Rational design of DKK3 structure-based small peptides as antagonists of Wnt signaling pathway and in silico evaluation of their efficiency

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Abstract

Dysregulated Wnt signaling pathway is highly associated with the pathogenesis of several human cancers. Dickkopf proteins (DKKs) are thought to inhibit Wnt signaling pathway through binding to lipoprotein receptor-related protein (LRP) 5/6. In this study, based on the 3-dimensional (3D) structure of DKK3 Cys-rich domain 2 (CRD2), we have designed and developed several peptide inhibitors of Wnt signaling pathway. Modeller 9.15 package was used to predict 3D structure of CRD2 based on the Homology modeling (HM) protocol. After refinement and minimization with GalaxyRefine and NOMAD-REF servers, the quality of selected models was evaluated utilizing VADAR, SAVES and ProSA servers. Molecular docking studies as well as literature-based information revealed two distinct boxes located at CRD2 which are actively involved in the DKK3-LRP5/6 interaction. A peptide library was constructed conducting the backrub sequence tolerance scanning protocol in Rosetta3.5 according to the DKK3-LRP5/6 binding sites. Seven tolerated peptides were chosen and their binding affinity and stability were improved by some logical amino acid substitutions. Molecular dynamics (MD) simulations of peptide-LRP5/6 complexes were carried out using GROMACS package. After evaluation of binding free energies, stability, electrostatic potential and some physicochemical properties utilizing computational approaches, three peptides (PEP-I1, PEP-I3 and PEP-II2) demonstrated desirable features. However, all seven improved peptides could sufficiently block the Wnt-binding site of LRP6 in silico. In conclusion, we have designed and improved several small peptides based on the LRP6-binding site of CRD2 of DKK3. These peptides are highly capable of binding to LRP6 in silico, and may prevent the formation of active Wnt-LRP6-Fz complex.
1. Introduction

Wnt signaling pathway regulates cell proliferation and embryonic patterning during development [1]. This signaling pathway has an important role in tumorigenesis of several human cancers and is most often deregulated in colorectal cancer (CRC) facilitating malignant development of polyps in the intestinal epithelium [2, 3]. Nowadays, inhibition of Wnt signaling pathway has been emerged as a promising therapeutic strategy in cancer treatment [4]. In vertebrates, Wnt signaling pathway dissemination is inhibited by two classes of extracellular antagonists: the first class which includes certain members of the DKK family, can bind and inhibit the LRP5/6 component of the Wnt receptor complex; while the members of second class which comprises secreted frizzled related protein (sFRP) family, WIF (Wnt inhibitory factor)-1 and Cerberus, can directly bind to the Wnt proteins or frizzled receptor [5].

While canonical and non-canonical pathway can be activated downstream to wnt signaling pathway, members of DKK family are highly capable of inhibiting canonical Wnt signaling by targeting LRP5/6, and therefore, hinder the formation of Wnt receptor complex. Among members of this family, DKK1 is the most widely studied member and exhibits a high potential of Wnt inhibition [6]. However, there is now compelling evidence suggesting that DKK3 can also be defined as a key inhibitor of Wnt signaling pathway [7]. A crystallographic study has elucidated that DKKs bind to an ectodomain of LRP5/6 which comprises four tandem β-propeller—EGF-like domain (PE) pairs. Also it was found that there is an overlapping between Wnt3a- and DKK-binding surfaces on the third PE pair of LRP5/6 [8]. Therefore, targeting this binding site may interfere with the formation of the active tri-complex (LRP6-Wnt-Frizzled) responsible for initiation of Wnt signaling pathway. Additionally, our in silico analyses established that DKK3, similar to other DKK family members, can bind to the third PE pair of LRP5/6 through its CRD2. Interestingly, we found that the CRD2 of DKK3, here referred to as DKK3C, is bound to the LRP5/6 receptor even tighter than the whole structure. Therefore, this ligand may be regarded as a therapeutic candidate in the cancers which are dependent on the activity of Wnt signaling pathway. Because of their small size and some unique physicochemical properties, small peptides have been widely proposed as therapeutic agents in cancer therapy [9, 10]. It has been demonstrated that use of ligand-derived C-terminal and internal peptides interfering with binding cleft of PDZ domain of Dishevelled (Dvl) proteins, key regulators of Wnt signaling pathway, may be an effective therapeutic strategy for inhibiting Wnt signaling pathway [11]. Nevertheless, due to some intrinsic weaknesses, such as poor physicochemical stability, poor solubility, and short circulating plasma half-life, natural peptides are often not efficiently applicable for use as therapeutic agents [12]. Thus, the weaknesses of initial peptides need to be resolved by computational improvement. Most recently, Zhang Y et al. rationally designed and improved several peptide ligands according to the structure-based information of CaMKIIα-MUPP1 PDZ interaction using a computational mutagenesis approach. The optimized peptides had a high binding affinity to MUPP1 PDZ 11 domain and could competitively disrupt the appropriate interaction between two proteins [13].

To the best of our knowledge, there is no published data reporting the rational peptide designing against Wnt signaling pathway based on the three-dimensional (3D) structure of DKK3. In this study, we have designed several potential inhibitory peptides against Wnt signaling pathway based on the LRP5/6-binding site of DKK3C and evaluated their binding energy, stability and other physicochemical properties in silico. In silico analysis showed that the designed peptides are readily bound to the Wnt- and DKK-binding interfaces of LRP6, and may be considered as possible therapeutic modalities for inhibiting Wnt-mediated cancer progression and invasion.
2. Results

2-1. Evaluation of three-dimensional (3D) structure of DKK3C

The 3D structure of DKK3C has not yet been determined experimentally, hence we predicted 3D structure of this domain using HM method. The quality of predicted 3D model of DKK3C was validated using several methods. Ramachandran plot analysis illustrated that 91%, 8% and 1% of residues were located in the most favored, additionally allowed and generously allowed regions, respectively. There was no residue in the disallowed region of Ramachandran plot (Fig 1A). In order to compare the resulted 3D model of DKK3C with the template structure (PDB ID: 2JTK) a structural alignment was performed using TM-align web tool. TM-score of DKK3C-2JTK structural alignment was computed to be 0.83 implicating a high accuracy of HM prediction method (two structures with TM-score = 1 have almost the same fold) (Fig 1B). The overall model quality computed by ProSA Z-score was calculated as -5.19 implicating a very high quality of the model when compared with experimentally validated protein structures (Fig 1C). The local model quality was also confirmed using ProSA web tool (Fig 1D). In parallel, a secondary structure alignment was conducted and the result manifested a significant secondary structure compatibility between DKK3C and the template model (Fig 1E). The compatibility of 3D-1D structures was evaluated using Verify 3D score. This score assesses the environment of each residue in a model with respect to the high resolution X-ray structures.

Fig 1. Validation of CRD2 model by several methods. (A) Ramachandran plot. The most favored, additionally allowed, generously allowed and disallowed regions are shown in red, yellow, beige and white colors, respectively. (B) Structural alignment of DKK3C (gray) and 2JTK pdb (blue). (C) ProSA Z-score plot of modeled 3D structure of DKK3C. The position of this model among experimentally solved protein structures is shown in an open red circle. (D) Local model quality by plotting energies as a function of amino acid sequence position. Generally, positive values correspond to problematic parts of the input structure. (E) Sequence and secondary structure alignment of DKK3C and mouse dkk2 (PDB ID: 2JTK) conducted by ESPript 3.0 (http://escript.ibcp.fr/EScript/EScript).

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and evaluates the compatibility of the model 3D structure with its sequence by assigning a compatibility score to each residue. Verify 3D was computed as 70.29% implicating a good 3D-1D compatibility of DKK3C model (>65% is considered as valid). ModFOLD results uncovered that the predicted model of DKK3C was significantly correct (p < 0.001). The global model quality score computed by ModFOLD was 0.7145 implicating the high confident model. The global model quality scores ranged between 0 and 1. Generally, scores less than 0.2 implicate the incorrectly modelled structures and scores greater than 0.4 are indicative of more confident models, which are highly similar to the native structure.

2–2. Comparison of LRP6-binding interface of CRD2 in DKK1 and DKK3

Comparison of each separately predicted 3D models of whole DKK and its CRD2, after refining and energy minimization steps, showed that DKK3C is embedded in the protein comprising from five β-strands along with several coils. Despite other DKK members, there is a region consisting of about 60 residues at the C-terminal of DKK3 that makes several small helices. This region may prevent the favorable binding of DKK3C to LRP6 (Fig 2A). As mentioned above, molecular docking studies showed that the binding affinity of DKK3C-LRP6 ($\Delta G_{\text{interaction}} = -14.6$ (kcal mol$^{-1}$)) was remarkably higher than whole DKK3-LRP6 complex ($\Delta G_{\text{interaction}} = -13.8$ (kcal mol$^{-1}$)). Although there was a substantial overlap between the LRP6-binding sites of DKK1 and DKK3, it was realized that a 7-mer box of DKK1 which was implicated in the DKK1-LRP6 interaction had no role in the binding of DKK3C to LRP6.
(Fig 2B and 2C). This may explain why the binding affinity of DKK1C to LRP6 complex was higher than DKK3C-LRP6 complex. According to the molecular docking results as well as literature-based information, two 11-mer boxes of DKK3C (residues 222–232 and 254–264) were chosen for peptide designing. Due to forming more H-bonds and non-bonded connections with LRP6, these two boxes could bind to the third PE pair of LRP6 even stronger than the corresponding LRP6-binding sites of DKK1. Further confirmation was achieved by using Meta Pocket 2.0 (MPK2) web tool [14]. This server utilizes a consensus method in which the binding sites are predicted by eight different methods including LIGSITECS, PASS, Q-Site Finder, SURFNET, Fpocket, GHECOM, Con-Cavity and POCASA. Indeed, several tools were applied to improve the accuracy of identification of putative binding pockets. Taken together, it was found that some specific residues of each mentioned box were actively involved in the DKK3C-LRP6 interactions. Therefore, these residues were not altered during the steps of affinity maturation and stability enhancement.

2-3. Peptide library screening

Based on molecular docking studies as well as literature-based data, two distinct boxes, located at positions 222–232 and 254–264 of DKK3, were chosen for generation of peptide libraries. Since, the residues 222, 226–229, 231–232 and 255–259 had a weak binding affinity to LRP6, they were substituted with favorable residues to improve the LRP-binding affinity of DKK3C. A peptide library was constructed for each box separately. After analysis of constructed libraries using R package and further affinity maturation steps, seven peptides were chosen (four peptides from Box-I and three peptides from Box-II) as the high-scored peptides in case of tolerance, affinity and physicochemical properties. The binding affinity and stability of selected peptides were improved using several repetitions of amino acid substitution, checking binding affinity, stability and other intended features. Only very few mutations on the 11th residue of tolerated peptides were favorable for LRP6 binding (ΔG_{interaction} < ΔG_{interaction} of input peptide). Fig 3 illustrates a schematic representation of the peptide optimization steps.

Furthermore, calculation of the surface potential of the LRP6-binding interface exhibited that DKK1 and DKK3 are mainly bound to a cavity of LRP6, a negatively charged site, through a hydrophobic pocket with a high positive surface potential (Fig 4). Consequently, the positive charge and hydrophobicity parameters were considered as important criteria for selecting desired peptides.

Final selected peptides were examined for water solubility and aggregation hot spots. Fortunately, only one of the peptides, named PEP-I4, had poor water solubility and an aggregation hot spot. The details of various calculated properties for each peptide are tabulated in Table 1.

2–4. The peptide-LRP6 complexes are highly stable and folded during MD simulation

The dynamics of the peptide-LRP5/6 complexes were evaluated by comparing the backbone root mean square deviation (RMSD) plots during simulations. RMSD can accurately show a quantitative expression of the conformational changes of peptide-LRP5/6 complexes during simulations. Protein backbone RMSD plots of peptide-LRP5/6 complexes are depicted in Fig 5A. The PEPI1-LRP5/6 and PEPII2-LRP5/6 complexes were stabilized at earlier time as compared to other peptide-LRP5/6 complexes. However, other peptide-LRP5/6 complexes were also stabilized after approximately 5 ns simulations. The lowest RMSD values were seen in PEPI1-LRP5/6 (~0.15 nm) and PEPII2-LRP5/6 (~0.2 nm) complexes, while the RMSD values of other peptide-LRP5/6 complexes were increased until ~0.25 nm and stayed around this value for a period of approximately 10 ns simulations. In parallel, radius of gyration (Rg)
values of each peptide–protein complex were also plotted to determine the folded state of each complex during 20 ns MD simulations. Except PEPI4-LRP5/6, other peptide-LRP5/6 complexes were highly compacted and folded during simulations (Fig 5B).

### 2–5. Structural fluctuations and surface accessibility of LRP6 binding site

To further assess the structural fluctuations of the peptide-LRP6 complexes, we calculated the root mean square fluctuations (RMSF) from the trajectories of each peptide-LRP6 simulations. Focusing on the Wnt-binding residues of LRP6, the results disclosed that the mobility of these residues was reduced upon peptide binding, specifically by PEP-I1 and PEP-II2. Indeed, the Wnt-binding site of LRP6 became more rigid and tended to be less flexible after peptide binding. Considering the different values of RMSF, LRP6 exhibited a different mobility when it bound to the peptides. Very low fluctuations of Wnt- and DKK-binding residues of LRP6 is most likely due to high stable conformation of peptide-LRP6 complexes. PEPI1-LRP6 and PEPII2-LRP6 complexes illustrated the lowest structural fluctuations throughout MD simulations (Fig 6A). In parallel, the solvent accessibility surface area (SASA) of each Wnt-binding residue was calculated from the trajectories of final 10 ns peptide-LRP6 simulations. The SASA of Wnt-binding residues in the peptide-LRP6 complexes were substantially lower than free LRP6 (except for K770 residue). This suggests that these residues are probably buried upon binding of designed peptides to LRP6. The surface accessibility of H834, a key residue of LRP6 which is involved in binding to Wnt proteins, was significantly decreased upon binding of PEP-I1 and PEP-II2 to LRP6. The average of SASA for Wnt-binding site of each complex was calculated as: free LRP6 (63.04 nm$^2$), PEPI1-LRP6 (41.61 nm$^2$), PEPI2-LRP6 (60.07 nm$^2$), PEPI3-LRP6 (50.63 nm$^2$), PEPI4-LRP6 (52.60 nm$^2$), PEPII1-LRP6 (52.54 nm$^2$), PEPII2-LRP6...
Fig 4. Binding model and surface potentials of DKK1/3 and LRP6. (A) DKK3C-LRP6 and (B) DKK1-LRP6 complexes. DKK1-LRP6 complex was obtained from PDB (ID: 3S2K), while DKK3C-LRP6 is the result of ClusPro docking server. The surface potential of binding sites (open circles) calculated by PyMOL software are shown in the bottom of figure. Blue, red and gray colors infer the positive, negative and hydrophobic regions, respectively.

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Table 1. Binding affinity energy, instability index and physicochemical properties of seven selected peptides.

| Peptide Name | Peptide Sequence | Binding Energy | Instability Index* | GRAVY ** | Net Charge at PH 7 | Water Solubility | Aggregation Hot Spot |
|--------------|------------------|----------------|--------------------|----------|--------------------|-------------------|---------------------|
| **Box-I**    |                  |                |                    |          |                    |                   |                     |
| Initial      | FQRGLLFVPVCT     | -822.2         | 66.03              | 0.836    | 0.9                | Poor              | No                  |
| PEP-I1       | CQRGWVAVRVC      | -1340.2        | -19.15             | -0.282   | 2.9                | Good              | No                  |
| PEP-I2       | CQRGWYGRVKC      | -1159.9        | -4.60              | -0.927   | 2.9                | Good              | No                  |
| PEP-I3       | CQRGWYRVDC       | -1217.3        | -34.52             | -1.173   | 0.9                | Good              | No                  |
| PEP-I4       | CQRGFWGAVRVC     | -1133.1        | -22.63             | -0.036   | 1.9                | Poor              | Yes                 |
| **Box-II**   |                  |                |                    |          |                    |                   |                     |
| Initial      | WELEPDGALDR      | -733.6         | 5.17               | -1.091   | -3                 | Good              | No                  |
| PEP-II1      | WRRVQEGALDR      | -1057.7        | 4.31               | -1.355   | 1.0                | Good              | No                  |
| PEP-II2      | WQKGKQGALDR      | -1295.7        | 0.61               | -1.718   | 2.0                | Good              | No                  |
| PEP-II-3     | WNRGRQGALDR      | -1143.3        | 15.53              | -1.827   | 2.0                | Good              | No                  |

* Instability index value <40 indicates that the peptide is stable.
** The positive and negative GRAVY measures infer the hydrophilicity and hydrophobicity, respectively

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(39.04 nm$^2$) and PEPI3-LRP6 (48.54 nm$^2$). Fig 6B represents the SASA values of Wnt-binding residues for each simulated peptide-LRP6 complex.

2–6. The peptides in complex with LRP6 produced high positive electrostatic potential and H-bonds

Electrostatic potential changes upon binding of the peptides were calculated by using normal mode analysis performed by PBEQ-Solver. The average conformation of the peptides was
extracted from the last 10 ns of each peptide-LRP6 simulations. Expectedly, we found a high positive potential around the peptides when bound to the LRP6 (Fig 7A). Arginine and lysine residues contributed to the high-volume positive coulomb cage bulb around the peptides. While, the negative lobes were attributed to the presence of negatively charged residues, aspartate and glutamate, in the peptide sequence. In addition, the formations of H-bonds between the peptides and LRP6 during MD simulations was identified. The number of peptide-protein H-bonds in PEPI3-LRP6 and PEPII2-LRP6 complexes were obviously higher than other peptides (Fig 7B).

Calculation of several components of peptide-LRP6 binding free energies obtained from the last snapshots of the MD trajectories showed a strong electrostatic interaction between the peptides and LRP6. The net sum of electrostatic interactions between the PEP-I1 and LRP6 \(\langle \Delta E_{\text{electrostatic}} \rangle = -349.13 \text{ kcal/mol} \) was more favorable than other peptide-LRP6 complexes. The obtained results from energetic analysis of 31 equally spaced snapshots taken from each peptide-LRP6 MD simulations are summarized in Table 2. Owing to the same structures of each peptide in the non-bound and bound states, the internal component of the \(\langle \Delta E_{\text{MM}} \rangle, \langle \Delta E_{\text{internal}} \rangle\), has zero contribution to the binding free energy \(\langle \Delta E_{\text{internal}} \rangle = 0 \) [15].

2–7. All peptides could efficiently occupy the Wnt-binding site of LRP6

As mentioned before, there is an overlap between the Wnt- and DKK-binding sites of LRP6. The putative residues of LRP6 which are involved in the binding of LRP6 to Wnt ligands include K770, N813, H834, Y875, M877, E708, E663, K662, Y706 and R1184. These residues are also bound to the members of DKK family. Analysis of peptide-LRP6 using Ligplot
(Dimplot) program revealed that the peptides can readily accommodate on Wnt-binding site of LRP6. However, only PEP-I1, PEP-I4 and PEP-II2 could form hydrogen bond with H834, the main residue involved in the Wnt-LRP6 interactions. All peptides bound to the LRP6’s third PE mainly via non-bonded contacts. More details about peptide-LRP6 interactions are shown in Table 3.

3. Discussion

Although, DKK3 was not previously thought to have interaction with LRP6 [16], emerging evidences indicate that this ligand can also bind to LRP6 via its CRD2 [17]. In another computational study, Fujii and colleagues reported that a unique 7-amino-acid insertion (L249-E255 in human DKK3) and P258 reduces the binding affinity of DKK3C to its receptor [18]. Consistent with this study, we found that a LRP-binding interface of DKK1 with several positively-charged residues (K222, H223, R224, R225, K226) have substituted to non-conserved residues in DKK3 (D243, P244, A245, S246, R247), which along with an insertion of a 4-amino-acid insertion (D250-L253 in human DKK3) may result in a weak binding of DKK3C to LRP6. Accordingly,
Table 2. Evaluation of peptide-LRP6 free energies, contacts and interacting surface.

| Energy  | PEP-I1 | PEP-I2 | PEP-I3 | PEP-I4 | PEP-II1 | PEP-II2 | PEP-II3 |
|---------|--------|--------|--------|--------|---------|---------|---------|
| ClusPro | -1340.2 | -1159.9 | -1217.3 | -1133.1 | -1057.7 | -1295.7 | -1143.3 |
| HADDOCK | -146.4±4.3 | -124.8±2.7 | -133.8±2.7 | -129.9±4.9 | -127.9±5.0 | -143.7±2.9 | -138.1±4.0 |
| ΔK_{Interaction} | -8.0 | -6.8 | -7.0 | -6.3 | -7.0 | -8.3 | -8.1 |
| ΔE_{electrostatic} | -349.13±16.08 | -180.47±8.34 | -174.23±7.43 | -192.49±10.31 | -138.61±4.3 | -163.08±6.98 | -233.28±12.1 |
| ΔE_{VDW} | -52.73±9.12 | -87.26±14.18 | -116.32±18.93 | -9.05±2.2 | -33.02±4.81 | -79.69±8.38 | -33.22±4.72 |
| ΔG_{solvation} | -373.90±21.43 | -398.68±23.1 | -324.21±18.6 | -318.32±14.21 | -318.08±13.98 | -496.38±24.69 | -285.25±12.43 |
| ΔE_{MM} | -401.86 | -267.73 | -290.55 | -201.54 | -171.63 | -242.77 | -266.50 |
| K_d (M) | 6.8e-06 | 1.1e-05 | 7.5e-06 | 4.4e-06 | 1.4e-06 | 8.2e-07 | 1.1e-06 |

Number of Interfacial Contacts (ICs)

| ICs ch-ch | 4 | 4 | 6 | 2 | 5 | 5 | 5 |
| ICs ch-po | 5 | 4 | 2 | 2 | 7 | 7 | 3 |
| ICs ch-ap | 16 | 14 | 15 | 11 | 11 | 15 | 13 |
| ICs po-po | 2 | 2 | 2 | 2 | 2 | 1 | 2 |
| ICs po-ap | 4 | 2 | 4 | 7 | 4 | 6 | 4 |
| ICs ap-ap | 13 | 15 | 13 | 16 | 15 | 18 | 16 |

Non Interacting Surface (NIS) per property

| %NIS ch | 27.54 | 28.99 | 28.17 | 30.54 | 29.52 | 30.32 | 29.80 |
| %NIS ap | 38.42 | 38.65 | 39.44 | 36.95 | 38.10 | 33.84 | 35.86 |

K_d: Dissociation constant
ch: charge, po: polar, ap: apolar

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we found that there are two distinct boxes in the DKK3C that are highly capable of binding to the third PE pair of LRP6. Therefore, several small peptides were designed based on these boxes of DKK3C. MD simulations revealed that the peptides can efficiently bind to Wnt-binding site of LRP6 and presumably block it.

Table 3. Residues of LRP6 interacting with the peptides as predicted by LIGPLOT. Bold residues: Wnt- and DKK-binding, bold and underline: DKK-binding.

| LRP6 Residues | Peptide Name | Hydrogen bonds forming AAs | Non-bonded contacts forming AAs |
|---------------|--------------|----------------------------|--------------------------------|
| PEP-I1        | L667, M710, D668, G795, E708, N794, Y706, E663, H834, Y875, L838, D878, I879, R886 | G709, A666, A752, W767, R751, I681, A664, A640, M877, F836, V876, W850, G837, L880, T797, T839, L796 |
| PEP-I2        | T724, E708, R751, Y706, N794, M877, E663, W850 | L796, A752, G795, S665, D878, F836, Y875, H834, L810 |
| PEP-I3        | G795, T765, W767, R792, R751, N794, E708, Y706, I681, H834, Y875 | L810, A752, A793, S665, A664, M877, F836, W850 |
| PEP-I4        | Y706, E708, R639, Y875, R792, R751 | M877, E663, W850, V876, F836, H834, L810, W767, F792 |
| PEP-I1        | R792, L810, D811, W850, Y875, Y786, M877, | E708, Y706, H834, F836, P835, E663, R639 |
| PEP-I2        | H834, W850, Y875, R751, E708, D878, S665, R792 | V876, M877, Y706, F836, N794, A666, L810, |
| PEP-I3        | A664, E663, S665, E708, D878, R751, R792, N794, L810 | M877, A666, W767, F836, V876, |

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According to the Chen et al. study, E663, E708, Y875, M877 and H834 residues of LRP6 are important in its binding to Wnt ligands. However, the impacts of E663 and H834 residues on the Wnt-LRP6 interaction are significantly higher than other aforementioned residues [8]. The lowest SASA measures for E663 and H834 residues of LRP6 were observed in PEPI1-LRP6 (E663 = 30.15 nm$^2$, H834 = 13.72 nm$^2$) and PEPII1-LRP6 (E663 = 26.31 nm$^2$, H834 = 1.56 nm$^2$) complexes compared to free LRP6 (E663 = 62.4 nm$^2$, H834 = 33.85 nm$^2$). This suggests that the binding of PEP-I1 and PEP-II2 to LRP6 may cause a meaningful conformational change in the 3D structure of LRP6 which results in a reduced availability of E663 and H834 residues for Wnt ligands. The SASA values of other Wnt-binding residues of LRP6 were also reduced through interaction of the peptides with LRP6 (Fig 6B). In all cases, except PEP-I4 and PEP-III1 peptides, the peptides were anchored by the hydrogen bonds with at least one of these critical Wnt-binding residues, E663 and H834. Among seven selected peptides, only PEP-I1 could form hydrogen bonds with both E663 and H834 residues simultaneously. The common Wnt- and DKK-binding residues of LRP6, which include E663, Y706, E708, K770, N813, H834, Y875 and M877 residues, are probably occupied by the peptides through hydrogen bonds (Fig 8). Studies have shown that I681, S749, R751, W767, G769, R792, N794, L810, D811, D830, F836 and W850 residues of LRP6 play a pivotal role in

Fig 8. (Up) Representation of third PE pair of LRP6. (Down) H-bond interactions between the designed peptides and third PE pair of LRP6. (A) PEP-I1, (B) PEP-I2, (C) PEP-I3, (D) PEP-I4, (E) PEP-II1, (F) PEP-II2 and (G) PEP-II3 with LRP6 surface. The common Wnt- and DKK-binding residues of LRP6 are shown in color. Non-bonded connections have been omitted to simplify the figure.

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the DKK-LRP6 interactions [19–21]. Our results indicated that all selected peptides could efficiently bind to at least five of these residues simultaneously (Table 3). On the whole, designed peptides may have the ability to selectively interact with Wnt- and DKK-binding residues of LRP6 and result in the prevention of Wnt signaling pathway through blocking Wnt-binding site of LRP6.

RMSD and Rg values are widely used for evaluating macromolecules stability and rigidity, respectively [22, 23]. A slight rise of RMSD values of peptide-LRP6 complexes implicate higher stability of peptide-protein complexes. The highest complex stability was observed in PEPI1-LRP6 and PEPII2-LRP6 complexes. Furthermore, Rg values of peptide-protein complexes were decreased during MD simulations. This elucidates that peptide-LRP6 complexes are highly compacted under physiological conditions. The structural fluctuations of peptide-LRP6 complexes were also assessed by measuring the RMSF values of each complex during MD simulations. RMSF is an indicator of the macromolecular flexibility and rigidity under thermal equilibrium, and the high fluctuating residues in the protein sequence are highly unstable presumably involved in promoting the protein structure to an unfolded state [24]. Pushie et al. demonstrated that the residues with higher RMSF values are associated with surface-exposed regions involved in protein-protein interactions [25]. PEP-I1, PEP-I3 and PEP-II2 could substantially reduce the RMSF measures of Wnt-binding residues compared to other peptides. The SASA evaluation of Wnt-binding site of LRP6 also confirmed the reduced accessibility of these residues upon peptide binding (Fig 6).

This is reasonable enough that the surface accessibility of functional residues is crucial for protein function. A reduced SASA value has been known to be associated with a significant reduction of protein activity [26]. Our SASA analysis revealed that Wnt-binding residues of LRP6 tended to be more buried upon peptide-LRP6 interactions. This suggests that the binding of designed peptides to LRPS/6 may result in the attenuation of Wnt-binding interface of LRP6 through burying the functional residues involved in Wnt-LRP6 interaction.

Nowadays, electrostatic interactions have been proven to be critical for the catalytic activity and protein—protein recognitions [27]. These energies stabilize the folded state of a protein and play a key role in fold specificity of macromolecules [28, 29]. Cheng et al. have reported that the DKK1-binding site of LRP6 is a hydrophobic patch with some polar residues. They found that the hydrophobic region of this binding site interacts with F205 and W206 residues of DKK1. Additionally, several polar interactions including D811 of LRP6 with R236 of DKK1, E708 of LRP6 with H204 of DKK1, R792 of LRP6 with E232 of DKK1, and H834 of LRP6 with S228 of DKK1 are actively involved in the maintenance of LRP6-DKK1 complex [21]. Moreover, Lin et al. found that several positively charged residues have a crucial role in the binding of Mesd to LRP6 [30]. These findings underscore that the binding cavities of LRP6 are largely charged with negatively charged residues. During the peptide binding affinity maturation steps, we considered the high positive surface potential of peptides as an important property for selecting the final peptides. Accordingly, peptides with higher positive surface potentials had a higher binding affinity to the LRP6 binding site.

**Conclusion**

In this study, we have proposed a therapeutic peptide design strategy to interrupt the Wnt-LRP6 interaction which was based on structural, dynamic and energetic analyses of the DKK3C-LRP6 interaction as well as the previous published data. The designed peptides are highly capable of binding to the Wnt- and DKK-binding sites of LRP6 in silico, in a selective manner. These peptides, especially PEP-I1 and PEP-II2, could induce a local conformational change in LRP6 structure leading to an increased buried surface area of Wnt-binding site. The
peptide-LRP6 complexes were highly stable and compact, and therefore, could be considered as possible therapeutic agents for hindering Wnt signaling pathway in the cancers which are dependent on function of this signaling pathway. However, the impacts of the selected peptides on Wnt signaling pathway need to be validated experimentally.

4. Materials and methods

4–1. Modeling, refinement and quantitative evaluation of DKK3C

Modeller v9.15 software was used for performing HM using the crystal structure of CRD2 of the mouse dkk2 (PDB ID:2JTK) as the template [31]. The HM method consists of the following steps: (i) template selection; (ii) target template alignment; (iii) model building; and (iv) model evaluation. These steps can be iteratively repeated, until a reasonably accurate model is generated. The sequence identity and similarity of this template with DKK3C were defined to be 30% and 73% respectively. Among 10000 generated models, 10 top models were chosen (based on their DOPE score) for further analyses. Evaluation of the models quality was carried out by VADAR, SAVES (https://services.mbi.ucla.edu/SAVES/), ProSA and ModFOLD web tools [32–34]. Parallel checking of model quality was conducted utilizing several tools to enhance the accuracy of validation. Finally, the best model was selected and considered for further analyses. GalaxyRefine and NOMAD-REF servers were used for refining and energy minimizing the selected model [35]. These servers rebuilt the model side-chains and performed overall structure relaxation using MD simulations. The low free-energy conformations were further refined by full-atomic simulations using ModRefiner method [36].

4-2. Construction of peptide library

The stability of initial peptide sequences was remarkably low; therefore, a focused peptide library was constructed based on all possible amino acid substitutions. According to the molecular docking studies and literature data mining, some residues were recognized as the key residues involved in DKK3-LRP6 interactions, and therefore, were not manipulated. A peptide library was constructed using Rosetta backrub and sequence tolerance protocols implemented in the Rosetta3.5 software [37]. Using these protocols, a given peptide sequence can be tolerated while still preserving its function at the defined level. The results were analyzed using R package and the tolerated peptide sequence was generated [38].

4-3. Optimization of binding affinity and stability of the peptides

First, the initial peptide sequences were tolerated utilizing Rosetta backrub and sequence tolerance protocols. However, the binding affinity of these peptides, both BoxI- and BoxII-derived peptides, to LRP6 were not satisfactory and needed to be improved. To this end, several steps of affinity maturation were performed by generating some logical substitutions. BLOSUM-62 scoring matrix, one of the most effective matrices for predicting the common amino acid substitutions, was used to generate non-deleterious substitutions [39]. Except the amino acid substitutions with score of -4, any residue was separately substituted with all other amino acids. Nevertheless, the main LRP6-DKK3C interacting residues which had been predicted by molecular docking studies as well as literature-based information did not change. Following each substitution, the binding affinity, stability and several physicochemical properties of the peptides were evaluated. At each position, the amino acid substitutions that lead to an increase in the binding affinity of the peptides to LRP6 were distinguished. All possible combinations of favorable substitutions were generated and their binding energy to LRP6 was calculated again. In this step, MD simulations were not carried out due to the high computational demand.
Finally, seven improved peptides (Box-I: four peptides, Box-II: three peptides) were selected for production of MD simulation trajectories.

4-4. Evaluation of binding affinity and stability of peptide variants

Any substitution was followed by evaluating peptide binding affinity and stability using ClusPro docking server and ProtParam (http://us.expasy.org/tools/protparam.html) web tool, respectively [40]. In parallel, a molecular docking was separately conducted using HADDOCK server to validate the peptide-LRP6 interaction mode. This program is a powerful docking tool which uses a data-driven approach supporting for a wide range of experimental data [41]. ClusPro assigns a weighted score for binding energy of a given protein complex. ProtParam calculates the instability index of the proteins and peptides. Instability index is a reliable measure that provides an estimate of the stability of a protein in a test tube by using statistical analysis of 12 unstable and 32 stable proteins. The proteins and peptides with instability index lower than 40 are classified as stable [42]. Prior to docking, the structure of each peptide was predicted by using PEP-FOLD server [43]. The initially docked peptide-protein complexes resulted from ClusPro server were refined using GalaxyRefineComplex web tool (http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=COMPLEX).

4-5. Calculation of physicochemical properties of peptides

Several physicochemical properties of the peptides including molecular weight, net charge at pH 7, pI, water solubility, aliphatic index, grand average of hydropathicity (GRAVY) and aggregation hot spots were calculated using ProtParam, PepCalc and Aggrescan tools [44, 45]. It is now widely accepted that a positive GRAVY value infers the hydrophobicity, while a negative GRAVY value indicates the hydrophilicity level of a protein [46]. Considering the binding affinity and stability, the aforementioned parameters were also computed to choose the best three peptides with completely matched properties for in vitro experiments.

4-6. MD simulations

In order to assess the conformational changes of the peptides, alone and in complex with LRP6, MD simulations were carried out using GROMACS version 5 for a period of 20 ns using the original GROMOS96 force field 43A1 [47]. MD simulation is a very efficient tool that is commonly used in the solid-state physics and materials community for modeling solids and liquids at the atomic level. Single peptides as well as peptide-LRP6 complexes were solvated in a solvation box using SPC/E water model with 10Å distance between the edges of the box and the peptides. The system was neutralized by replacing solvent molecules with Cl⁻ and Na⁺ ions. Subsequently, the system was relaxed using steepest descent algorithm followed by conjugate gradient algorithm. After several energy minimization steps, the entire system was equilibrated for 100 ps using canonical (NVT) and the isothermal—isobaric (NPT) ensembles. Finally, the equilibrated system was simulated for a period of 20 ns using NPT ensemble to understand the structural dynamics of the peptides undergoing interaction with LRP6.

4-7. Visual presentations

All protein figures were created using PyMOL and VMD programs [48, 49]. The graphs obtained from MD simulations were mainly plotted using the Grace software (GRACE: http://plasma-gate.weizmann.ac.il/Grace/).
4-8. Calculation of the electrostatic potential around the peptides

The electrostatic potential around the selected peptides was calculated for the extracted structures of the peptides derived from peptide–LRP6 MD simulations. We used the average coordinates of peptide structures extracted from the final 10 ns simulations (10–20 ns) for the electrostatic potentials calculations. The PBEQ-Solver [50] was used for calculating electrostatic potential surface around each peptide by solving the nonlinear Poisson-Boltzmann (PB) equations with a grid spacing of 1 Å [27].

4-9. Energetic analysis

To calculate the binding energy of each peptide–LRP6 complex, an average structure was prepared from the last 10 ns MD simulations. The electrostatic and van der Waals (VdW) energies were calculated for the last snapshot configurations taken from the MD trajectories of peptide–LRP6 complexes and then averaging the values. Unbound peptides and LRP6 snapshot configurations were also prepared from the peptide–LRP6 complex trajectories. The coordinates of 31 snapshots were taken at 100-ps intervals during the last 3 ns simulations of each peptide–LRP6 complex, where the complexes appeared to acquire a stable configuration, and used for energetic analysis.

4-9-1. Gibbs free energy of the peptide–LRP6 interaction. The Gibbs free energy change (ΔG) for all selected peptides undergoing the interaction with LRP6 were calculated from the atomic structures of peptides and LRP6 receptor. The interaction energy calculations for each peptide–LRP6 complexes were computed for their average structures extracted from the last 10 ns of MD production phase. PRODIGY web tool was used for measuring ΔG of peptide–LRP6 interactions as well as their polar and nonpolar contacts [51]. This server computes the number of interfacial contacts (ICs) and percentage of non-interacting surface (NIS) in a protein-protein complex which have been demonstrated to be the key parameters in the protein-protein binding energy prediction [52]. Free energy of interaction was calculated as follow:

\[
\Delta G_{\text{interaction}} = -0.09459 \text{ ICs}_{\text{charged}/\text{charged}} - 0.10007 \text{ ICs}_{\text{charged}/\text{apolar}} + 0.19577 \text{ ICs}_{\text{polar}/\text{polar}} - 0.22671 \text{ ICs}_{\text{polar}/\text{apolar}} + 0.18681 \%\text{NIS}_{\text{polar}} + 0.3810 \%\text{NIS}_{\text{charged}} - 15.9433
\]

Where, ICs_{xxx/yyyy} is the number of Interfacial Contacts found between the interface of the first and the second interactors classified based on the polar/apolar/charged nature of the interacting residues (i.e. ICs_{charged/apolar} is the number of ICs between charged and apolar residues). The contacts between two residues were defined if any of their heavy atom was within a distance of 5.5 Å.

4-9-2. Solvation energy. The solvation energy consists of the electrostatic (ΔG_{polar, solvation}) and nonpolar (ΔG_{apolar, solvation}) contributions. Solvation energies of the peptides were presented as the average values of the energies calculated for the snapshot configurations at the last 3 ns MD simulations. The PBEQ-Solver has been used for calculating the solvation energy of each peptide.

4-9-3. Molecular Mechanics (MM) calculations. The free binding energies of peptide–LRP6 complexes have been computed with the MM according to over snapshots from MD trajectories. The 31 equally spaced snapshot configurations extracted from the last 3 ns peptide–LRP6 simulations were used for energy calculations. \(\langle E_{\text{MM}} \rangle\) is generally calculated as follow:

\[
\langle E_{\text{MM}} \rangle = \langle E_{\text{internal}} \rangle + \langle E_{\text{electrostatic}} \rangle + \langle E_{\text{VdW}} \rangle
\]

Where, the \(\langle E_{\text{internal}} \rangle\) value includes bond, angle, and torsional angle energies, \(E_{\text{electrostatic}}\) and \(E_{\text{VdW}}\) values refer to the non-bonded electrostatic and van der Waals interactions of the peptide–LRP6 complex.
peptides and proteins. The value $E_{\text{electrostatic}}$ was calculated using APBS [53] as implemented in PyMOL. The $E_{\text{VdW}}$ value was calculated using a standard GROMACS utility with the same force field applied in the MD simulations (No cutoff was defined for the evaluation of non-bonded interactions).

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**References**

1. Clevers H. Wnt/β-catenin signaling in development and disease. Cell. 2006; 127(3):469–80. doi: 10.1016/j.cell.2006.10.018 PMID: 17081971

2. Polakis P. Wnt signaling in cancer. Cold Spring Harbor perspectives in biology. 2012; 4(5):a008052. doi: 10.1101/cshperspect.a008052 PMID: 22438566

3. Myant K, Sansom OJ. Wnt Signaling and Colorectal Cancer. Wnt Signaling in Development and Disease: Molecular Mechanisms and Biological Functions. 2014: 357–67.

4. You L, He B, Xu Z, Uematsu K, Mazieres J, Mikami I, et al. Inhibition of Wnt-2-mediated signaling induces programmed cell death in non-small-cell lung cancer cells. Oncogene. 2004; 23(36):6170–4. doi: 10.1038/sj.onc.1207844 PMID: 15208662

5. Cruciat C-M, Niehrs C. Secreted and transmembrane wnt inhibitors and activators. Cold Spring Harbor perspectives in biology. 2013; 5(3):a015081. doi: 10.1101/cshperspect.a015081 PMID: 23085770

6. Kawano Y, Kypta R. Secreted antagonists of the Wnt signalling pathway. Journal of cell science. 2003; 116(19):2627–34.

7. Veeck J, Dahl E. Targeting the Wnt pathway in cancer: the emerging role of Dickkopf-3. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer. 2012; 1825(1):18–28.

8. Chen S, Bubeck D, MacDonald BT, Liang W-X, Mao J-H, Malinauskas T, et al. Structural and functional studies of LRP6 ectodomain reveal a platform for Wnt signaling. Developmental cell. 2011; 21(5):848–61. doi: 10.1016/j.devcel.2011.09.007 PMID: 22000855
9. kafshdouziam M, Poorebrahaim M, Najafi S, Rahimi H, Karimipoor M, Gheibi N. Computational Design of TrkB Peptide Inhibitors and Their Biological Effects on Ovarian Cancer Cell Lines. Int J Pept Res Ther. 2016:1–11.

10. Maryam I, Seyede SM, Seyed SA, Sadegh Azimzadeh SA, Sadegh A, Mansour P, HOXB7 and Hsa-miR-222 as the Potential Therapeutic Candidates for Metastatic Colorectal Cancer. Recent Patents on Anti-Cancer Drug Discovery. 2016; 11:1–10. http://dx.doi.org/10.2174/1574892811999160628114857.

11. Zhang Y, Appleton BA, Wiesmann C, Lau T, Costa M, Hannounsh RN, et al. Inhibition of Wnt signaling by Dishevelled PDZ peptides. Nature chemical biology. 2009; 5(4):217–9. doi: 10.1038/nchembio.152 PMID: 19252499

12. Fosgerau K, Hoffmann T. Peptide therapeutics: current status and future directions. Drug discovery today. 2015; 20(1):122–8. doi: 10.1016/j.drudis.2014.10.003 PMID: 25450771

13. Zhang Y-L, Han Z-F, Sun Y-P. Structure-based identification of CaMKIIa-interacting MUPP1 PDZ domains and rational design of peptide ligands to target such interaction in human fertilization. Amino acids. 2016; 48(6):1509–21. doi: 10.1007/s00726-016-2211-6 PMID: 26984442

14. Zhang Z, Li Y, Lin B, Schroeder M, Huang B. Identification of cavities on protein surface using multiple computational approaches for drug binding site prediction. Bioinformatics. 2011; 27(15):2083–8. doi: 10.1093/bioinformatics/btr331 PMID: 21636590

15. Huo S, Wang J, Cieplak P, Kollman PA, Kuntz ID. Molecular dynamics and free energy analyses of cathepsin D-inhibitor interactions: Insight into structure-based ligand design. Journal of medicinal chemistry. 2002; 45(7):1412–9. PMID: 11906282

16. Mao B, Wu W, Davidson G, Marhold J, Li M, Mechler BM, et al. Kremen proteins are Dickkopf receptors that regulate Wnt/β-catenin signalling. Nature. 2002; 417(6889):664–7. doi: 10.1038/nature756 PMID: 12050670

17. Mohammadpour H, Pourfathollah AA, Zarif MN, Khalili S. Key role of Dkk3 protein in inhibition of cancer cell proliferation: An in silico identification. Journal of theoretical biology. 2016.

18. Fujii Y, Hoshino T, Kumon H. Molecular simulation analysis of the structure complex of C2 domains of DKK family members and β-propeller domains of LRP5/6: explaining why DKK3 does not bind to LRP5/6. Acta medica Okayama. 2013; 68(2):63–78.

19. Ahn VE, Chu ML, Choi HJ, Tran D, Abo A, Weis WI. Structural basis of Wnt signaling inhibition by Dickkopf binding to LRP5/6. Developmental cell. 2011; 21(5):862–73. Epub 2011/10/18. doi: 10.1016/j.devcel.2011.09.003 PMID: 22000856

20. Bao J, Zheng JJ, Wu D. The structural basis of DKK-mediated inhibition of Wnt/LRP signaling. Science signaling. 2012; 5(224):pe22. Epub 2012/05/17. doi: 10.1126/scisignal.2003028 PMID: 22589387

21. Cheng Z, Biechele T, Wei Z, Morrone S, Moon RT, Wang L, et al. Crystal structures of the extracellular domain of LRP6 and its complex with DKK1. Nature structural & molecular biology. 2011; 18(11):1204–10.

22. Simmerling C, Strockbine B, Roitberg AE. All-atom structure prediction and folding simulations of a stable protein. Journal of the American Chemical Society. 2002; 124(38):11258–9. PMID: 12236726

23. Lobanov MY, Bogatyreva N, Galzhitskaya O. Radius of gyration as an indicator of protein structure compactness. Molecular Biology. 2008; 42(4):623–8.

24. Monhemi H, Housaindokht MR, Moosavi-Movahedi AA, Bozorgmehr MR. How a protein can remain stable in a solvent with high content of urea: insights from molecular dynamics simulation of Candida antarctica lipase B in urea: choline chloride deep eutectic solvent. Physical Chemistry Chemical Physics. 2014; 16(28):14882–93. doi: 10.1039/c4cp00503a PMID: 24930496

25. Pushie MJ, George GN. Active-site dynamics and large-scale domain motions of sulfite oxidase: A molecular dynamics study. The Journal of Physical Chemistry B. 2010; 114(9):3266–75. doi: 10.1021/jp908731f PMID: 20158265

26. Padhi AK, Jayaram B, Gomes J. Prediction of functional loss of human angiojenin mutants associated with ALS by molecular dynamics simulations. Scientific reports. 2013; 3.

27. Sheinerman FB, Norel R, Honig B. Electrostatic aspects of protein—protein interactions. Current opinion in structural biology. 2000; 10(2):153–9. PMID: 10753808

28. Lumb KJ, Kim PS. A buried polar interaction imparts structural uniqueness in a designed heterodimeric coiled coil. Biochemistry. 1998; 37(37):13042–3.

29. Xiao L, Honig B. Electrostatic contributions to the stability of hyperthermophilic proteins. Journal of molecular biology. 1999; 289(5):1435–44. doi: 10.1006/jmbi.1999.2810 PMID: 10373377

30. Lin C, Lu W, Zhang W, Londoño-Joshi AI, Buchsbaum DJ, Bu G, et al. The C-terminal region Mesd peptide mimics full-length Mesd and acts as an inhibitor of Wnt/β-catenin signaling in cancer cells. PloS one. 2013; 8(2):e58102. doi: 10.1371/journal.pone.0058102 PMID: 23469146
31. Martí-Renom MA, Stuart AC, Fiser A, Sánchez R, Melo F, Šali A. Comparative protein structure modeling of genes and genomes. Annual review of biophysics and biomolecular structure. 2000; 29(1):291–325.

32. Willard L, Ranjan A, Zhang H, Monzavi H, Boyko RF, Sykes BD, et al. VADAR: a web server for quantitative evaluation of protein structure quality. Nucleic acids research. 2003; 31(13):3316–9. PMID: 12824316

33. Wiederstein M, Sippl MJ. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic acids research. 2007; 35(suppl 2):W407–W10.

34. McGuffin LJ, Buenavista MT, Roche DB. The ModFOLD4 server for the quality assessment of 3D protein models. Nucleic Acids Res. 2013; 41(Web Server issue):W368–72. Epub 2013/04/27. doi: 10.1093/nar/gkt294 PMID: 23620298

35. Heo L, Park H, Seok C. GalaxyRefine: protein structure refinement driven by side-chain repacking. Nucleic acids research. 2013; 41(W1):W384–W8.

36. Xu D, Zhang Y. Improving the physical realism and structural accuracy of protein models by a two-step atomic-level energy minimization. Biophysical journal. 2011; 101(10):2525–34. doi: 10.1016/j.bpj.2011.09.024 PMID: 22098752

37. Lauck F, Smith CA, Friedland GF, Humphris EL, Kortemme T. RosettaBackrub—a web server for flexible backbone protein structure modeling and design. Nucleic acids research. 2010; 38(suppl 2):W569–W75.

38. Ihaka R, Gentleman R. R: a language for data analysis and graphics. Journal of computational and graphical statistics. 1996; 5(3):299–314.

39. Henikoff S, Henikoff JG. Amino acid substitution matrices from protein blocks. Proceedings of the National Academy of Sciences. 1992; 89(22):10915–9.

40. Comeau SR, Gatchell DW, Vajda S, Camacho CJ. ClusPro: an automated docking and discrimination method for the prediction of protein complexes. Bioinformatics. 2004; 20(1):45–50. PMID: 14693807

41. De Vries SJ, van Dijk M, Bonvin AM. The HADDOCK web server for data-driven biomolecular docking. Nature protocols. 2010; 5(5):883–97. doi: 10.1038/nprot.2010.32 PMID: 20431534

42. Guruprasad K, Reddy BB, Pandit MW. Correlation between stability of a protein and its dipeptide composition: a novel approach for predicting in vivo stability of a protein from its primary sequence. Protein engineering. 1990; 4(2):155–61. PMID: 2075190

43. Maupetit J, Derreumaux P, Tuffery P. PEP-FOLD: an online resource for de novo peptide structure prediction. Nucleic acids research. 2009:gkp323.

44. Lear S, Cobb SL. Pep-Calc. com: a set of web utilities for the calculation of peptide and peptoid properties and automatic mass spectral peak assignment. Journal of computer-aided molecular design. 2016; 30(3):271–7. doi: 10.1007/s10822-016-9902-7 PMID: 26909992

45. Conchillo-Solé O, de Groot NS, Avilés FX, Vendrell J, Daura X, Ventura S. AGGRESCAN: a server for the prediction and evaluation of "hot spots" of aggregation in polypeptides. BMC bioinformatics. 2007; 8(1):1.

46. Kyte J, Doolittle RF. A simple method for displaying the hydrophilic character of a protein. Journal of molecular biology. 1982; 157(1):105–32. Epub 1982/05/05. PMID: 7108955

47. Abraham MJ, Murtola T, Schulz R, Páll S, Smith JC, Hess B, et al. GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. SoftwareX. 2015; 1:19–25.

48. DeLano WL. The PyMOL molecular graphics system. 2002.

49. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. Journal of molecular graphics. 1996; 14(1):33–8. PMID: 8744570

50. Jo S, Vargyas M, Vasko-Szedlar J, Roux B, Im W. PBEO-Solver for online visualization of electrostatic potential of biomolecules. Nucleic acids research. 2008; 36(suppl 2):W270–W5.

51. Vangone A, Bonvin AM. Contacts-based prediction of binding affinity in protein-protein complexes. 2015; 4:e07454.

52. Kastritis PL, Rodrigues JP, Folkers GE, Boelens R, Bonvin AM. Proteins feel more than they see: fine-tuning of binding affinity by properties of the non-interacting surface. Journal of molecular biology. 2014; 426(14):2632–52. Epub 2014/04/29. doi: 10.1016/j.jmb.2014.04.017 PMID: 24768922

53. Baker NA, Sept D, Joseph S, Hose M, McCammon JA. Electrostatics of nanosystems: application to microtubules and the ribosome. Proceedings of the National Academy of Sciences. 2001; 98(18):10037–41.