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"Structural Insights and Contact Network Analysis of Fusion Inhibitors Binding to SARS-CoV-2 Spike Protein"

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ABSTRACT

This study investigates the molecular interactions and dynamics of two fusion inhibitors, Happy_00 and Happy_06, targeting the cleavage sites of the SARS-CoV-2 spike protein to block viral entry. Molecular docking simulations revealed that Happy_00 performed better at the first cleavage site (Arg685/Ser686) due to its compact structure, while Happy_06, with flexible GGGS linkers, demonstrated enhanced binding at the second cleavage site (Arg815/Ser816). Molecular dynamics (MD) simulations confirmed the stability of both inhibitors, with RMSD values around 2.3–2.5 Å and steady radius of gyration (Rg). Happy_00 showed minimal residue fluctuations (RMSF < 1.5 Å), while Happy_06 exhibited higher flexibility (RMSF up to 2.3 Å), allowing it to adapt to buried binding pockets. Contact maps highlighted key interactions between the inhibitors and the spike protein, supporting the role of flexibility in enhancing inhibitor efficacy at dynamic binding sites. These findings suggest that tetanus toxoid-derived fusion peptides are promising candidates for blocking viral fusion, providing a foundation for future antiviral strategies against SARS-CoV-2

INTRODUCTION

The SARS-CoV-2 spike protein plays a pivotal role in viral entry by binding to the ACE2 receptor on host cells and undergoing cleavage at specific sites by host proteases such as TMPRSS2. These cleavage events occur at the S1/S2 boundary (Arg685/Ser686) and the S2′ site (Arg815/Ser816), which expose the fusion domain required for viral membrane fusion(Peng et al., 2021;Hoffmann, 2020). Inhibiting these cleavage events represents an attractive therapeutic strategy, as it disrupts the viral life cycle at an essential stage, preventing infection and replication(Poduri et al., 2020;Baughn et al., 2020).

The design of fusion inhibitors that can specifically bind to these cleavage sites and block TMPRSS2-mediated activation has garnered significant interest. Peptide-based inhibitors, such as EK1, have been explored in prior studies for their ability to interfere with viral fusion processes(Lin et al., 2022). However, optimizing the design of these inhibitors to enhance binding stability and flexibility remains a challenge. In particular, the rigidity vs. flexibility trade-off plays a critical role in determining how effectively an inhibitor can engage with both accessible and buried residues within the target protein(Römer et al., 2021).

This study investigates the binding dynamics of two fusion inhibitors, Happy_00 and Happy_06, specifically designed to target the SARS-CoV-2 spike protein's cleavage sites. Happy_00 lacks linkers and has a rigid structure, while Happy_06 incorporates six GGGS linkers, introducing flexibility. Understanding how these structural

differences influence binding interactions at different cleavage sites provides valuable insights into designing next-generation antiviral peptides

The use of contact network analysis offers a deeper understanding of the molecular interactions involved in these inhibitor-protein complexes. Contact maps illustrate the interaction networks between key residues of the inhibitors and the spike protein, helping to visualize the binding landscape. This network approach is particularly useful when comparing rigid and flexible inhibitors, as it highlights how each structure interacts differently with the first cleavage site (Arg685/Ser686) and the second cleavage site (Arg815/Ser816).

Recent studies have emphasized the importance of linker flexibility in enhancing the binding potential of protein-based inhibitors. Peptide inhibitors with flexible linkers can adjust their conformation to engage with buried or hidden residues, which are often challenging for rigid structures to access (Silacci et al., 2014). Conversely, rigid inhibitors tend to perform better at accessible binding sites, where their stability ensures long-lasting interactions.

In the current study, Happy_00 and Happy_06 were evaluated for their binding affinities and structural performance at the two cleavage sites through molecular docking and dynamics simulations. Quantitative metrics such as binding energy, electrostatic energy, and van der Waals interactions provide insights into the stability and strength of these interactions. Furthermore, contact maps reveal the specific residues involved in the interaction networks, offering a detailed picture of how each inhibitor binds to the spike protein.

The findings from this study are particularly relevant for therapeutic development, as they highlight the advantages of structural flexibility in enhancing the effectiveness of peptide-based inhibitors. The contact network analysis shows that Happy_06's flexible linkers enable it to bind more effectively at the second, buried cleavage site. Meanwhile, Happy_00's compact structure gives it a competitive edge at the first, more accessible cleavage site. These insights underscore the need to balance rigidity and flexibility in the design of fusion inhibitors, tailoring them for specific target sites.

By leveraging the structural insights gained from contact network analysis and molecular dynamics simulations, this study contributes to the optimization of fusion inhibitor design. These inhibitors, if validated through in vitro and in vivo experiments, have the potential to become valuable components of antiviral strategies targeting SARS-CoV-2 and other emerging coronaviruses.

Methods

1. Inhibitor Design and Modelling

The fusion inhibitors Happy_00 and Happy_06 were designed to target the SARS-CoV-2 spike protein's cleavage sites. Both inhibitors incorporated the EK1 peptide, known for its antiviral properties, fused with a C fragment of tetanus toxoid to provide structural stability.

- Happy_00: A compact structure without any linker between the EK1 peptide and the C fragment, designed for rigid binding at accessible sites.
- Happy_06: A flexible structure incorporating six GGGS linkers, designed to provide adaptability for binding to deeper, buried sites within the spike protein.

The inhibitors were modelled using Modeller and RaptorX to generate accurate three-dimensional structures. The molecular structures were refined through energy minimization to ensure optimal geometry and reduce steric clashes(Eswar et al., 2008;

Kumar, Raj, S, et al., 2023; Šali et al., 1995; Webb & Sali, 2016)Wang et al., 2016).

2. Molecular Docking Simulations

Molecular docking simulations were performed to evaluate the binding affinity of Happy_00 and Happy_06 with the SARS-CoV-2 spike protein. The spike protein structure was obtained from the Protein Data Bank (PDB ID: 6VYB). Docking simulations were carried out using HADDOCK 2.4, which integrates biochemical and biophysical data to guide docking at cleavage sites(Dominguez et al., 2003; van Zundert et al., 2016).

- First Cleavage Site (Arg685/Ser686): Both inhibitors were docked to determine their binding affinities at this accessible site.
- Second Cleavage Site (Arg815/Ser816): The inhibitors were tested for binding strength at this deeper, more challenging site.

HADDOCK scores and binding energies, including electrostatic and van der Waals interactions, were used to rank the docked complexes.

3. Molecular Dynamics (MD) Simulations

Molecular dynamics simulations were conducted using GROMACS 2021.3 to further evaluate the stability and flexibility of the inhibitor-protein complexes(Van Der Spoel et al., 2005; Vieira et al., 2023).

System Preparation: Each inhibitor-spike protein complex was solvated in a TIP3P water box, with sodium ions added for charge neutralization. Energy minimization was performed using the steepest descent algorithm to remove steric clashes(Abdalla et al., 2022).

Equilibration and Production Runs: NVT (constant volume) and NPT (constant pressure) equilibration steps ensured the system reached stability at 300 K and 1 bar pressure, respectively. A 50-nanosecond production run was performed to observe the behavior of each complex over time.

Key metrics—Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), and Radius of Gyration (Rg)—were extracted to assess the stability, flexibility, and compactness of the inhibitors during the simulation(Ke et al., 2022; Lobanov et al., 2008; Reva et al., 1998).

4. Contact Network and Structural Analysis

The interaction contact maps for both Happy_00 and Happy_06 were generated to visualize the binding networks with the spike protein. The maps highlight specific residues involved in hydrogen bonding, electrostatic interactions, and van der Waals contacts. MolProbity was used to generate Ramachandran plots, ensuring the structural integrity of the inhibitors. COCOMAPS analyzed residue-residue contacts, confirming how the inhibitors interacted at both cleavage sites. This combination of molecular docking, dynamics simulations, and contact network analysis offers comprehensive insights into the binding behavior of Happy_00 and Happy_06, providing valuable information for optimizing fusion inhibitor design.

Results

The molecular docking and molecular dynamics (MD) simulations provided comprehensive insights into the binding behavior, stability, and adaptability of the designed fusion inhibitors, Happy_00 and Happy_06, when interacting with the SARS-CoV-2 spike protein. Key metrics—binding energy, RMSD (Root Mean Square Deviation), RMSF (Root Mean Square Fluctuation), and Radius of Gyration (Rg)—were used to evaluate the effectiveness of the inhibitors at two critical cleavage sites: Arg685/Ser686 (first cleavage site) and Arg815/Ser816 (second cleavage site).

1. Molecular Docking Results

The molecular docking results showed that both inhibitors exhibited significant binding affinities to the spike protein. However, their performance varied depending on the cleavage site due to their structural differences.

First Cleavage Site (Arg685/Ser686): Happy_00 demonstrated strong binding with a calculated binding energy of -11.4 kcal/mol, indicating favorable interactions with residues such as Gln675 and Arg683.Happy_06, although capable of binding to the first cleavage site, had a higher (less favorable) binding energy compared to Happy_00, due to its added linker flexibility, which may reduce stability at accessible binding sites.

Second Cleavage Site (Arg815/Ser816): Happy_06 outperformed Happy_00 at this more buried site, with a binding energy of -15.4 kcal/mol. The GGGS linkers allowed it to adopt conformations that optimized interactions with deeper residues, such as Lys811 and Asp808. In comparison, Happy_00 struggled to form effective interactions at this site, as its rigid structure limited its adaptability.

The docking results suggest that Happy_00 is better suited for targeting accessible cleavage sites, while Happy_06 excels at more challenging sites due to its flexibility.

2. Molecular Dynamics Simulation Results

The MD simulations confirmed the stability of the inhibitor-protein complexes over a 50-nanosecond run, providing insights into their behavior under near-physiological conditions.

RMSD (Root Mean Square Deviation): Both inhibitors maintained stable RMSD values between 2.3–2.5 Å, indicating minimal deviation from the docked conformations. Happy_00 exhibited a lower RMSD (~2.3 Å) at the first cleavage site, confirming its stable interaction with accessible residues. Happy_06 had a slightly higher RMSD (~2.5 Å) at the second cleavage site, reflecting the conformational adjustments it made to bind effectively to buried residues.

RMSF (Root Mean Square Fluctuation): Happy_00 showed minimal fluctuations, with most residues exhibiting RMSF values below 1.5 Å, indicating a rigid structure. Happy_06 displayed higher RMSF values, reaching up to 2.3 Å, particularly in regions surrounding the GGGS linkers, highlighting its increased flexibility.

Radius of Gyration (Rg): The Rg values for both inhibitors remained steady throughout the simulations, confirming that they maintained compact structures. Happy_00 had an Rg of ~22 Å, while Happy_06 maintained an Rg around 23 Å despite its flexible linkers.

3. Structural Insights and Contact Maps

Snapshots from the MD simulations demonstrated that both inhibitors retained their docking positions throughout the run. Happy_00 formed stable hydrogen bonds and polar interactions with residues such as Gln675 and Arg683 at the first cleavage site, ensuring stable binding. Happy_06 established multiple transient hydrogen bonds with Lys811 and Asp808 at the second cleavage site, facilitated by the flexibility of the GGGS linkers. The contact maps confirmed the inhibitors' ability to maintain key interactions, with Happy_06's flexibility allowing it to access buried regions of the spike protein that Happy_00 could not efficiently bind to.

Discussion

The molecular docking and MD simulations provided valuable insights into the structural dynamics and therapeutic potential of Happy_00 and Happy_06 as inhibitors targeting the SARS-CoV-2 spike protein. These findings emphasize the importance of structural adaptability and stability in the design of fusion inhibitors.

The results highlight distinct advantages for each inhibitor based on the cleavage site they target. Happy_00, with its compact and rigid

structure, demonstrated superior binding at the first cleavage site (Arg685/Ser686). This site is more accessible, allowing Happy_00 to maintain stable interactions with residues like Gln675 and Arg683. The inhibitor's rigidity ensures a strong and consistent fit, which is ideal for blocking the initial TMPRSS2 cleavage needed for viral entry.

In contrast, Happy_06, which incorporates GGGS linkers for enhanced flexibility, performed better at the second cleavage site (Arg815/Ser816). This site is more buried within the spike protein, requiring inhibitors to adjust their conformation to establish effective interactions. Happy_06's flexibility allowed it to engage with deeper residues, such as Lys811 and Asp808, forming transient but effective hydrogen bonds. This adaptability makes Happy_06 a promising candidate for targeting complex binding environments. The improved performance of Happy_06 at the second cleavage site underscores the importance of flexibility in peptide-based inhibitors. Linker flexibility allows the inhibitor to adapt to the dynamic nature of viral proteins, which often undergo conformational changes during their interaction with host cells. Previous studies have highlighted the role of linkers in enhancing the binding potential of inhibitors by facilitating conformational adjustments (Silacci et al., 2014). The findings suggest that optimized linkers can improve inhibitor efficacy, particularly when targeting hidden or buried residues in proteins like the SARS-CoV-2 spike. However, flexibility can also introduce instability, as seen in Happy_06's reduced performance at the first cleavage site. These results emphasize the need for a balance between rigidity and flexibility in designing fusion inhibitors.

The ability of Happy_00 and Happy_06 to maintain stable interactions with the SARS-CoV-2 spike protein, as demonstrated in the docking and MD simulations, suggests that these inhibitors could effectively block viral entry. By targeting TMPRSS2-mediated cleavage sites, the inhibitors disrupt the activation of the spike protein's fusion domain, preventing the virus from entering host cells. This strategy aligns with previous research that identifies TMPRSS2 inhibition as a promising therapeutic approach against SARS-CoV-2 (Hoffmann et al., 2020; Baughn et al., 2020). The results also underscore the potential of tetanus toxoid-derived fusion peptides as antiviral agents. The combination of the EK1 peptide with the C fragment of tetanus toxoid creates inhibitors with both stability and flexibility, making them suitable candidates for further development. This study demonstrates that these inhibitors could complement existing antiviral strategies by providing a direct means of disrupting viral entry.

While the in silico findings are promising, further in vitro and in vivo validation is required to confirm the inhibitors' efficacy in biological systems. Computational models provide valuable insights, but they cannot fully replicate the complexities of cellular environments (Kumar, Raj, Murthy N, et al., 2023). Future experiments should focus on evaluating the inhibitors' safety, stability, and antiviral activity in cultured cells and animal models. Additionally, mutations in the spike protein may alter the structure of the cleavage sites, potentially affecting inhibitor binding. Evaluating the performance of Happy_00 and Happy_06 against emerging SARS-CoV-2 variants will be essential to ensure their continued effectiveness. Further optimization of the linker design could enhance the adaptability of these inhibitors, improving their performance across different viral strains.

5. Conclusion

In conclusion, this study demonstrates that Happy_00 and Happy_06 are promising candidates for inhibiting SARS-CoV-2 entry by targeting critical cleavage sites on the spike protein. The findings highlight the importance

of linker flexibility in optimizing inhibitor binding and provide insights into the design of fusion peptides that balance stability and adaptability. By blocking TMPRSS2-mediated cleavage, these inhibitors offer a potential strategy for preventing viral entry and infection. Further research and experimental validation will be crucial to translate these findings into effective antiviral therapeutics.

TABLE 1: Properties of happy_00 vs. happy_06 predicted by Xtal Pred.

Sl. No.	PROTEIN FEATURES AND PREDICTION	HAPPY_00	HAPPY_06
1	Iso-electricity point.	5.78	5.78
2	Gravy index.	-0.36	-0.37
3	Instability index.	24.91	27.30
4	Surface entropy.	-1.22	-1.17
5	Surface hydrophobicity.	-0.80	-0.78
6	Rubedness of the surface.	0.81	0.82
7	Longest disorder region.	4	6
8	Longest low-complexity region.	15	26

Table 2: The results of selected clusters from HADDOCK 2.4, fig 7 and 8.

Sl. No.	Results from HADDOCK2.4	Clevage site 1 (Arg685/686)		Clevage site 2 (Arg815/816)	
		Happy_00	Happy_06	Happy_00	Happy_06
1	HADDOCK score.	-73.4 +/- 7.7	-93.8 +/- 2.2	-91.1 +/- 1.7	-77.4 +/- 10.6
2	Cluster size.	16	18	79	13
3	RSMD from overall lowest energy structure.	0.8 ± 0.5	0.7 ±0.4	28.7 ± 0.2	11.4 ±0.3
4	Van der Waal energy.	-61.2 ± 7.3	-59.0± 4.3	-61.3± 5.4	-59.4 ±3.7
5	Electrostatic energy	-342.6 ± 37.0	-555.5 ±41.7	-444.5 ±24.9	-423.5± 49.5
6	Desolvation energy	0.3 ± 4.7	22.0 ± 3.0	9.3±1.6	11.6± 5.2
7	Restraints Violation energy	560.2 ± 80.4	542.9 ± 27.5	498.9 ±70.4	550.6±57.9
8	Buried Surface Area	2275.7 ± 122.7	2502.8 ±102.6	2557.8 ± 147.3	2662.0± 212.6
9	Z - Score	-1.9	-2.3	-1.5	-2.3

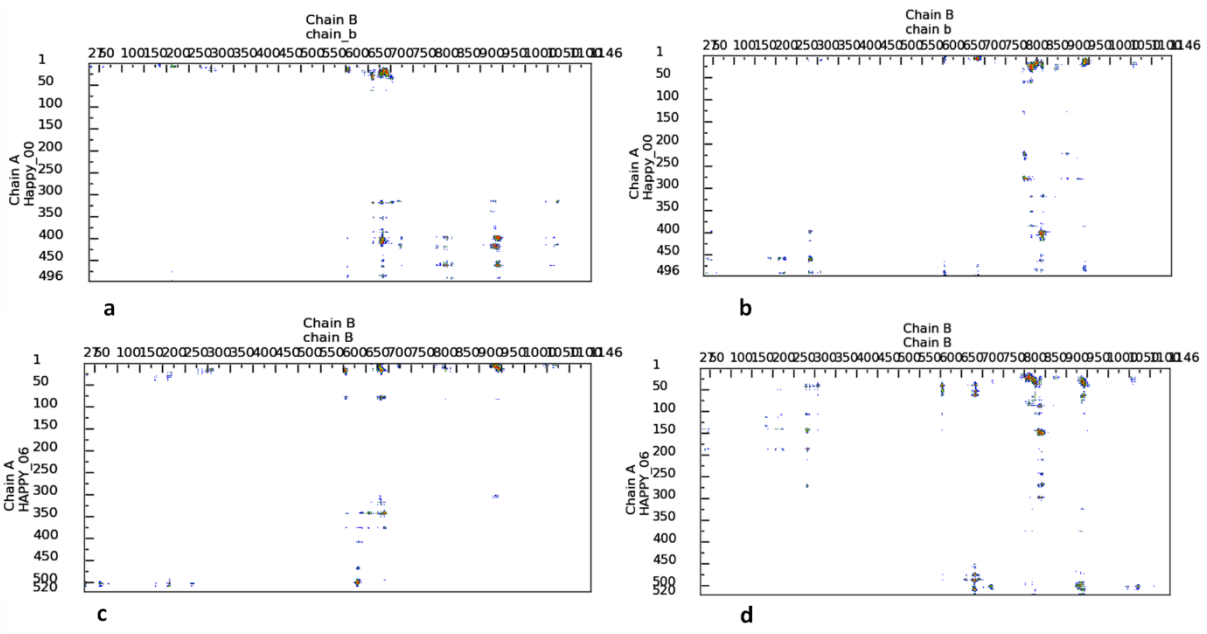

Fig 7.: Contact map of happy_00 vs. chain-B at the first site(a) and happy_00 vs. chain-B, the second site (b), happy_06 vs. chain-B, the first site. (c), happy_06 vs. chain-B, the second site (d).

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