules (IL-20R1/IL-20R2 and IL-22R1/IL-20R2) present on MCF-7 cells which initiates signal transduction pathway (JAK-STAT signal pathway) (Kisseleva et al. 2002; Lubkowski et al. 2018).

In traditional medicines, the honeybee products are extensively used to reduce toxicity of other drugs including anti-viral, anti-parasitic, anti-bacterial and anti-tumor activity against breast cancer (Lim & Lee 2015; Lyu, Fang & Li 2019). Melittin, a peptide derived from honeybee venom is an amphoteric, highly water soluble and cationic polypeptide of 26 amino acids comprised of 40-50% of total dry weight of honeybee venom. Its anti-tumor activity as a killing peptide had been reported previously against breast cancer cells (Ip et al. 2008; Mir

Hassani et al. 2021). Melittin disrupts phospholipids in cell membrane of cancerous cells by activating the enzymes phospholipase-A_2 and phospholipase-D. This upsets the membrane integrity due to pore formation and eventually cell lysis occurs (Lee et al. 2001). Beside cell membrane disruption, melittin attacks on tumor cells by inhibiting calmodulin. This disturbs polyamine transport into the cell and affects physiological mechanisms of membrane potential, protein transport, pH and transmembrane ion gradient (Lyu, Fang, and Li 2019).

Fusion of cytokine with a killing peptide may enhance the antitumor activity due to synergic effect against breast cancer. Therefore, in this study, we aimed to design theoretically a bifunctional peptide containing a targeting domain and a killing domain. For this, a killing peptide called melittin was computationally joined with a targeting peptide IL-24 to generate a melittin-IL24 fusion peptide. This peptide was docked to heterodimer receptor (Interleukin 22 Receptor Subunit Alpha-Interleukin 20 Receptor Subunit Beta (IL22RA-IL20RB)) present on breast cancer cells. Finally, after performing the simulation, the results were analyzed to evaluate its therapeutic potential.

Materials and Methods

Construction of melittin- IL-24 fusion protein

The amino acid sequence of human IL-24 in FASTA format was retrieved from NCBI database (https://www.ncbi.nlm.nih.gov/protein/AAH09681.1?report=fasta). For melittin, FASTA format of amino acid sequence was obtained from Universal Protein Knowledgebase (UniProtKB, http://www.uniprot.org/) database. The three dimensional (3D) crystalline structure of soluble IL-24 heterodimer receptor (PDB ID: 6DF3, IL-22RA and IL-20RB) was retrieved in PDB file format from Protein Data Bank (PDB) online server (https://www.rcsb.org/structure/6df3) (Table 1).

To design the fusion construct, we need mature polypeptide chain of IL-24 which was generated by removing the signal peptide (1-52 amino acids) from N-terminal and the remaining chain (53 to 207 amino acids) was fused with melittin. Both peptides were linked via a rigid linker reported by Zhao et al. (2019).

Secondary structure prediction of fusion construct

FASTA format of newly constructed fusion peptide was submitted to online server GOR IV (http://gor.bb.iastate.edu/) for secondary structure prediction. Functional characteristics including alpha helix, beta-sheets, coiled-coil domain, low-complexity sections, regions with abnormal structure, disulfide bridges location and solvent accessible area were also assessed as described by Moghadam et al. (2019).

TABLE 1. Primary sequence of proteins

Peptide	Amino acid sequence	No of Amino acid	Reference
Interleukin-24	QEFHFGPCQVKGVVPQK	155	https://www.ncbi.nlm.nih.gov/protein/
	WEAFWAVKDTMQAQDNIT		(GenBank: AAH09681.1)
	SARLLQQEVLQNVSDAES		
	CYLVHTLLEFYLKTVFKN		
	YHNRTVEVRTLKSFSTLA		
	NNFVLIVSQLQPSQENEM		
	FSIRDSAHRRFLLFRRAFK		
	QLDVEAALTKALGEVDI		
Linker	EAAAKEAAAKEAAAK	15	Zhao, Liu et al. 2019)
Melittin	GIGAVLKVLTTGLPALIS	26	http://www. uniprot.org/
	WIKRKRQQ		(PDB ID: 6DST)

HOMOLOGY MODELLING FOR 3D STRUCTURE PREDICTION, VALIDATION AND QUALITY ASSESSMENT

Primary sequence of fusion protein in FASTA format was submitted to I-TASSER online server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Roy, Kucukural & Zhang 2010; Yang et al. 2015; Zhang 2008) to generate a reliable 3D structure. For quality assessment and structure validation, a series of online servers were used including Verify3D (http://services.mbi.ucla.edu/Verify_3D) (Bowie, Lüthy & Eisenberg 1991) to verify the 3D atomic model compatibility with its corresponding amino acid, ERRAT2 (https://saves.mbi.ucla.edu/results?job=1033540&p=errat) to get the overall quality factor (OQF), ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php) (Wiederstein & Sippl 2007) to calculate the Z-score and local quality estimate and finally Rampage server (http://mordred.bioc.cam.ac.ukrapper/rampage.php) (Pourhadi et al. 2019) to generate the Ramachandran plot.

STRUCTURE REFINEMENT

The 3D structure of melittin-IL-24 fusion protein was refined by Galaxy Refine online server (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE). For protein side chain reconstruction, repacking, and 3D structural relaxation, this server uses molecular dynamic (MD) simulations and the CASP10 refinement approach. Structures refined by this server has improved local and global residue quality and can be proceeded to further analysis (Ko et al. 2012).

PHYSIOCHEMICAL PARAMETERS AND SOLUBILITY OF MELITTIN-IL-24 FUSION PROTEIN

Physiochemical properties were calculated on Protparam (https://web.expasy.org/protparam/). This online server deduces the properties using FASTA amino acid sequence of proteins. Parameters including molecular weight, isoelectric point (pI), aliphatic index, extinction coefficient, half-life and average hrdopathicity (GRAVY) were estimated to predict the fusion protein behavior for *in vitro* studies (Gasteiger et al. 2005). For solubility prediction, FASTA sequence was submitted to proSol online server (https://prosa.services.came.sbg.ac.at/prosa.php) (Hebditch et al. 2017).

TOXICITY, ANTIGENICITY AND ALLERGENICITY PREDICTION

The toxic nature, allergenic property and antigenic behavior of melittin-IL-24 fusion protein were calculated by online servers of ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/), AlgPred (https://webs.iiitd.edu.in/raghava/algpred2/) and VaxiJen (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html), respectively. ToxinPred estimates the total number of toxic regions with amino acid sequence in submitted FASTA sequence. AlgPred is a kind of BLAST that works on the principle of searching similarity between submitted sequence and known epitope database. VaxiJen server predicts antigenicity based on the physico-chemical parameters of fusion protein. A threshold of 0.5 was selected for antigenicity prediction (Doytchinova & Flower 2007; Saha & Raghava 2006).

MOLECULAR DOCKING AND INTERACTIONS

To understand the interactions and binding orientation between melittin-IL-24 fusion protein and its heterodimeric cognate receptor (IL22R1-IL21R2), we engaged ClusPro 2.0 (https://cluspro.bu.edu/login.php) online server for docking (Kozakov et al. 2017). For docking study, the crystalline structure of heterodimer receptor was downloaded in PDB format (PDB ID: 6DF3). The downloaded protein structure was purified on Pymol graphic system by removing the attached IL24, ligands and water molecules. 3D structure of fusion protein and receptor, in PDB format, was submitted to ClusPro server using default settings. The finally obtained docked complex was analyzed for protein-protein interaction on PDBsum, PDBePISA and PRODIGY. These software identify salt bridges, hydrogen bonds, interacting interfaces, binding affinity, solvation energy (kcal/mol), tunnels and pores present in the docked complex (DeLano 2002; Laskowski 2009). Finally for binding affinity calculation, prodigy online server (https://wenmr.science.uu.nl/prodigy/) was used (Vangone & Bonvin 2015).

MD SIMULATION

Molecular dynamics (MD) simulation is a powerful computational technique used to study the behavior and properties of molecules and materials at the atomic level. Desmond is a software package developed by Schrödinger for MD simulations of biological systems, including proteins, nucleic acids, and membranes.

The stability of protein-protein interactions was investigated through molecular dynamics (MD) simulations. Specifically, MD simulations were carried out on the most stable complexes with high binding energies. To determine the dynamic binding behavior and binding stability of protein-inhibitor complexes in their

docked poses, MD simulations were performed using the Desmond module of Schrödinger. Simulation was run for 100 ns at 1 atm and 300k with NPT ensembles. The RMSD, RMSF, total energy of the complex, Rg and SASA values were analyzed. A detailed description of the methodology can be found elsewhere (Gul et al. 2022; Rehman et al. 2023a, 2023b).

SOLUBLE EXPRESSION PREDICTION IN *E. coli*

To predict the soluble expression of fusion protein in *E. coli*, the FASTA sequence was submitted to online server of SoluProt (https://loschmidt.chemi.muni.cz/soluprot/) (Hon et al. 2021). Ghomi, Kittilä and Welner (2020) claimed that the performance and accuracy of SoluProt exceeds all other currently available tools for soluble expression prediction.

Results and Discussion

According to World Health Organization, breast cancer is among the top ranked of cancers which cause the highest number of deaths annually (Lischer et al. 2021). Available treatment options for cancer are limited and will be less accessible in future to public due to annual sharp rise in cases and expansion in cancer mechanism. Hence, a specific drug candidate with enhanced targeting as well as killing property is required (Trinidad-Calderón, Varela-Chinchilla & García-Lara 2021). Among novel therapeutic drugs, the fusion proteins derived from natural sources are particularly important due to their domain modulation option with different architecture for desired functional characteristics as observed in natural proteins during phenomena of evolution (Long 2000; Yu et al. 2015). Recent advances in bioinformatics have made possible to evaluate the therapeutic potential of fusion proteins against bimolecular targets for cancer treatment through their *in silico* studies (Maurya, Kushwaha & Mani 2019).

Cytokines are natural peptides that make chemical bridges between different cells of immune system by working as molecular messenger. They generate a robust, self-limited, and synchronized response to propagate efficient immune signals in target cells via specific receptors. In the last two decades, the dynamics of cancer research has been diverted to explore the characteristics and molecular signaling networks of cytokines (Shafique et al. 2021). Previously, interleukin-peptide fusion proteins have successfully been evaluated for structural and functional analysis via computational approach which lead to their *in vitro* expression and purification. For example, significant activities have been reported by interleukin 24-BR2 peptide against MCF-7 (Pourhadi et al. 2019), interleukin 24-LK6 fusion peptide against breast cancer (Muhammad Rehman et al. 2023), interleukin 24-p28 peptide against breast cancer (Ghavimi et al. 2020; Jahanian-Najafabadi, Ghavimi & Akbari 2020), interleukin 24-NRC peptide against breast cancer (Soleimani et al. 2016), TAT- interleukin 24-KDEL peptide for tumor apoptosis and interleukin 24-RGD peptide against MCF-7 cancer cells (Xiao et al. 2009). Yang et al. (2007) found a high yield of interleukin-24 soluble expression in pET32a vector instead of pET28a and 22b.

Melittin is a honey bee venom derived natural peptide previously reported with many biological activities (Mir Hassani et al. 2021). Melittin alone or in fusion with other peptide has shown potent anti-tumor activity against astrocytoma cells (Hait et al. 1985), leukemic cells (Killion & Dunn 1986), lung cancer cell (Zhu et al. 1991), osteosarcoma cell lines (Chen et al. 2004), hepatocellular carcinoma (Hu et al. 2006), ovarian cancer cells (Jo et al. 2012; Li et al. 2004), melanoma cancer cells (Liu et al. 2002), glioma cells (Yang et al. 2007) and breast cancer (Lischer et al. 2021). In this study, we computationally fused human interleukin 24 as a targeting domain with melittin as a cytolytic domain to construct a novel peptide and evaluated it *in silico* activity against breast cancer cells.

CONSTRUCTION OF FUSION PROTEIN

The primary sequence of fusion protein is composed of a total of 196 amino acid residues and encoded by a gene length of 588 nucleotides. Homo sapiens IL-24 and *Apis mellifera* (honeybee) venom derived melittin peptide are consist of 155 and 26 amino acids, respectively. The mature part of IL-24 and melittin were computationally fused via a rigid liker of 15 amino acids. Figure 1(A) and 1(B) represents the FASTA sequence and schematic diagram of amino acid residues in fusion protein which was further modified at C and N terminal for its *in vitro* expression studies.

SECONDARY STRUCTURE PREDICTION

The secondary structure of fusion protein was predicted using GOR IV to identify the regions of alpha-helix and beta-sheets in sequences. Based on the result, melittin-IL-24 fusion protein consisted of 54.59% (107 residues) alpha helix, 17.35% (34 residues) extended strands and

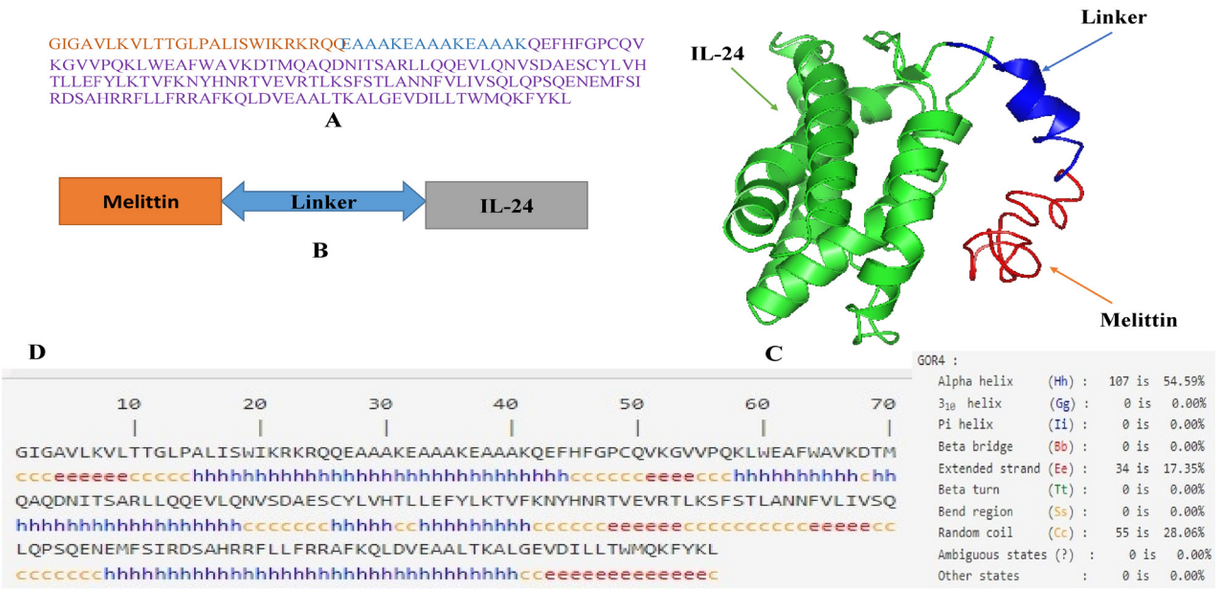

FIGURE 1. Structural illustration of melittin-IL-24 fusion protein

28.06% (55 residues) random coils (Figure 1(D)). The secondary structure analysis indicated the presence of more alpha helix conformation in fusion complex which imparts more stability (Sakurai et al. 2005). The linker was predicted to attain an alpha helix conformation that may impart additional stability to the fusion protein.

TERTIARY STRUCTURE EVALUATION AND QUALITY ASSESSMENT

The 3D conformation of fusion protein was predicted on I-TASSER online server. It works on the principle of segment matching and structural complementary of templates for improved model quality (Chakravarty et al. 2008). I-TASSER constructed 5 top models depending on their c-score (Table 2). A higher c-score value signifies a more valid structure with high confidence (Zheng et al. 2021). The first model with highest C-score of -1.06, TM-score of 0.58 and RMSD of 7.6 was designated as the most reliable structure and that top construct was proceeded further for quality and validation evaluation (Figure 1(C)).

The created template chimera was passed through quality and validation checks by multiple programs including ERRAT2, Verify 3D, ProSa-Web and Ramachandran plot. The 3D structure of newly constructed fusion protein was submitted for quality validation through Ramachandran plot. The protein model of melittin-IL-24 fusion showed 87.6% (162 residues) of residues were in allowed region, 8.6% (16 residues) of residues were in additionally allowed region, 1.6% (3 residues) of residues were in generously allowed region and 2.2% (4 residues) of residues were in disallowed region. Total number of glycine and proline residues in primary sequence was 6 and 4, respectively (Figure 2(A)). Quality assessment graph by Verify 3D program designated a status of 'PASS' for the designed fusion protein. The results showed that 87.24% of amino acid residues have 3D-1D score ^{3} 0.2 which predicts a good fusion protein for its further evaluation (Figure 2(E)).

ERRAT2 program calculated the score of 87.23 for the modelled fusion protein. Figure 2(D) shows the yellow and red areas which exceed the confidence limits of 95% and 99% of error, respectively. Moreover, the graph of local model quality illustrates that most of amino acid residues presence on positive side with (Figure 2(B)) Z-score graph of -5.27 (Figure 2(C)) validates and predicts the well-defined protein structure.

PREDICTED PHYSICOCHEMICAL PARAMETERS AND SOLUBILITY OF FUSION PROTEIN

Protparam computed the physiochemical properties of melittin-IL-24 fusion protein as summarized in Table 3. The total number of basic amino acids, arginine and

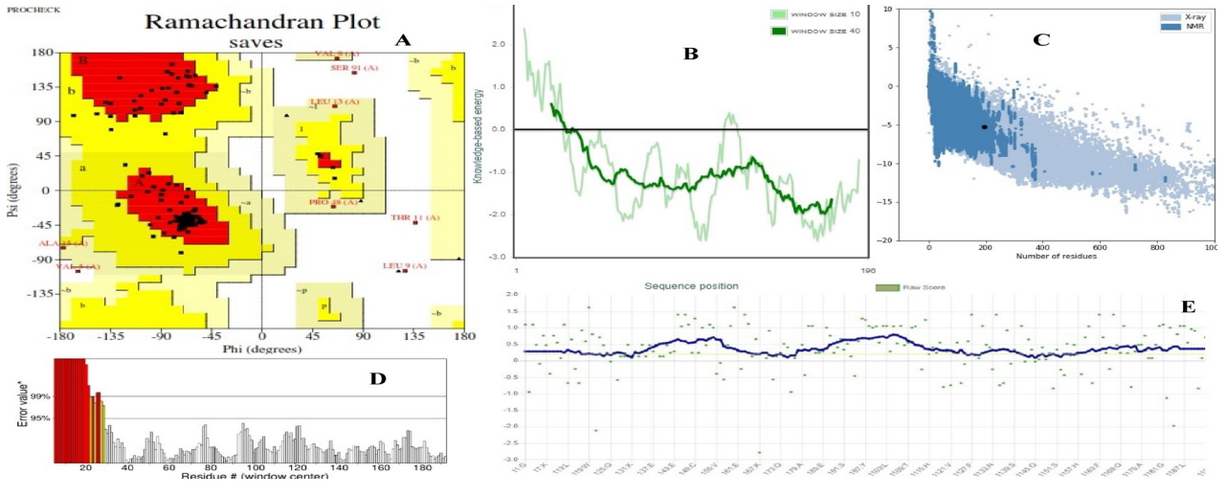

FIGURE 2. Quality assessment and validation graph of the melittin-IL24 fusion protein

TABLE 2. C-score of top 5 models predicted by I-TASSER

Model	C-sore	TM-value
1	-1.06	+0.58
2	-1.79	-
3	-2.55	-
4	-2.51	-
5	-2.23	-

lysine residues, was 24 while acidic amino acids, aspartic acid and glutamic acid was 16 with isoelectric point of 9.46 imparting alkaline characteristics to melittin-IL-24 fusion protein. Leucine (12.2%) was found to be the highest number of amino acid. Other calculated parameters included molecular weight, extinction coefficient, predicted half-life in prokaryotic cell, GRAVY, aliphatic index and instability index. ProtParam predicted the hydrophilic nature of fusion protein due to abundance of polar amino acid residues. The extinction coefficient (ε_{280}) for protein-protein interaction and aliphatic index which is an indicator of protein stability, were 28085 ($M^{-1}cm^{-1}$) and 96.58, respectively. Additionally, the ability of fusion complex to react in water or GRAVY index was -0.118. The isoelectric point which describes the zero net charge state of fusion protein was 9.46. Moreover, the estimated half-life in prokaryote, yeast and eukaryotic cells was >10h, >20h and 30h, respectively. Reasonable half-life in prokaryotes, increased solubility and good soluble expression prediction in *E. coli* proposes *in vitro* evaluation of melittin-IL-24 protein by gene cloning and expression studies.

To estimate solubility, the primary FASTA amino acid sequence was processed by protein-sol program. ProSol web server calculates protein solubility by comparing the query sequence predicted solubility with the average solubility of population dataset in *E. coli*

(Hebditch et al. 2017). It compares the estimated results with its database whereby predicting any solubility value greater than 0.45 (threshold) is considered soluble (Hebditch et al. 2017). The theoretical solubility value of under consideration protein was 0.552.

TOXICITY, ALLERGENICITY AND ANTIGENICITY EVALUATION

Melittin-IL-24 fusion protein was predicted as non-toxic, non-antigenic and non-allergen by ToxinPred, VaxiJen and AlgPred programs, respectively. No peptide residues exhibited toxicity and overall protective antigen score was 0.4137. For docking, 3D structure was subjected to structure perturbation, overall structural relaxation, and energy minimization by MD simulation on GalaxyRefine online server.

DOCKING ANALYSIS

Docking was performed on ClusPro online portal to elucidate the interaction mechanism between melittin-IL-24 protein with its cognate heterodimer receptor. The major types of interactions involved in protein-protein docked complexes are non-covalent bonds including electrostatic and van der Waals forces which mutually contribute to maintain the stereochemistry of protein (Mandell et al. 2001). ClusPro works on the principle of Fast Fourier Transform Correlation method which computes and ranks the docked complexes in three steps. Firstly, with the help of PIPER, rigid docking is performed by sampling the conformations in billions. Secondly, it generates the clusters of one thousand lowest energy structures based on RMSD (root mean square deviation) to predict the largest cluster and presents most likely docked complex. Thirdly, the selected docked model is stabilized through energy minimization step (Kozakov et al. 2017). The output page of ClusPro displayed ten (10) different docked complexes starting from 0 to 9 which were ranked on the basis of energies and cluster size. Table 4 shows the energies of all 10 models along with cluster size while Figure 3(B) shows the stable docked complex of fusion protein with IL-24 heterodimer receptor. The negative energy values in kcal/mol are related to stability of complex as proteins attain most stable structure at their lowest energy state. Thus, the most negative value of top ranked model for electrostatic and van der Waals forces is depicted as the most stable pose. Moreover, two (2) factors are important in binding strength of complex that are total area of interface and number of amino acid residues involved in interactions. Therefore, the complex with the lowest minimum energy (Model No. 0) was selected for further interaction studies.

TABLE 3. Melittin-IL24 fusion protein's physical and chemical properties

Physiochemical properties	IL24-Linker-LK6
Number of amino acids	196
Theoretical pI	9.46
Molecular weight	22404.00
Instability index	42.17
Aliphatic index	96.58
Total positively charged amino acids	(Arg + Lys): 26
Total negatively charged amino acids	(Asp + Glu): 19
Grand average of hydropathicity (GRAVY)	-0.118
Coefficient of extinction (in M^{-1} cm^{-1} at 280 nm)	28085
Half-life in prokaryotic cell	>10 Hrs

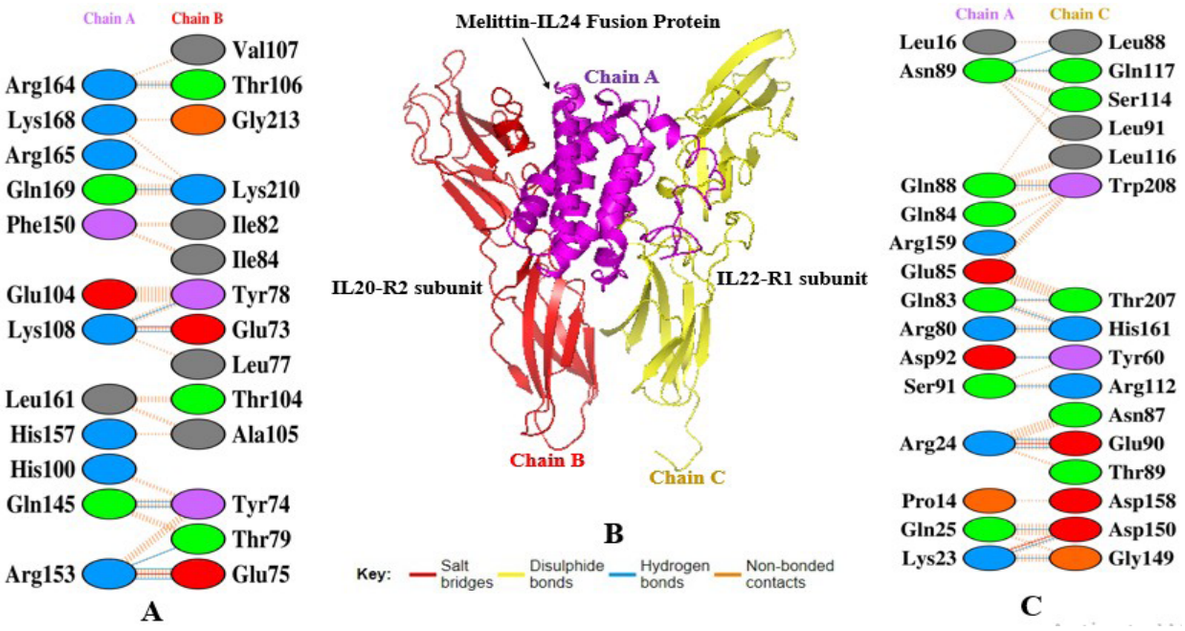

FIGURE 3. Docked complex of melittin-IL-24 fusion protein with receptor and binding residues

TABLE 4. Cluster scores of docked complexes

Cluster	Member	Representative	Weighted score
0	91	Center	-684.8
		Lowest Energy	-845.1
1	52	Center	-706.4
		Lowest Energy	-844.1
2	51	Center	-675.6
		Lowest Energy	-829.6
3	47	Center	-740.2
		Lowest Energy	-917.8
4	46	Center	-716.1
		Lowest Energy	-799.2
5	40	Center	-744.3
		Lowest Energy	-904.6
6	36	Center	-684.1
		Lowest Energy	-803.6
7	35	Center	-749.4
		Lowest Energy	-755.2
8	33	Center	-701.7
		Lowest Energy	-809.2
9	33	Center	-745.5
		Lowest Energy	-809.1

RESIDUE INTERACTION ANALYSIS

Theoretical estimation of binding energies between amino acid residues of protein-protein interface could be a challenging task. With advances in bioinformatics algorithms, today many software and online servers are available working by empirical and analytical approaches. However, the results of each module is somehow different from the other and the results depends on the submitted 3D structure of a particular protein (Lubkowski et al. 2018). The binding strength of fusion protein with its receptor depends on the length of interface area and number of amino acid residues contributed by both moieties for making non-covalent interactions i.e., more number of amino acids with longer interface area depicts stronger interaction and predicts stable conformation (Khan et al. 2021). According to PDBsum and PDBePISA, the primary complex formation is contributed by hydrogen bonds between fusion protein and heterodimer receptor as reported previously by Lubkowski et al. (2018) for IL-24 and its cognate receptor. We engaged PDBePISA and PDBsum programs to see insight of docked complex for analyzing the forces of attraction at molecular level (Figure 3(A) and 3(C)). Further, the energy of solvation during complex formation was calculated. A total of 34 interactions were predicted mediated by 28 hydrogen bonds and five (5) salt bridges (Table 5). The total interface area between IL20-R2 receptor subunit residues and fusion protein residues involved in interaction was 734.3 Å^2 with stabilizing energy of -7.3 Kcal/M including nine (9) hydrogen bonds contributed by Arg^{164}, Gln^{169}, Lys^{108}, Gln^{145}, Arg^{153} and Val^{107}, Thr^{106}, Lys^{210}, Tyr^{78}, Glu^{73}, Tyr^{74}, Thr^{79}, Glu^{75}, respectively. The stabilization via disulphide bonds were provided by Lys^{108}, Arg^{153} and Glu^{73}, Glu^{75} contributed by IL20-R2 receptor subunit and fusion protein, respectively. Additional complex stability was gained via binding interactions between IL20-R1 receptor subunit residues and fusion protein residues extended to an interface area of 891.6 Å^2 stabilizing energy of -6.2 Kcal/M. A total of 13 hydrogen bonds were predicted between IL22-R1 receptor subunit residues and fusion protein residues involving Asn^{89}, Gln^{88}, Gln^{83}, Arg^{80}, Asp^{92}, Ser^{91}, Arg^{24}, Gln^{25}, Lys^{23} and Leu^{88}, Gln^{117}, Trp^{208}, Thr^{207}, His^{161}, Tyr^{60}, Arg^{112}, Glu^{90}, Asp^{150}, Gly^{149}, respectively. The two (2) disulphide linkages between IL22-R1 receptor subunit and fusion protein was provided by Arg^{24}, Lys^{23} and Glu^{90}, Asp^{150}, respectively. Figure 4(A) and 4(B) illustrate the interacting surfaces and binding residues. PRODIGY online web service was employed to predict the binding energy (ΔG) and dissociation constant (K_d) of docked complex. The binding affinity decreases dramatically as Kd value increases with rise in temperature from 25 °C to 40 °C (Table 5).

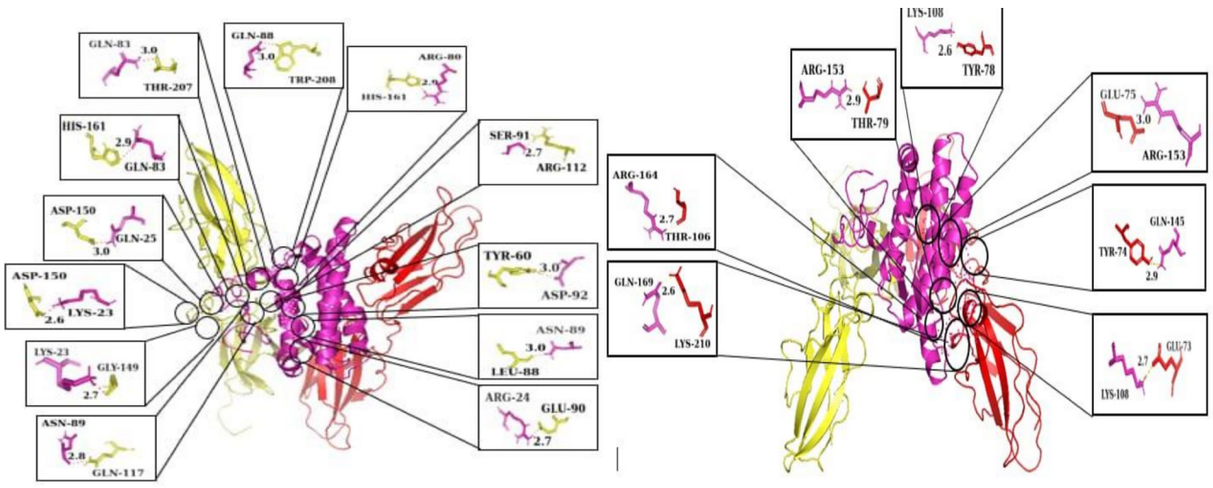

FIGURE 4. Interaction between fusion protein and heterodimer receptor chains

TABLE 5. Melittin-IL24 binding affinities and interface statistics of model No. 0

Chains	IA	IR	SB	DS	HB	NBC	Tunnel	Binding affinities	
A:B 13.8	688:662	12:14	2	-	9	58	-	ΔG **(kcal mol^{-1})**	-
A:C E-10	824:841	14:16	2	-	13	107	1	**K_d (M) at 25.0 °C**	7.6
B:C E-10	456:463	8:8	1	-	6	38	1	**K_d (M) at 40.0 °C**	2.3

IA-interface area (Å^2), IR-No of interface residues, DS- No. of disulphide bonds, SB-No. of salt bridges, HB- No. of hydrogen bonds, NBC- No. of non-bonded contacts

PREDICTION OF PROTEIN EXPRESSION IN *E. coli*

The online server of SOLUPROT 1.0 is a sequence-based prediction tool which estimates the soluble expression of protein in *E. coli.* Melittin-IL-24 fusion protein indicated a very high stable expression of fusion protein with solubility score of 0.860. The default threshold value of solubility is 0.5 with any value higher than 0.5 indicates soluble expression.

MD SIMULATION

Molecular dynamics simulations were performed on the top hits containing high binding energies. Over the simulation period, the projected conformational changes from the initial structure were presented in terms of root mean square deviation (RMSD). Moreover, structural stability, atomic mobility, and residue flexibility at times of interaction of protein-hit were expressed with root mean square fluctuation (RMSF) values (Figures 5 & 6). The peaks of RMSF graph represents the fluctuation portion of the protein through the simulation. The N- and C-terminal show more changes than any other portion of the protein. Alpha helices and beta strands show less fluctuation, as they are stiffer than the unstructured part of protein, than loop portion. The RMSD of the IL24-Melittin docked complex showed small deviation at almost 60 ns to 75 ns and then the system was converged throughout the simulation. It indicates the stability of the protein-protein complex and whether the simulation has equilibrated (Figure 5(A)). Similarly, the Root Mean Square Fluctuation (RMSF) is useful for characterizing local changes along the protein chain. For RMSF of IL24-Melittin docked complex, the fluctuations were in range of 3Å which indicated the stability (Figure 5(B)). The analyses of energy parameters for complex indicates that the total energy of the system was almost consistent and hence the complex get stable (Figure 5(C)). The solvent-accessible surface area (SASA) correlates for the molecular surface area being assessable to solvent molecules providing a quantitative measurement about the extent of protein/solvent interaction. The SASA values were from 28252 Å^2 to 31571 Å^2. There was rise in SASA initially indicating expansion of protein value in initial phase and after that it was consistent (Figure 6(A)). The radius of gyration (Rg) for the simulation trajectory provides information regarding the compressed nature of the protein, in which a higher Rg profile denotes less rigidity in the biological system. The value of Rg initially increased from 28 to 29.6 till 25 ns and after that it was consistent (Figure 6(B)).

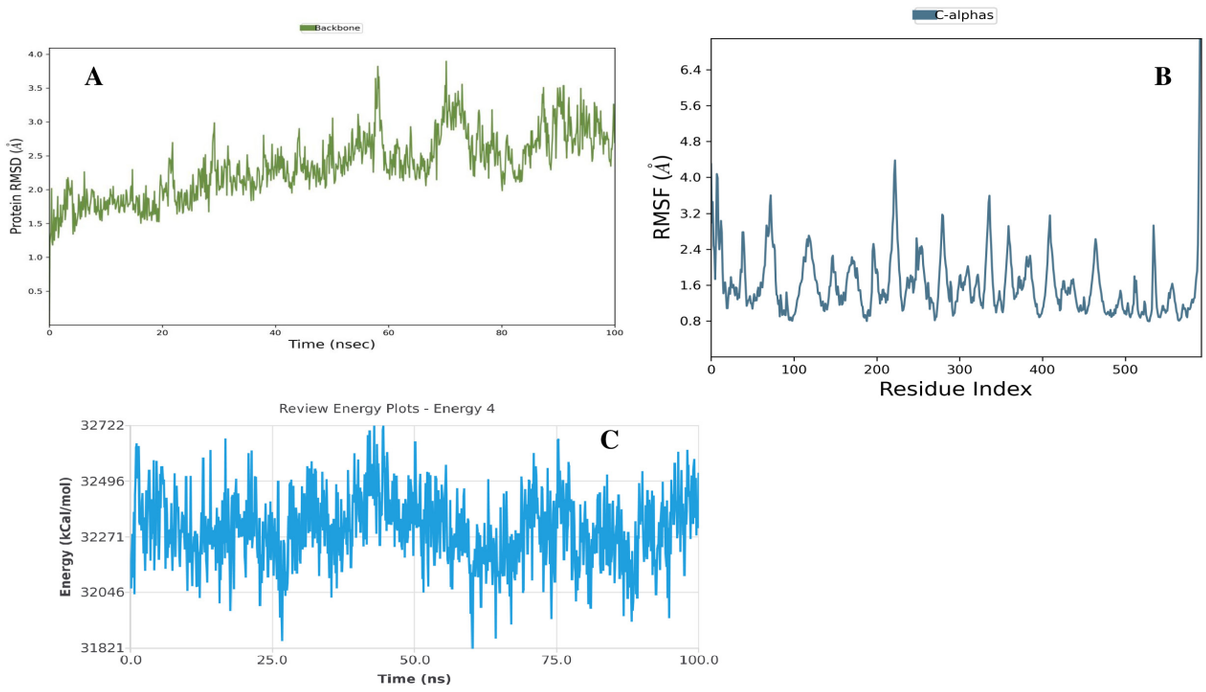

FIGURE 5. Simulation graphs of RMSF, RMSD and energy

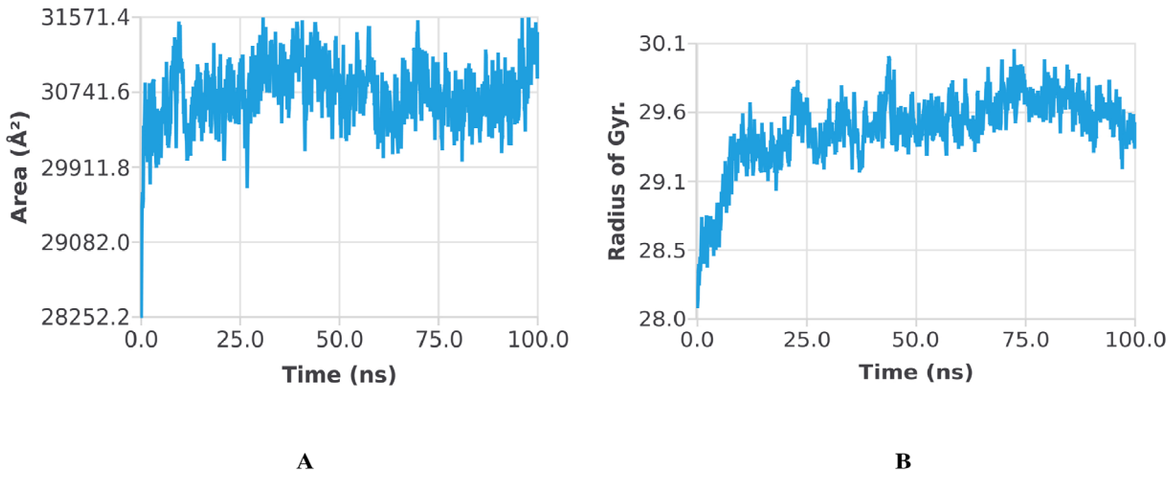

FIGURE 6. Simulation graphs of SASA and Rg

Conclusion

In conclusion, the present study highlighted the *in silico* prediction of the melittin-IL-24 fusion protein. Our findings showed that melittin-AEAAAKEAAAKA-IL-24 has a reliable 3D structure of fusion protein with good quality score, validated amino acid residues, enhanced solubility, good predicted *E. coli* expression, acceptable physiochemical properties, stable docked complex, multiple strong interactions, high receptor affinity and promising dynamics characteristics. Therefore, this fusion protein is anticipated to show better anticancer efficacy. However *in vitro* and *in vivo* studies are needed to assess their biological activity and cytotoxic effects. Studies are undergoing for further assessments of the melittin-IL-24 fusion proteins.

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