RESEARCH ARTICLE

IN SILICO STUDIES OF TUMOUR TARGETED PEPTIDE DERIVED FROM PROTEASE PRODUCED BY BACILLUS CEREUS HN FOR TARGETING WNT SIGNALLING PATHWAY USING MOLECULAR DOCKING AND MOLECULAR DYNAMICS

Sumaiya Hasnain[1] and Dr. M. Shaialaja Raj[2]
1. Student, Dept. of Microbiology, St. Francis College for Women, Uma Nagar, St. no. 6, Begumpet, Hyderabad - 500019, Telangana, India.
2. Head, Dept. of Microbiology, St. Francis College for Women, Uma Nagar, St. no. 6, Begumpet, Hyderabad - 500019, Telangana, India.

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Abstract

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Background: In today's world, it's becoming increasingly difficult to ignore the role of cancer in social health. Breast cancer remains the second global cause of death in women. Alterations disrupt normal cell function, cause cancerous cells to over-proliferate, and avoid mechanisms that might typically control their growth, division, and migration. A disruption in signalling pathways, a mutation that might lead to overexpression of certain genes that can, in turn, activate the signalling pathway and send it into an override mode that leads to diseases such as cancers. These signalling pathways can be targeted not only for use as biomarkers for the early detection of certain cancers but also for targeted treatment of cancers. The prospect of the development of new substances with antitumoral potential is of great importance for thetreatment of breast cancer.
Result: The objective of this work is to design a peptide from a laboratory-produced Protease enzyme isolated from *Bacillus cereus HN*and use that as a drug or ligand that could potentially bind to the LRP5 protein and inactivate the Wntsignalling pathway in triple-negative breast cancer cells.
Conclusion: The work was aimed at computational modelling of a safe peptide which followed the pharmacokinetic parameters and conducting docking analysis to assess the binding free energy between LRP 5 and the designed peptide. The docking results show that the ligand is a good candidate for novel drug development. The models created and analysed in this work will most certainly help in future research on targeting the Wntsignalling pathway and its components.

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Introduction:

Cancer is characterized by uncontrolled and rapid division of the cells. The current treatment modalities include the use of chemotherapeutics, radiation and surgery. These are not only cost-effective methods of treatment but also ones that can cause an array of side effects. The advent of next-generation sequencing and multi-omics analysis has given insight into the signalling pathways and their vast array of interactions and circuitries with the cells.

Corresponding Author:- Dr. M. Shaialaja Raj
Address:- Head, Dept. of Microbiology, St. Francis College for Women, Uma Nagar, St. no. 6, Begumpet, Hyderabad - 500019, Telangana, India.

Selective delivery of therapeutic agents to cancer cells will ensure the effectiveness of treatment while minimizing the damage to the surrounding healthy tissue. The abnormal proliferation of cells and decreased apoptosis can be consequences of a dysregulated signalling pathway. Targeting the specific receptors of these signalling pathways can lead to targeted drug delivery.

The Wingless-related integration site (Wnt)/β-catenin signalling pathway is a conserved axis that participates in various physiological processes such as proliferation, differentiation, apoptosis, migration, invasion and tissue homeostasis (Choi B, Cave C, Na C, Sockanathan S. 2020). Increasing evidence suggests that impairment of the regulatory mechanism of the Wnt/β-catenin pathway contributed to the progression and development of some solid tumours andblood malignancies (Zhang M., Weng, W., et. al 2018, Gajos-Michniewicz A, Czyz M. 2020, and Ge X, Wang X. 2010). Abnormal transcriptional regulation of β-catenin leads to early events in carcinogenesis. The β-catenin-dependent signalling pathway can be triggered following the interactionof Wnt ligands to the LDL Receptor Related Protein 5/6 (LRP-5/6) receptors and Frizzled(FZD) receptors. The binding of Wnt ligand and receptors induces dishevelled (DVL), which causes the aggregation of the complex composed of AXIN, Glycogen synthase kinase-3 beta (GSK3β),casein kinase 1(CK1), andAdenomatous Polyposis Coli protein (APC) to the receptor. Thereafter, the phosphorylation and inhibition of GSK3βensures an increase in the concentration of cytosolic β-catenin. Following this, the un-phosphorylated β-catenin present in the cytosol then migrates to the nucleus and accumulates, which then interacts with T cell-specific factor (TCF)/lymphoid enhancer-binding factor (LEF) and co-activators, such as Pygopus and B-cell CLL/lymphoma 9 (Bcl-9), to trigger the Wnt target genes like Cellular myelocytomatosis oncogene (c-Myc), cyclin D1 and cyclin-dependent kinase inhibitor 1A (CDKN1A), resulting in the upregulation ofT-cell factor/lymphoid enhancer factor(TCF/LEF) target gene (Zhang, Y., Wang, X; 2020)

Extensive research on peptides indicates their wide application across the therapeutic spectrum and their potential application in treating and diagnosing multiple cancers and tumour progressionas peptide therapy. These peptides are also used in imaging as well as in targeted drug delivery. Anticancer peptides are a good choice for selectively targetingtumour-specific receptors. Tumour-targeted peptides are known to have higher tissue penetrance than antibodies or their chemical modificationsalong with the benefit of increased stability and pharmacokinetics. (Thundimadathil J. 2012). Peptides can be used in a variety of ways for the treatment of cancers. They can be used fordrug delivery to cancer cells, upregulating or downregulating natural proteins to enhance or inhibit signal transduction. The swift resynthesis of peptides using automated methods along with the availability of rapid testing methods makes these peptides quite adaptable and desirable in the current therapeutic industry. (Sato, A. K. Viswanathan et. al., 2006)

The production of peptides is comparatively less complicated than that of proteins and antibodies. They are easier to transport and are simple, they can also be easily modified by coupling to different substances and therefore have a longer shelf life. (Tsuji A. 2005)

Studies are being conducted on the possible use of proteolytic enzymes to solubilize and necrotize the tumour tissues. In this study, we have used the in-silico method to study the anti-tumour property of protease. The analysis of antitumour activity is performed by targeting the Wnt/ β-catenin pathway with the peptide of isolated protease. The Wnt/β-catenin signalling pathway is associated with regulating the pluripotency, self-renewal of stem cells and differentiation ability (Takabe N et al., 2011). Abnormal activation of the Wnt/β-catenin pathway promotes Cancer stem cell (CSC) progression and thus leads to the deterioration and metastasis of cancer (Takahashi et al., 2010). LDL receptor-related proteins 5 and 6 are single transmembrane proteins that act as co-receptors of Wnt ligands which are crucial for Wnt transduction. Signal transduction is mediated by LRP 5 and LRP 6. It begins with Wnt binding to the frizzled receptor which leads to the recruitment of Dvl and Axin/GSK3 complex which triggers phosphorylation of LRP5/6 (Qian Ren et al., 2021). Thus, the blockade of LRP5/6 and Fzd complex formation may lead to new strategies in cancer treatment (Li, Y., & Bu, G. 2005). LRP5 acts as a co-receptor to Wnt with Frizzled family members and transduces Wnt-canonical signals which can be antagonized by the LRP5 ligand, Dickkopf 1 (Dkk1). Dickkopf (DKK)–related proteins inhibit the Wnt signalling pathway by directly binding to the ectodomains of LRP5.

The current study uses the protease isolated from Bacillus cereus HN.The bacteria were subjected to 16s rRNA molecular characterization for the identification of the bacteria. The retrieved sequence is then used to identify the protein and design the peptide for the study.

Methodology:

In silico molecular docking studies were performed on LRP 5 protein against the designed peptide of the Gamma-D-glutamyl-L-lysine dipeptidyl-peptidase (an acid endopeptidase; PDB ID 3H41)

Target Protein Selection and Homology Modelling

The NGS data of Homo sapiens is retrieved from NCBI. Upon studying the Wnt Signalling pathway proteins participating in the pathway are searched through Pubmedand the target protein is selected. The structure of LRP 5was downloaded from the protein database UniProt (UniProt accession no 017595). Swiss-PDB Visualizer was used to build missing side chains and residues.

Active Site Analysis of the target proteins

The target proteins were uploaded on CavityPlus, a web server that analyses and detects the potential active sites for ligand binding on the protein structure's surface (Xu, Y., Wang, S., Hu, et al., 2018).

Preparation of Ligands

The sequence attained from the 16S rRNA molecular typing was run through BLASTX. Gamma-D-glutamyl-L-lysine dipeptidyl-peptidase (an acid endopeptidase; PDB ID 3H41) was found to show an 89.2% identity score. A peptide (seq. L-ser – L- cys- L- alanine – D- Glu) belonging to the conserved DomainNlpC/P60 was designed using ACPred (Schaduangrat N, Nantasenamat C et. al, 2019) and ToxinPred(Gupta et. al. 2013)

In silico prediction of pharmacokinetic parameters

The designed peptide was analyzed to study the pharmacokinetic parameters such as Absorption, Distribution, Metabolism, Excretion and Toxicity on Drug Likeness Tool; DruLiTo (Geete A., Damre M., et. al.),pKCSM (Pires, D. E., et al., 2015)and OSIRIS property explorer (Sander, T. 2001). The peptide was evaluated based on Lipinski's rule of five for drug-likeness and graph-based signatures to study ADMET properties for drug development.

MolecularDocking Studies

Docking studies were carried out using Flare V4.0 (Cresset®, Litlington, Cambridgeshire, UK). The protein was uploaded and prepared. Hydrogen atoms were added, Gasteiger charges were assigned and the non-polar hydrogen atoms were merged. The peptide was loaded on the software and its role was assigned as the test ligand. Tamoxifen was taken as a reference ligand.

The active site is picked and the grid was built using a grid spacing of 10 A$^{\circ}$. Normal docking of the target protein with test and reference ligands is performed. The best poses are analyzed for the binding free energy (kcal/mol). The molecular interactions are studied using BIOVIA new discovery studios. (BIOVIA, Dassault Systèmes, [Discovery studios], San Diego: Dassault Systèmes, 2021).

Molecular dynamics simulation

The molecular dynamic simulation was done using the webserver WebGro (Jamroz, M., Kolinski, A. and Kmiecik, S. 2013) and CABS-Flex 2.0 (Jamroz, M., Kolinski, A. and Kmiecik, S. 2013) and CHARMM m27 forcefield is applied.The apo-forms of the protein structures and also the protein-ligand complexes were first put in the centre of a box with at least a 1.5 nm distance from the edges to the surface of the proteins. Then, The TIP4P system is used to fill thebox with water molecules. Sodium and chloride ions were added to neutralize the system with 150 nM of natural salt maintaining the physiological concentration. The temperature and pressure (Bussiet al., 2007 and Parrinello& Rahman, 1981) were maintained at 310K and 1 Bar respectively to provide stable environmental conditions. The number of cycles was set to 50. The solvated system is energy minimized by applying the steepest descent algorithm for 10000 steps

Results and Discussion:

Studies are being conducted on the possible use of proteolytic peptides to solubilize and necrotize the tumour tissues. In this study,in silico tools were used to design, characterize and dock the peptide against LRP 5 protein. The structure of the target protein was retrieved from the protein databank, optimized and the active sites were analyzed. The peptide was designed using machine learning (ML)and Deep learning (DL) toolsACPred and ToxinPredthat utilizes various classification, regression and clustering algorithms such as Support Vector Machine

(SVM)to obtain the best model. The prepared ligand was characterized and docked against the target protein followed by the analysis of system stability and fluctuation.

Active Site Analysis of the target protein.
Cavities were detected on LRP 5. Fig 1 shows**Cavity site 1**(SER:105:A, GLU:133:A, ARG:142:A, LYS:143:A,GLY:326:A, VAL:327:A, CYS:336:A, LYS:337:A, ALA:338:A, GLY:339:A, ALA:340:A, GLU:341:A, GLU:342:A, ILE:365:A, VAL:366:A, LEU:367:A, GLN:368:A, TYR:380:A, ASP:381:A, PRO:382:A, GLU:384:A)and **Cavity site 4**(ARG:349:A, THR:350:A, ASP:371:A, ILE:372:A, ARG:373:A, HIS:374:A, ALA:375:A, ILE:376:A, ASP:391:A, ASP:392:A, GLU:393:A, VAL:394:A, ARG:395:A, ALA:396:A, ARG:398:A, ASN:412:A, THR:435:A, GLY:436:A, THR:437:A, ASP:438:A, ARG:439:A, GLU:460:A, ARG:462:A, TRP:478:A, LEU:1472:A, TYR:1473:A, ASP:1474:A, ARG:1475:A, ASN:1476:A, HIS:1477:A, VAL:1478:A, THR:1479:A, GLY:1480:A) having a Medium and Strong druggability. The predicted Maximum pKd value indicates the ligandability of the cavity binding site. A value less than 6 suggests that this may not be a suitable binding site. Cavity site 1 and Cavity site 4 have Pred. Max. pKd value of 10.00 and 9.67 respectively. The drug - score of each site is 81.00 and 1907.00. The best pocket was found to be one of the two main b-propeller domains in the crystallographic structure. LRP5 contains four b-propeller domains thatinteract with themselves and other components and these domains are considered to be of great significance.
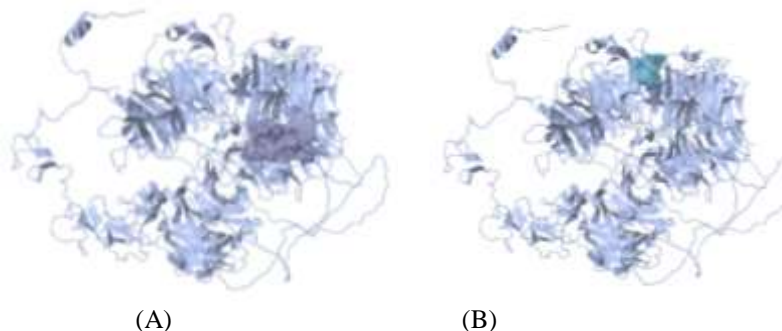

(A)   (B)
Fig 1: (A) Cavity Site 4; (B) Cavity site 1

In Silico Prediction of pharmacokinetic parameters
Drug-likeness properties such as Molecular weight (MW), partition coefficient (log P), Topological Surface area (tPSA),Hydrogen bond donors (HBD), Hydrogen Bond Acceptors (HBA), number of rotatable bonds (NRB), hERG I inhibitor, hERG II inhibitor, Hepatotoxicity,Oral rat Acute Toxicity (LD 50), Carcinogenicity, Immunogenicity, Cytotoxicity and Max. tolerated dose (humans) etc. were predicted on the DruLito tool for the designed peptide for docking studies as represented in Table 1. The peptide was found to be in accordance with Lipinski's rule of five(implemented from Lipinski CA. et al., 2001. Adv Drug Deliv. Rev. MW ≤ 500, LogP≤ 4.15, N or O ≤ 10, NH or OH ≤ 5)

Table 1: Drug likeness prediction results.

S. No	Ligands	Mol. Wt.	NRB	HBA	HBD	tPSA	LogP	Lipinski's Rule of Five Violations
1.	Peptide (ser-cys-ala-glu)	279.32	11	9	5	166.1	-3.704	No
2.	Tamoxifen	371.22	8	2	0	12.47	6.342	Yes

Pharmacokinetic properties such as absorption, distribution, metabolism, excretion and toxicity studies have been performed for the designed peptide. With the idea of converting the peptide into an oral drug, GI absorption and BBB permeability indicate the absorption and distribution of the designed peptide. The in-silico prediction results are shared in Table 2.

Asseen,the pkCSM ADME prediction parameters indicated that ser-cys-ala-glu peptide showed high gastrointestinal (GI) absorption compared to the reference drug tamoxifen,which showed low absorption.Metabolism of the drug is also regulated by a range of cytochromes (CYPs) such as CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4

are important for the in vivo biotransformation of the drug. Acute toxicity prediction results, such as LD50 values suggest that the designed peptide has no acute toxicity. The results also help us predict the ligands' hepatotoxicity, carcinogenicity, mutagenicity, immunotoxicity, and cytotoxicity as shown in Table 3. Based on ADMET prediction result analysis, the designed peptide may be a good choice for use as an oral drug.

Table 2: ADME predictions of the designedpeptide and Standard compound Tamoxifen.

S.No	(logKp) cm/s	GI Absorption	BBB Permeability	Total Clearance (Log ml/min/kg)	Inhibitor Interaction					
					p-gp	CYPIA2	CYP2CI9	CYP3A4 Inhibitor	hERG I	hERG II
1.	-10.68	High	No	0.438	No	No	No	No	No	No
2.	-2.737	Low	No	0.556	Yes	Yes	No	Yes	Yes	Yes

Table 3: Toxicity predictionof the designedpeptide and Standard compound Tamoxifen.

S.NO	LD$_{50}$ (mol/kg)	Max. Tolerated Dose	Synthetic accessibility	Organ Toxicity				
				Hepatotoxicity	Carcinogenicity	Immunotoxicity	Teratogenicity	Cytotoxicity
1.	1.771	1.554	2.24	Inactive	Inactive	Inactive	Inactive	Inactive
2.	2.285	0.313	3.01	Inactive	Active	Inactive	Active	Active

Molecular Docking prediction
The test ligand and the reference ligand were allowed to dock to LRP 5. The best pose with the minimum binding free energy was analyzed. The docking scores are given in Table 4.

Table 4: Docking Scores of the designed peptide and Tamoxifen with LRP 5.

Ligand	Protein	MW	#Atoms	sLopP	tPSA	Flexiability	Lf rank score	LfdG	LfVscore	Total charge	RMSD
(Ser-cyc-ala-glu)	LRP 5	320.3	21	-1.8	166.1	10	-4.84	-6.83	-9.224	-1	0
Tamoxifen	LRP 5	372.5	28	6	13.7	5.3	-2.8	-5.35	-6.855	-1	0

Molecular Interaction Analysis
The study of molecular interactions between the ligand and the residues within the binding pocket of protein is an important step in choosing the potential compounds. Binding energy provides a summary of favourable and unfavourable interactions in the binding pocket. The designed peptide turns out to be a favourable inhibitory compound. The higher free binding energy is a result of Pi-Alkyl, salt bridges and hydrogen bonds to the residues in the binding pocket. Since the designed peptide obeys Lipinski's rule of five, it can be considered safe for human use. As seen in Fig 2, the interactions show us that the peptide can make favourable bonds with the residues inside the pocket and can therefore stay longer in the binding pocket.

In this case,the Peptide-LRP5 complex, Pi- Alkyl interaction is formed with Ile 376. Also, conventional hydrogen bonds and salt bridges are formed with Asp 417, Asp 419, Thr 435, Arg 462, Arg 1475, Val 1478, and Arg 349, His 374, Asp417, Asp 419, Arg 462, respectively.The binding interactions of the drug Tamoxifen which is used to prevent breast cancer show that tamoxifen also interacts with the same amino acids in the same position. Tamoxifen forms strong hydrophobic interactions with Asp 417, Asp 419, and Arg 462. However, tamoxifen forms weaker Van der Waal interactions with Thr 435,Asp 417, and Arg 1475, which makes it easier to leave the binding pocket. This when compared to the designed peptide that forms Van der Waal interactions with only His 547 along with its strong Pi- Alkyl, salt bridges and hydrogen bonds allows the peptide to stay in the binding pocket longer.

The peptide binds with the residues found in Cavity Site 4 of the protein which is the conserved β-propeller domain of LRP5 showing that it is capable of altering the backbone of the domain and interfering with the structural dynamics which may lead to the inhibition of the signal transmission. The peptide interferes with the structure and

the folding of the domains and inhibits any transmission of the signals through the LRP5 receptor protein. The peptide is seemingly having the same effect as DKK according to another study conducted (Ju Bao et al., 2012).
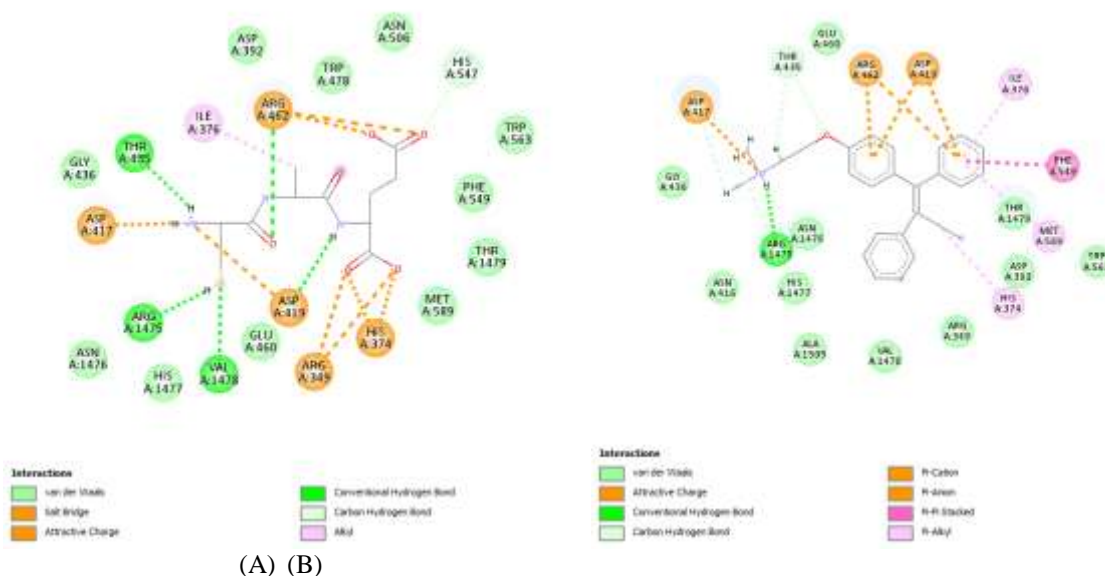

(A) (B)

Fig 2: Molecular interactions (A) LRP 5 with the designed peptide (B)LRP 5 with tamoxifen.

MD simulations: System Stability and fluctuation analysis.
The molecular dynamic simulation was performed for 50 cycles. To relax the crystallographic structures the simulation was done for 100 ns. The MD simulation was carried on to evaluate protein-ligand complexes under a more realistic environment. Interactions between the ligand and the protein inside the binding pocket can be visualized under realistic conditions during the simulation.

The stability of the systems is checked and Root Mean Square Distance (RMSD) analysis is performed. As shown in Figure 3 (A), the system was seen to reachdecent stability. Moderate deviations were noticed for tamoxifen between 2 and 6 A°. This deviation could be a result of the nonbonded water molecule present in the active site. The RMSD value depicts that there might be a change in protein structure due to the inhibitory activity of the ligand. Thus, by changing the folding of a protein to the point that it will lose the ability to interact with the previously recognized target site, FZD, in this case. Hence the dimerization of Wnt ligands with frizzled receptor and its co-receptor can be inhibited.Another important analysis presenting the ability of the ligand to change the secondary structure of the protein is Root Mean Square Fluctuation (RMSF) as shown in Figure 3 (B). In the complex state of protein with the peptide, fluctuations of the backbone of the protein are extremely higher compared to the apo form of the protein (absence of any ligand). This shows that compounds have dramatically changed the structure of the protein.
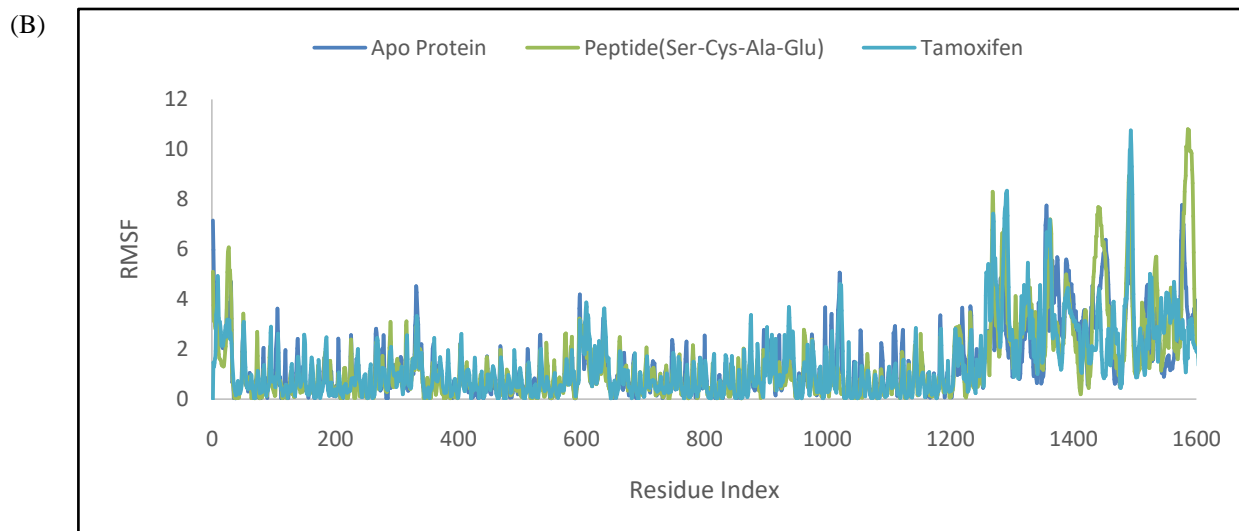
(A)

(B)
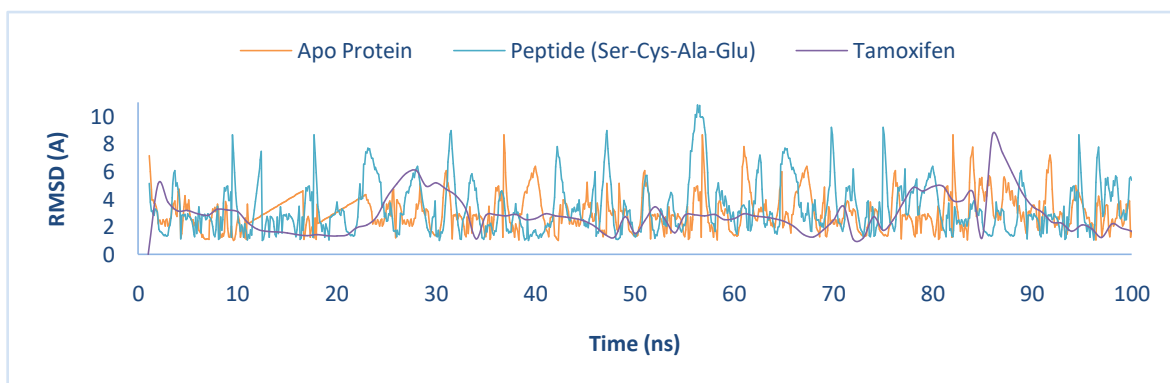
Fig 3: (A) Root Mean Square Deviation (RMSD) and (B) Root Mean Square Fluctuation (RMSF) values of the LRP 5 complexes in 100 ns production runs

Research shows that the binding of DKK to LRP 5 can cause its dimerization thus inhibiting the Wntsignalling pathway (Brott BK et al., 2002). So far with the results of RMSD and RMSF, the fluctuations indicating that there might be a structural change could very well mean that peptide binding to LRP 5 following the same mechanism as DKK can lead to LRP 5 dimerization and thus blocking the Wnt signally pathway. The RMSD and RMSF values show that the ligand can change the secondary structure of the protein, and the binding free energies show that it can bind to the binding pocket properly and will not leave the binding pocket easily.

Discussion:

Triple-negative breast cancer is the worst prognosis. To this date, there is a lack of molecular markers in the cells and there has been no significant development in the treatment modality. There is a dire need for new, specific and less toxic drugs, however, the development of newer drugs is slowed down by the lack of molecular targets for this type of cancer. For a better understanding of the underlying mechanisms involved in development and progression is required for the development of newer drugs and therapies. Over the years, a lot of studies have been performed to understand the wntsignalling pathway. In silico tools for molecular docking and MD simulation have helped achieve a great understanding of the structures involved in the pathway.The relationship between the Wntsignalling pathway and the development and progression of tumours has been studied for a long time (Nusse and Varmus, 2012). Currently, no available drugs can inactivate this signalling pathway, and no studies, to the best of our knowledge, identify inhibitors that target the co-receptor LRP 5.

A study performed by (Zhang et al. 2013; Lu et al. 2011; Lu et al. 2012) identifies inhibitors of co-receptor LRP6. The Zinc compounds were searched through the ZINC database, this study was performed with 10 compounds and examined in MD simulations to see if the compounds interact strongly with the binding pockets. Another study by Yooinet. al., 2020 and Yu, S., Li, D., Zhang, N. et. al, 2022predicted the binding capability of 29 herbal compounds against sclerostin, an antagonist of the Wntsignalling pathway. The study concluded that potential inhibitors could interact with the critical regions of the protein.In 2019, Sadeghi et. al studied the DKK family, one of the critical antagonist families against the Wntsignalling pathway. Using protein structure prediction tools, Molecular Dynamic simulation studies, and molecular docking, they characterized the interaction profile to find the details of this signalling pathway. Unravelling these interaction details has been beneficial for understanding the underlying mechanisms of cancers such as colorectal cancer (Sadeghi et al., 2019).

Apart from biological and chemical agents, large antibodies as well as small peptides have also been developed to target the Wntsignalling pathway. A peptide similar to our work was developed using a stabilized alpha helix of BCL-9 (SAH-BCL9) which selectively suppressed the Wntsignalling by causing disruption of the BCL9 - β- catenin complex. However, even after multiple modifications the peptide was found to be non-soluble and showed non-

specific toxicity which was likely due to aggregation and precipitation (SA Kawamoto, 2010), in contradiction, our peptide was shown to be soluble and had no overt pharmacokinetic consequences.

The tools and techniques employed in this work have been proven to be accurate and precise, and are capable of finding potential compounds for inhibiting the desired molecular targets. Designing a new inhibitor for the LRP5 protein target can be a good breakthrough for several human diseases. Moreover, in 2013, Yan Ni Lohet al., performed in vitro studies and provided data that supported the role of the Wntsignalling pathway in acquired resistance to Tamoxifen, our standard for comparison in the current study. This study further proves that Tamoxifen resistance is acquired due to the upregulation of the Wntsignalling pathway and our study supports this theory with the hypothesis that tamoxifen binds weakly to the binding pockets and leaves the pocket quickly. This study also provides the basis for the development of a newer drug that can inhibit the Wntsignalling pathway by binding strongly to the target protein.

Conclusion:
These data support that inhibiting LRP 5 can have a great impact on controlling tumour metagenesis. In this work, a peptide was designed from a naturally produced protein. The validity of the peptide was confirmed by applying Lipinski's rule of five, toxicity predictions and molecular docking. The peptide was further examined by performing MD simulations to study the interactions between the ligand and the protein. The binding free energy of the peptide was superior to the control molecule. By analyzing the details of interactions of the protein with the ligand it was revealed that interactions involving Pi-Alkyl, conventional hydrogen bonds and salt bridges seem to play an important role in the binding pocket. The results presented here should serve as a starting point for future studies involving the Wntsignalling pathway, the molecular interaction between its components, and the development of new drugs that target this pathway and studying them in vitro and in vivo.

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