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Docking studies reveal zerumbone targets *β*-catenin of the Wnt–*β*-catenin pathway in breast cancer

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Abstract: Breast cancer is the second most common cancer among women worldwide. The Wnt–*β*-catenin pathway appears to be deregulated in most cancer cells including breast cancer. The role of zerumbone, the active sesquiterpene from *Zingiber zerumbet* Roscoe, on the Wnt–*β*-catenin pathway is relatively unknown, especially detailed molecular studies have yet to be published. Using the Chemistry at HARvard Macromolecular Mechanics (CHARMm) force field-based docking protocol, CDOCKER, the molecular interactions between zerumbone and key proteins of the Wnt–*β*-catenin pathway were evaluated in this study. The results suggest that zerumbone has a strong affinity for free *β*-catenin in the cytoplasm, as well as the *β*-catenin–transcription factor 4 complex in the nucleus. The overall hydrophobic nature of zerumbone allowed its interaction with other hydrophobic residues, such as Trp383, while its active *α*,*β*-unsaturated carbonyl facilitated its interaction with positively charged residues, such as Lys345, Arg386 and Asn415 in the *β*-catenin binding pocket. However, the Wnt protein and its frizzled receptor showed no attraction to zerumbone.

Keywords: zerumbone; Wnt–*β*-catenin pathway; frizzled (Fzd) protein; *β*-catenin–transcription factor 4 complex; molecular docking.

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INTRODUCTION

The Wnt–*β*-catenin pathway (Fig. 1A and B) is pivotal in cell genesis, proliferation, migration and body axis patterning.[1–3] It is also associated in various cancers, including, colon cancer, liver cancer, breast cancer, *etc*.[4–7] Nineteen fully characterized Wnt family of secreted proteins are essential for effecting the signalling of the pathway. These proteins are 330–450 amino acids long and several proteins of the family especially have been found to be overexpressed in cancer.[3,8] In the canonical pathway, the secreted Wnt proteins bind to the constitutively expressed extracellular portion of the cysteine-rich domain of the frizzled (Fzd) transmembrane receptor. Another transmembrane protein, the low--density lipids-related protein (LRP) 5 or 6 also binds to the complex along with a dishevelled (DSH) protein. This quaternary complex produces significant changes in the cytosol, the most significant being the release of *β*-catenin from its destruction complex.[8–12]

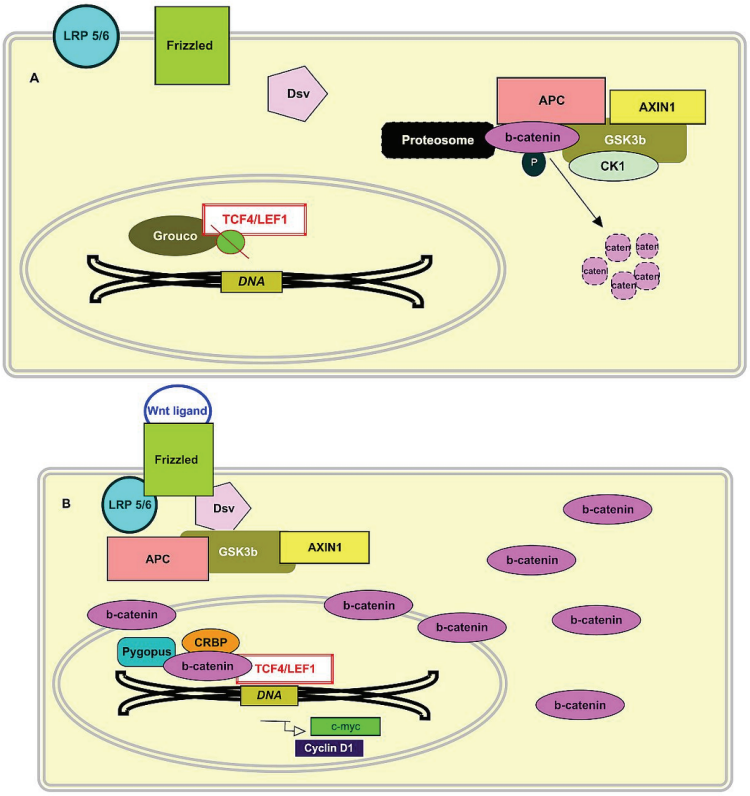

Fig. 1. The canonical Wnt–*β*-catenin pathway. A) In the absence of the Wnt ligand, the pathway is in its "off" state, *β*-catenin is controlled. B) In the "on" state, *β*-catenin binds to TCF–Lef in the nucleus to induce transcription. The double line frame represents the cell wall; b-catenin refers to β-catenin.

β-Catenin is a protein of the armadillo repeat family having two important functions. One function is the facilitation of cadherins in forming cell–cell adhesion. *β*-Catenin interacts with cadherin as an adaptor protein along with *α*-catenin, forming a ternary complex that creates strong cell–cell attachments.[13] The second function is the transcription of the cell proliferating proteins. When the signal is "off" (Fig. 1A), *i.e.*, Wnt is not bound to the Fzd receptor, the adenomatous polyposis coli (APC), axin and the glycogen synthase kinase 3 *β* (GSK3*β*) form the *β*-catenin destruction complex that degrades any excessive quantity of cytosolic *β*-catenin by phosphorylating and marking it for ubiquitination.[14] When the signal is turned on (Fig. 1B), the APC, axin and GSK3*β* release *β*-catenin and it translocates to the nucleus. In the nucleus, the protein binds selectively to two important proteins, the T-cell factor (TCF) or lymphoid enhancer binding factor (Lef), a group of transcription proteins, forming a transcription complex. Among the protein targets, the oncogenic proteins cyclin D1, c-myc and c-Jun remain the most significant.[3,5,7,15,16]

Aberrations in the pathway's core components, such as adenomatous polyposis coli (APC), axin and *β*-catenin proteins, have been found in several cancers. In most cases, the APC is truncated at the *β*-catenin binding site. In breast cancers, however, Wnt-signalling can be activated in the absence of mutations in any of the above proteins.

Studies indicate that, in most cases, there is overexpression of the Wnt ligands. This leads to significantly high levels cytoplasmic and nuclear *β*-catenin.[12] In 46–80 % of breast cancers, there is a loss of expression of secreted frizzled-related protein 1 (sFRP1), a negative Wnt pathway regulator, and predictably sFRP1 loss is a result of poor prognosis.[12] Due to its importance in cancer, several inhibitors of the pathway have been proposed. *β*-Catenin has been of special interest because of its dual action on cell–cell adhesions and the facilitation of the transcription factor 7-like 2 (TCF4) in the nucleus. The available options of anticancer drugs are usually non-selective and toxic. The use of alternate medicine has always been an interesting area in medical explorations as alternative or combined with existing therapeutic options to inhibit the progression of the disease.[17–22]

Zerumbone (Fig. 2), the sesquiterpene from *Zingiber zerumbet* Roscoe, is known for its anti-cancer activity against several cancers by modulating several proteins to induce apoptosis.[24] Several articles have identified key proteins that could be inhibited by zerumbone for arresting cancer cell growth.[25–35] Reports

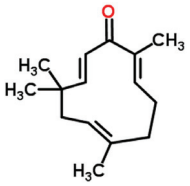

Fig. 2. Chemical structure of zerumbone.[23]

have also been published on the apoptotic activity of zerumbone on liver, ovary and cervix cancers, as well as leukaemia.[25–27,31,32] It was reported to act as a modulator of osteoclastogenesis induced by the receptor-activated nuclear factor kappa-light-chain-enhancer of the activated B cells (NF-κB) ligand, the receptor activator of the nuclear factor kappa B ligand (RANKL).[36]

Several researchers concentrated on the Wnt-pathway for finding druggable targets. Finding inhibitors for the protein and the pathway were studied.[6,9–11,37–40] Hallett *et al.* (2012) showed that successful binding of small molecules with a predominantly hydrophobic nature to the armadillo region of β-catenin would be useful in preventing β-catenin–(TCF4)/lymphoid enhancing factor (Lef) interaction, thereby causing decreased growth of breast tumour initiator cells.[38] Quercitin, silibinin and naringin were investigated as compounds from natural sources that inhibit the β-catenin pathway in breast cancer cells by arresting the cell cycle in the G1 phase.[41–43] Silibinin inhibition of the low density lipoprotein related protein 6 (LRP6) was observed and a consequential blockade of the Wnt signal was detected.[42] The small size of zerumbone (MW = 218 g mol^{-1}) and its hydrophobic character makes it an interesting molecule for inhibiting β-catenin.

Zerumbone possesses an α,β-unsaturated carbonyl group that is known to form Michael adducts. Its most important reaction is with glutathione that leads to an increased intracellular redox potential of cancerous cell, as compared to that of normal cells, ultimately leading to apoptosis of the cancer cells.[27,44] Despite this information, knowledge of the exact mechanism of action is unknown.

In this study, binding of zerumbone to the proteins of Wnt–β-catenin pathway was explored. The Wnt-pathway was selected for its established role in several cancers, including breast and colonic cancers and its key protein, β-catenin. To the best of our knowledge, there is no published study of inhibitory effect of zerumbone on the Wnt–β-catenin pathway in cancerous cells.

Ligand–protein interactions can be studied easily using docking programs. These techniques provide useful insights of the molecular events occurring at the binding interface of the ligand–protein interaction site. Their utility is vital in complementing and supplementing experimentally determined data. CHARMm force field-based docking software, CDOCKER, of the Discovery Studio 2.5.5 (Accelrys, Inc., San Diego, CA, USA) suite of programs was used for the docking studies to evaluate the most likely target proteins of zerumbone and determine the binding modes of the molecule with its target proteins. CDOCKER applies grid-based molecular dynamics simulated annealing protocol by using CHARMm force field while devising the appropriate position of the ligand in the active pocket. The algorithm offers flexible ligand docking where the non-bonded interactions are softened during the docking procedure but removed during the final minimisation process.[45] The protein was held rigid during the entire process.

EXPERIMENTAL

Protein and ligand starting structures

Three dimensional structures of the β-catenin–TCF4 proteins complex (1JDH.pdb) were obtained from the Protein Data Bank (PDB).[46] The structure of zerumbone was obtained from the Pubchem database (structure ID: 5470187).[47] Due to the unavailability of the β-catenin––bound inhibitor complex, molecules with known activity against the protein were used. The three dimensional structure of the human Fzd protein and Wnt were not available in the PDB. The Basic Local Alignment Search Tool (BLAST), from www.ncbi.nlm.nih.gov, revealed the availability of the *Xenopus* Wnt8–Fzd8 complex (4F0A.pdb) in PDB.[48,49] Due to the 98 % sequence similarity between human and Xenopus Fzd, the docking studies were realised using the *Xenopus* Wnt8–Fzd8 complex.

Preparation of structures for docking

Discovery Studio 2.5.5 (Accelrys, Inc., San Diego, CA, USA) was used to prepare the protein and ligand as inputs based on the CDOCKER protocol.[45] PKF118-310, reported as an inhibitor in the literature,[50] was used for control docking to compare with zerumbone. CDOCKER adds and minimizes the energy after adding the polar hydrogens on the protein while keeping the heavy atoms fixed. All-atom representation was applied to assign formal and partial charges to the ligand. The formal charges were assigned to match the protonation state of the atoms at pH 7, while the partial charges were based on Momany–Rone force field.[51] The binding site was detected from the coordinates of the protein–protein complex of the PDB file or by using the *find sites from receptor cavities* in the Tools section under Define and Edit Binding Sites in the Discovery Studio.

Docking with CDOCKER[45]

The docked poses were ranked according the lowest CDOCKER energy, which was calculated based on the internal ligand strain energy and the receptor–ligand interaction energy and the lowest CDOCKER Interaction Energy, which is a measure of the potential energy from non-bonded interactions (van der Waals, polar or non-polar) between the ligand and protein. All the docked complexes were further used to calculate the binding energies using the Calculate Binding Energies protocol in the Discovery Studio. The binding energies are calculated based on the following general equation:

$$E_{\text{binding}} = E_{\text{complex}} - E_{\text{protein}} - E_{\text{ligand}} \tag{1}$$

where E is the energy. The method combines the CHARMm-based energies with implicit solvation methods to calculate the potential energy of the complex, receptor and the ligand. The ligand was minimized *in situ* before calculation of the final binding energy to remove the ligand conformational strain.

The CDOCKER program uses CHARMm force-field energy calculations represented by the equation:

$$\begin{aligned} V = & \sum_{\text{bonds}} k_b(b-b_{\text{o}})^2 + \sum_{\text{angles}} k_\theta(\theta-\theta_{\text{o}})^2 + \sum_{\text{dihedrals}} k_\phi[1+\cos(n\phi-\delta)] + \\ & + \sum_{\text{impropers}} k_\omega(\omega-\omega_{\text{o}})^2 + \sum_{\text{Urey–Bradley}} k_u(u-u_{\text{o}})^2 + \\ & + \sum_{\text{non-bonded}} \varepsilon\left[(R_{\min_{i,j}}/r_{i,j})^{12} - (R_{\min_{i,j}}/r_{i,j})^6\right] + \frac{q_i q_j}{\varepsilon r_{ij}} \end{aligned} \tag{2}$$

CDOCKER uses the grid-based docking method to calculate the ligand potentials and combines them with molecular dynamics to obtain the final minimized ligand pose in the receptor. The specific docking protocol implemented in CDOCKER involves the generation of ligand poses in the protein binding site that is followed by several cycles of molecular dynamics.[45] The energy of the final protein–ligand complex is obtained from a modified CHARMm param19/toph19 energy function.[52]

The pathways in Fig. 1 (A and B) were drawn using Pathvisio beta-3.0 software,[53] LIGPLOT[54] was used to illustrate the interaction chart of the ligand and protein and Pymol was used to visualize the docked ligands.[55]

RESULTS AND DISCUSSION

Binding energy and CDOCKER interaction energy

The values obtained for the binding energies and the CDOCKER interaction energy profiles of known inhibitor molecules with target proteins or from a literature review were used as control dockings to compare the results with those of zerumbone to the transmembrane Fzd protein and *β*-catenin protein in the Wnt pathway that are presented in Fig. 3. The Wnt ligand binds to the Fzd protein in a thumb-index finger fashion. Docking was realized on both binding sites that occur on either side of the Fzd protein. Due to lack of information about any inhibitor of the complex, the results with zerumbone are presented here. The *β*-catenin molecule is the most researched molecule in the Wnt pathway. PKF118-310, extensively reported as an inhibitor of the *β*-catenin–TCF4 complex,[37,50,56] was used as a control ligand to compare with zerumbone. Two potential target sites on the complex were observed with comparable binding energy for both inhibitory molecules.

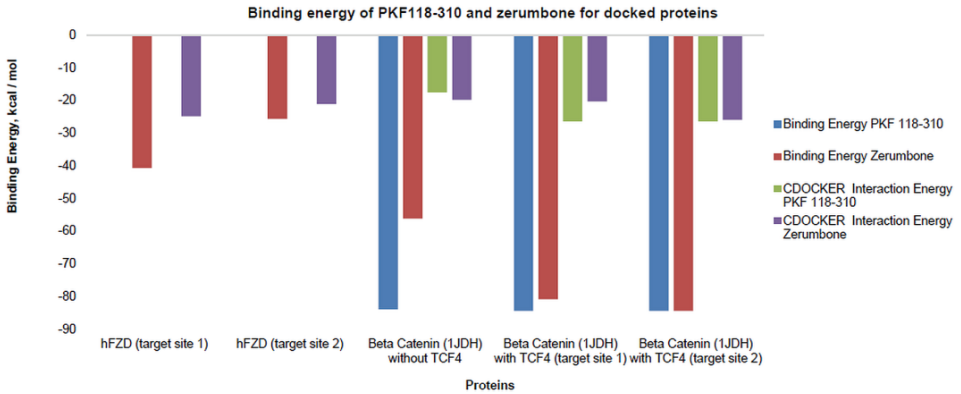

Fig. 3. Energy profile of known inhibitor molecules and zerumbone from CHARMm-based docking software (CDOCKER).

In the case of zerumbone, van der Waals forces, π–π interactions and electrostatic charges contributed by the *α*,*β*-unsaturated carbonyl group are the major contributors to the binding energy, while the control PKF118-310 has two car-

bonyl groups and nitrogen atoms that contribute highly to the electrostatic forces responsible for the strong binding of the molecule. The overall results indicated that zerumbone and the PKF118-310 were able to bind to β-catenin. Zerumbone showed stronger interaction to the complex of β-catenin and TCF4, while PKF118-310 showed that it could dock effectively to β-catenin both with and without TCF4, which confirmed the experimental evidence presented by Lepourcelet *et al*. that PKF118-310 is a strong inhibitor of the formation of the β-catenin–TCF4 complex.[50]

Docking of Wnt–frizzled complex with zerumbone

Studies by Janda *et al.*[48] and Bienz and He[57] suggested that Wnt binds to Fzd in a thumb-and-index finger like conformation covering both sides of the Fzd protein. Since this conformation is essential for the binding of the Wnt ligand, inhibiting complete or partial binding could prove the usefulness of this target for zerumbone. Using both sites as potential hotspots, the docking results showed that zerumbone had a weak affinity for the Fzd protein. The researchers[48,57] also suggested that the Wnt ligand had a palmitoleic acid chain attached to its serine 187 residue that inserted itself between the amino acids of the frizzled receptor, called Site 1. This is essential for proper binding of a protein to the receptor as well as facilitating the anchoring of Wnt into the cell membrane.

Analysis of the site revealed that Site 1, shown in Fig. 4A and B, is made from residues Gln37, Phe38, Pro40, Leu41, Met88, Tyr91 and Phe93. These refer to Gln71, Phe72, Pro74, Leu75, Met122, Tyr125 and Phe127 of the murine template. This is the same site where the lipid attachment of Wnt penetrates the Fzd--cysteine-rich domain, providing support for the Wnt–Fzd binding model. The binding energy of –44.35 kcal* mol^{-1} suggested that zerumbone could modify or partially inhibit the association of Wnt and may be useful in preventing the anchoring of Wnt to Fzd if the cancer cells were exposed to the drug before the formation of the Wnt–Fzd complex, since docking with the Wnt–Fzd complex structure was unable to give any pose for zerumbone.

Site 2, shown in Fig. 4A and C, is near the other binding area of the Wnt to Fzd protein. The site consists of Thr39, Val40, Pro41, Leu42, Gln62, Asp63, Gly66, Leu67, His70 and Trp71. According to Janda *et al.*,[48] the residues Tyr48, Phe86, Ile95, Leu97, Tyr100, Leu104, Leu147 and Met149 of the frizzled structure are engaged in the binding of the C-terminal loop of Wnt in the Fzd–Wnt complex. The Wnt finger loop engages with residues 116–118 and Phe52.[48]

Zerumbone binds in the region just outside the binding area of Wnt with a binding energy of –26.25 kcal mol^{-1} and hydrogen bond formation between the zerumbone carbonyl oxygen and the Fzd residue Leu42. Combined, these results

* 1 kcal = 4184 J

indicate that the frizzled receptors could be a target for inhibiting the Wnt signalling with zerumbone, which needs further investigation.

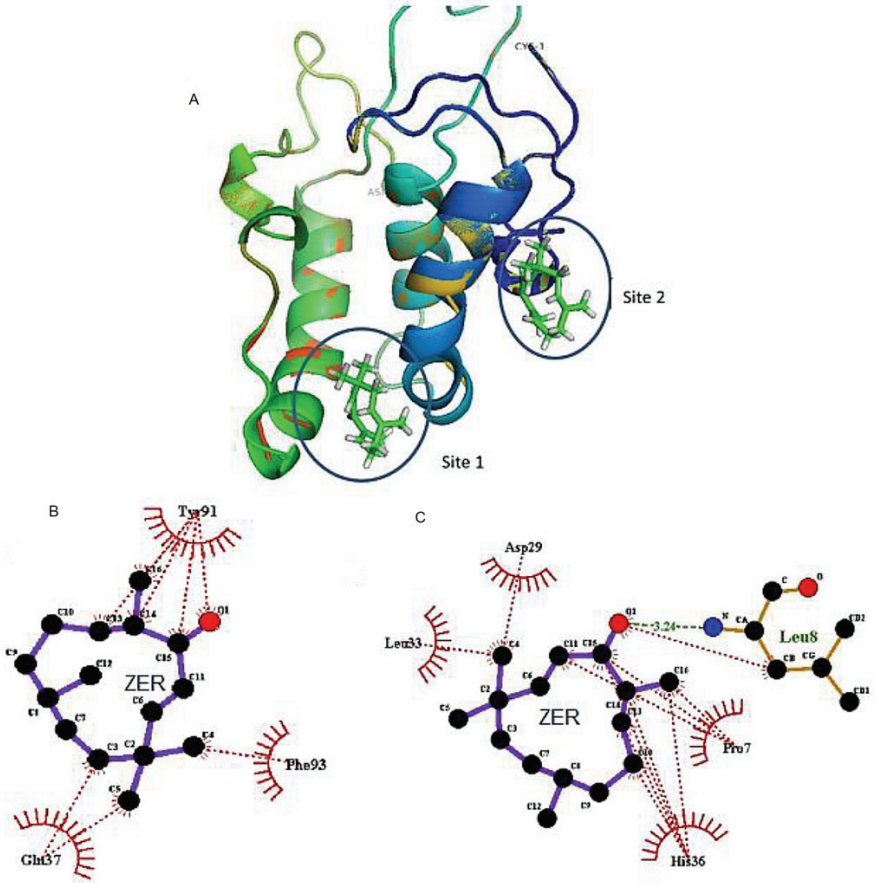

Fig. 4. Binding of zerumbone to Fzd8. A) Zerumbone bound to Fzd8 Site 1 and Site 2. B and C) LIGPLOT results of Fzd8–zerumbone complex at Site 1 and Site 2.

β-*Catenin*

β-Catenin owes its importance to the fact that it is responsible for cell proliferation in the Wnt signalling pathway. Once it is free from the binding complex of axin–APC–GSK3*β*, it translocates to the nucleus and forms the transcription complex with TCF4 or Lef. This also makes it a prime target for tumour cells as its concentration is unusually higher in cancerous cells as compared with normal cells.[4,5,7,9,11–13,15,16,58,59] In the case of breast cancer, *β*-catenin is over-expressed in the almost 80 % of cases.[12,38,60,61] The 3D structures of *β*-catenin complexes available in the PDB database do not have a small molecule or drug inhi-

bitor bound to it. Hence, the available protein–protein complexes were used as targets to determine the site and mode of zerumbone binding.[56,62–65]

β-*Catenin–TCF/Lef transcriptional complex*

The β-catenin–TCF/Lef transcriptional complex is important in cancerous cell survival. The inhibition of the complex has been the interest of several researchers.[38,41,50,66–68]

Structural analysis of β-catenin, solved by Huber *et al.*,[13] revealed that the protein possesses α-helical arm repeats that pack against one another to form an elongated superhelix of α-helices. This creates a groove in the twisted superhelical structure that is highly charged and was proposed to be the interacting surface for β-catenin targets. According to studies by Graham *et al.*,[64] TCF also recognizes and binds specifically to the same region. Docking was performed using an experimentally determined β-catenin–hTCF4 complex inhibitor molecule PKF118-310[37,50] and zerumbone using the 1JDH structure that forms the hTCF4 complex with β-catenin.[56]

In the first part of the modelling experiment, the TCF was removed and docking was performed on β-catenin only. The results shown in Fig. 5 indicated the binding pocket of PKF118-310 was composed of Lys345 and Val346 and a binding energy of –83.94 kcal mol^{-1}.

However, the binding pocket of zerumbone consisted of the amino acids Trp338, Arg342, Lys 345, Arg376, An380 and Trp383 residues in the same binding region. Zerumbone exhibited a binding energy of –60.58 kcal mol^{-1}, which is comparatively higher than that of the inhibitor. According to Graham *et al.*,[64] TCF binding was to the pocket lined by the residues Tyr306, Gly307, Lys312, Lys 345 and Asn387. From the present docking studies, it was found that PKF118-310 occupied the vital binding site of the complex with Gln 302, Tyr306, Gly307, Lys312 and Arg342 within 4Å distance of the molecule. Additionally, an analysis of the binding site showed that PKF118-310 formed a hydrogen bond with Lys345. These results also confirmed the experimental results of Lepourcelet *et al.*,[50] that PKF118-310 was a strong inhibitor of the β-catenin–TCF4 complex. Their results demonstrated an IC_{50} value between 1 and 30 μmol (measured as the amount of compound required to reduce at A_{490} to 50 % MTS assay). These amounts were sufficient to inhibit the interaction of β-catenin with TCF4. Meanwhile, the zerumbone binding pocket was further away and only Lys345 and Trp383 were directly near the TCF binding pocket where it formed hydrophobic interactions with most residues. The docked structures and LIPGPLOT results are illustrated in Fig. 5A–D.

In the second part of the modelling, docking was performed with the β-catenin–TCF4 complex. The results shown in Fig. 6A and B indicated that in the presence of TCF4, the position of the PKF118-310 molecule that showed strong

binding to the *β*-catenin without TCF4 was shifted slightly. However, the position showed that it is lodged between the two proteins. The binding pocket showed Lys345, Val349, Arg386 and Asn 387 of *β*-catenin and Glu24, Gly25, Gln27, Glu28 and Glu29 of TCF4. The LIGPLOT (Fig. 6B) also showed that the molecule engaged hydrophobically with all the molecules with which it was hydrogen bonded except Arg386. This result was again in agreement with the results of Lepourcelet *et al.*[50] that PKF118-310 could disrupt the complex. Combining both results, it could be hypothesised that since the compound physically positions itself between the two proteins and its carbonyl oxygen engages the *β*-catenin residues with itself, the interactions of the two proteins could be weakened.

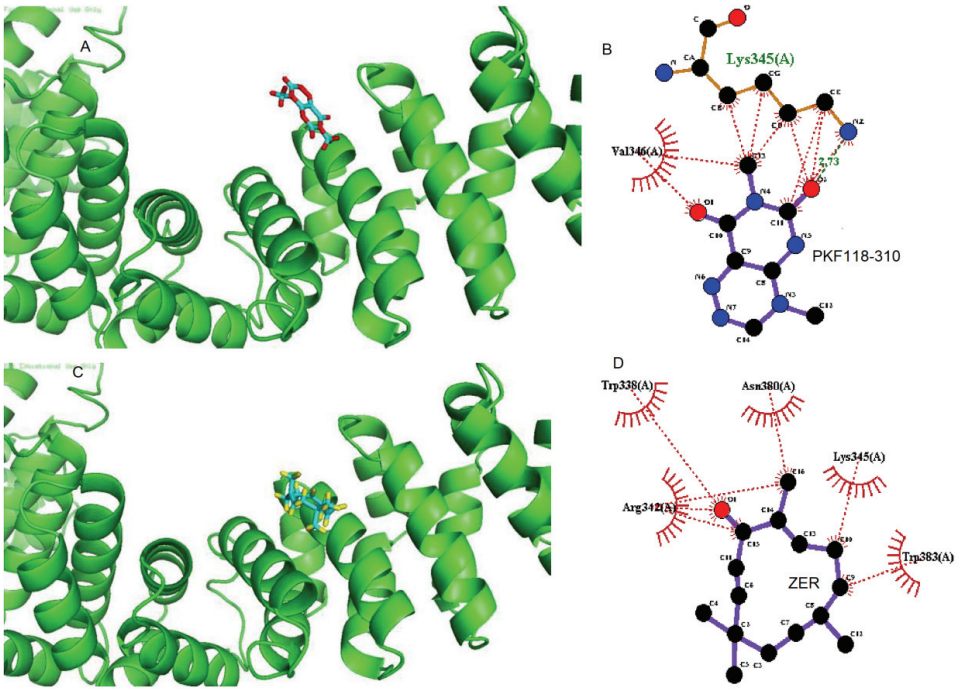

Fig. 5. PKF118-310 and zerumbone binding with *β*-catenin in the absence of TCF4. A) PKF118-310 bound to *β*-catenin. B) LIGPLOT result of PKF118-310 binding to *β*-catenin. C) Zerumbone binding to *β*-catenin. D) LIGPLOT results show zerumbone forms hydrophobic interactions with molecules of the binding site.

Two different positions were obtained for the protein–ligand complex in case of zerumbone. According to the results shown in Fig. 7A–D, it was possible for zerumbone to be sandwiched between *β*-catenin and TCF4. The calculated binding energies of –80.8 (site 1 in Fig. 3) and –84.4 kcal mol^{-1} (site 2 in Fig. 3) for both positions indicated the formation of a possibly strong complex when

compared to zerumbone binding in the absence of TCF4. Analysis of the bound structure revealed that zerumbone could interact with both proteins and hence, a low binding energy was obtained. It seems that zerumbone re-arranges itself and forms hydrogen bonds with Lys345 (site 1) or Lys270 (site 2) of β-catenin.

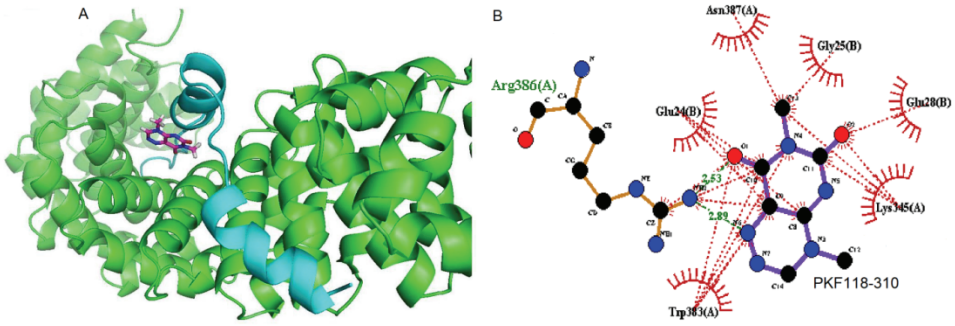

Fig. 6. PKF118-310–β-catenin complex II in the presence of TCF4. A) Docked position of PKF118-310 in the complex. The TCF chain (cyan) and β-catenin (green) can be seen. B) LIGPLOT diagram indicates the hydrogen bond between Arg386 of β-catenin and PKF118-310.

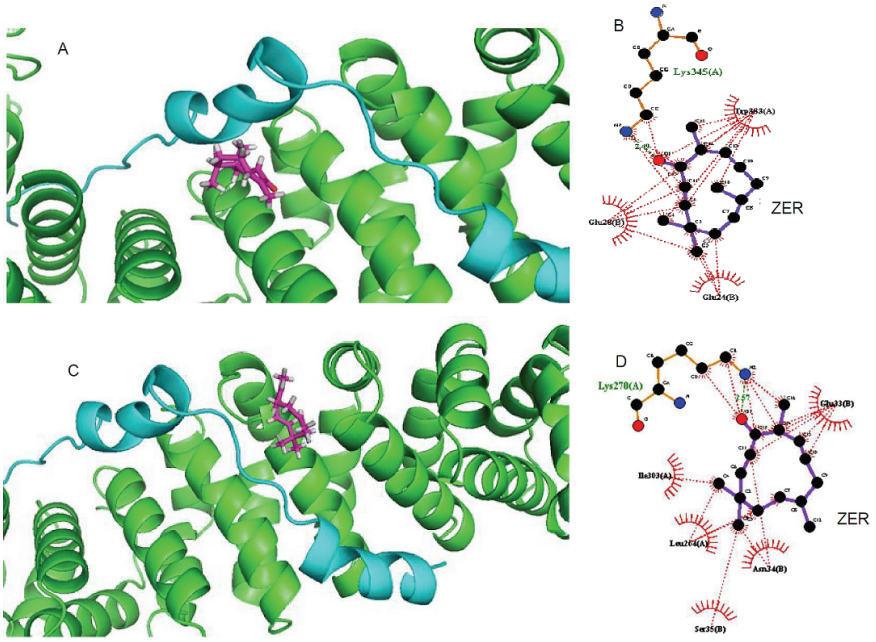

Fig. 7. Zerumbone (ZER)–β-catenin–TCF4 complex at site 1 A) A docked position of zerumbone in the complex. The TCF chain (cyan) and β-catenin (green) can be seen. B) LIGPLOT diagram indicates the hydrogen bond between Lys345. Zerumbone–β-catenin––TCF4 complex at site 2. C) Docked position of zerumbone in the complex with TCF chain (cyan) and β-catenin (green). D) LIGPLOT diagram indicates the hydrogen bond between Lys270.

The ligand being a hydrophobic molecule binds to the centre of the complex, which appears to have acidic residues of TCF and hydrophobic residues of *β*-catenin. In the *β*-catenin–TCF–zerumbone complex, residues Lys345, Trp383, Arg386, Asn387 and Asn415 of *β*-catenin, and residues Glu24, Gly25, Gln27 and Glu28 of TCF were involved in the interaction with zerumbone. The carbonyl group formed a hydrogen bond with *β*-catenin residue Lys345. The *β*-catenin region involved is the armadillo repeat 5 (Lys 345) and repeat 6 (Trp383, Arg386 and Asn387),[13] which is consistent with studies that showed this region to be the crucial region of interaction. The most important residues involved in the interaction are shown in Fig. 7B.

The *β*-catenin groove comprised of armadillo repeats 5–9 forms the core binding regions for TCF. According to Xu and Kimelman,[69] the first 50 residues of the TCF molecule insert themselves between the positively charged groove of the protein. Studies from the interaction of TCF/LEF with *β*-catenin revealed that the human TCF4 residues 16–32 containing residues $Dx\theta\theta x\phi x_{2\text{-}7}E$ interacted with the armadillo repeat 5–9. Residues 40–50 of the TCF engaged more strongly *via* salt bridges between the two proteins at K312 and K435. Graham *et al.*[64] added that K312 interacts with Glu24 or Glu29. Mutational analysis revealed that any change in the glutamic acid residues weakens the binding between the complexes.

Fig.7 (C and D, referred to as site 2 in Fig. 3) revealed a docked position in which the carbonyl group of zerumbone forms a hydrogen bond with Lys270. Leu263, Leu264, and Ile303 of *β*-catenin, and Glu33, Asn34, Ser35 and Ser36 for TCF were the remaining residues lining the binding pocket of zerumbone. Here, the region of *β*-catenin involved was the armadillo repeat 4.[13] Due to the hydrophobicity of repeat 4 and the positivity of the residues in both regions, it was found that zerumbone could position itself into the space such that its negative carbonyl is instrumental in bond formation with *β*-catenin. The docking results indicated that residues of the armadillo repeats 4 and 5 were possible binding targets for zerumbone. Armadillo repeat 5 and 6 were the major areas in contact with TCF4. In either case, zerumbone appeared to be placed between the kink region of the TCF protein and above the *β*-catenin molecules. The calculated binding energy of these complexes (–80.8 to –84.4 kcal mol^{-1}) was lower than that of the *β*-catenin–zerumbone complex, –65.58 kcal mol^{-1}. From the figures, it could be observed that zerumbone is in close contact with residues of both proteins.

Yu *et al.*[68] highlighted three hotspots that could be targeted for rational drug design of inhibitors against the complex. These residues were the interacting spots of Ly435/Lys508 of *β*-catenin and Asp16/Glu17 of TCF4. Another one was Lys312/Lys345 of *β*-catenin and Glu24/Glu29 of TCF4 binding in an alternative conformation and the last one was a hydrophobic pocket lined with Phe253,

Ile256, Phe293, Ala295 and Ile296 of β-catenin creating a binding pocket for Val44 and Leu48 of TCF4. The present docking results indicated that zerumbone preferably docked in the region with residues Lys312/Lys345 of β-catenin and Glu24/Glu29 of TCF4. Graham *et al*.[64] in their study also indicated two critical interactions between the two molecules. According to their study, the salt bridge between Asp16 and Lys 435 was important for the stability of the complex. The other critical area is the region with Lys312 and its surrounding residues that provide the first anchorage to TCF4. They concur that the area is comparatively less dynamic as compared with the Lys435/Asp16 contact point and that the Lys312/Glu29 is another critical point of interaction between the two molecules. Besides these crucial residues of the region, Glu26 and Glu28 of TCF4 interact with the neighbouring residues of Lys312 to enhance the interaction with Glu28 of TCF4 interacting with the side chain of Lys345.[64] The N-terminal residues of TCF4 are vital for recognizing Lys435 of β-catenin. This interaction is critical for the correct initial positioning of TCF4 with β-catenin. The glutamate cluster 23–29 of TCF4 is significant in providing the enhanced binding energy to interact with K312 and its surrounding area for a firm transcription complex. Targeting any of these two areas with inhibitors would prove useful.

The present results showed that the common site of interaction between the PKF118-310/zerumbone and the β-catenin/TCF complex is with β-catenin residues Lys345 and Arg386, and Glu24, Gly25, Gln27 and Glu28 of TCF4. The commonality of Lys345 and Glu24 and 28 indicate the importance of these residues in the discovery of potential inhibitors.

The β-catenin–TCF complex is essential for the transcription of cancer cell proliferating proteins. Hence, inhibition of the complex is critical for stopping cancerous cells. The docking results are consistent with wet lab experiments that indicate a favourable binding of β-catenin with zerumbone in breast cancer cells with an IC_{50} value of 12±2 μg mL^{-1}.[70] The effect of zerumbone in inhibiting β-catenin appears to be time dependent. It induces cell cycle arrest at 48 hours and apoptosis begins even at 24 h. Combining these experimentally determined results and modelling, it could be proposed that the binding of zerumbone to β-catenin in the β-catenin–TCF complex can disrupt the proper docking of the proteins in the complex by breaking the interaction of important residues, such as Lys345 of β-catenin and Glu24 of TCF4 that is necessary for stabilizing the complex for effectual binding of the complex to DNA for transcription of proliferation genes in cells. Since docking experiments were performed in a way that the β-catenin–TCF complex was held rigid, molecular dynamic simulations could be a supplementary way to confirm for how long and how deep zerumbone can affect the binding of the two proteins.

CONCLUSIONS

Breast cancer has the second highest mortality rate among women worldwide. The importance of aberrant signalling pathways cannot be undermined. In this study, molecular docking was used as a method to understand the probable specific targets of zerumbone, an important anticancer agent, on the TNF and Wnt-pathways. The docking results could suggest that zerumbone does not have just one target protein. It targets at least two pathways to establish its action. From the obtained results, it could be proposed that zerumbone does not inhibit *β*-catenin from translocating to the nucleus. The most probable action of zerumbone is to inhibit the transcription of the cell proliferating genes for c-myc, cyclin D1, by the *β*-catenin–TCF complex. Due to its strong binding to the complex, it is quite possible that the complex does not dock to the DNA strongly, and the genes are not transcribed. The obtained docking results verify the computational evidence that zerumbone is a potential anticancer drug that specifically targets the *β*-catenin–TCF complex with more specificity and resilience than the tumour necrosis pathway proteins. Its small size and hydrophobic nature make it easier to fit into the small crevice created between *β*-catenin and TCF.

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ИЗВОД

СТУДИЈЕ ДОКИНГА ОТКРИВАЈУ ДА ЗЕРУМБОН ЦИЉА *β*-КАТЕНИН У Wnt–*β*-КАТЕНИН СИГНАЛНОМ ПУТУ КОД РАКА ДОЈКЕ

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Рак дојке је други најчешћи рак код жена у свету. Wnt–*β*-катенински пут изгледа да је дерегулисан код већине канцерских ћелија, укључујући рак дојке. Улога зерумбона, активног сесквитерпена из *Zingiber zerumbet* Roscoe, у Wnt–*β*-атенинском сигналном путу је релативно непозната, поготово што још нису публикована детаљнија молекуларна проучавања. Користећи на Chemistry at HARvard Macromolecular Mechanics (CHARMm) пољу сила засновани докинг протокол, CDOCKER, ова студија израчунава молекулске интеракције између зерумбона и кључних протеина у Wnt–*β*-катенинском

сигналном путу. Наши резултати сугеришу да зерумбон има јак афинитет ка слободном *β*-катенину у цитоплазми као и ка комплексу *β*-катенина и транскрипционог фактора 4 у језгру. Претежно хидрофобна природа зерумбона дозвољава његову интеракцију и са другим хидрофобним остацима као што је Trp383, док његов активни *α,β*-незасићени карбонил олакшава његову интеракцију са позитивно наелектрисаним остацима попут Lys345, Arg386 и Asn415 у *β*-катенинском везивном џепу. Међутим, Wnt протеин и његов укрућени рецептор не показују привлачност ка зерумбону.

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