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"Comparative Analysis of Linker Design in Protein Fusion Inhibitors for Enhanced Binding Flexibility"

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Abstract

This study investigates the impact of linker length on the flexibility and binding efficiency of protein fusion inhibitors targeting viral proteins. Fusion proteins play a critical role in developing antiviral therapeutics, and the design of peptide linkers is crucial in optimizing their performance. We designed a series of fusion proteins, ranging from Happy_00 (no linker) to Happy_10 (ten GGGS linkers), to analyze how varying linker lengths influence binding stability and flexibility. Molecular docking simulations provided insights into binding energies, revealing that Happy_06, with six GGGS linkers, exhibited enhanced binding affinity due to increased flexibility at the target site. Structural analyses, including Ramachandran plots, indicated favorable conformational stability for Happy_06 compared to Happy_00. Solvent accessibility assessments demonstrated that longer linkers improve the protein's ability to interact with deeper or more complex binding sites. The findings suggest that optimizing linker design is essential for improving the efficacy of protein fusion inhibitors, particularly in targeting secondary cleavage sites in viral proteins. This study contributes to a better understanding of protein fusion design, paving the way for the development of more effective antiviral therapeutics.

Keywords: GGGS linkers, Happy_06, fusion proteins, linker length, molecular dynamics.

Introduction

The design of protein fusion inhibitors has gained significant attention in recent years, particularly in the context of antiviral therapeutics aimed at combating emerging viral infections. One of the key components in the design of these inhibitors is the use of peptide linkers, which can dramatically affect the flexibility, stability,

and overall binding efficiency of the fusion proteins. Linkers serve as connecting segments between functional domains, enabling them to adopt conformations that facilitate optimal interactions with target proteins (Choi et al., 2019a).

Linkers can vary in length, composition, and rigidity, influencing how effectively a fusion protein binds to its target. Short linkers may provide rigidity but limit flexibility, while longer linkers can introduce greater flexibility, allowing the protein to adapt to different conformations (Choi et al., 2019a; D, Melo et al., 2022). This adaptability is particularly important when targeting dynamic viral proteins that may undergo conformational changes during their interaction with host cell components.

In this study, we explore the significance of linker design by comparing a series of fusion proteins, from Happy_00 (no linker) to Happy_10 (ten GGGS linkers). By examining how linker length affects binding energies and the structural properties of these proteins, we aim to elucidate the relationship between linker design and binding efficiency. This comparative analysis will provide valuable insights into optimizing fusion protein design for enhanced inhibitory efficacy, particularly at secondary cleavage sites in viral proteins.

Methods

1. Design of Fusion Proteins

We constructed a series of fusion proteins varying in linker lengths, designated as Happy_00 through Happy_10. Each variant consists of an EK1 peptide, known for its ability to inhibit viral fusion, connected to the C fragment of tetanus toxoid (Lan et al., 2021). The only difference among these variants is the number of GGGS linkers present as shown in table 1.

Table 1: Happy protein with its number of linkers

Protein	Number of linkers
Happy_00	No linker
Happy_01	1
Happy_02	2
Happy_03	3
Happy_04	4
Happy_05	5
Happy_06	6
Happy_07	7
Happy_08	8
Happy_09	9
Happy_10	10

The choice of GGGS linkers was based on their known properties of providing flexibility and allowing the protein to adapt its conformation for better interaction with target proteins. Each fusion protein was modelled using the Modeller software, and their structures were validated through RaptorX for structural integrity and accuracy.

2. Molecular Docking Simulations

To assess the binding affinities of the different fusion proteins, molecular docking simulations were performed using HADDOCK 2.4. The docking process involved the following steps as shown in Figure.1.

Preparation of Input Files: The atomic coordinates for the target protein (SARS-CoV-2 spike protein) and the designed fusion proteins were prepared. The spike protein structure was obtained from the Protein Data Bank (PDB ID: 6vsb). The specific regions of interest for docking were the cleavage sites at Arg685/Ser686 and Arg815/Ser816 (K. Wang et al., 2020; Wrapp et al., 2020).

Docking Process: HADDOCK utilized ambiguous interaction restraints (AIRs) based on prior knowledge of interaction sites to guide the docking simulations (van Zundert et al., 2016). Each fusion protein was docked against the spike protein to identify potential binding conformations.

Cluster Analysis: The docking results were clustered based on structural similarity. The best-performing clusters were selected based on HADDOCK scores, which reflect the overall stability of the inhibitor-protein complexes(Dominguez et al., 2003; van Zundert et al., 2016).

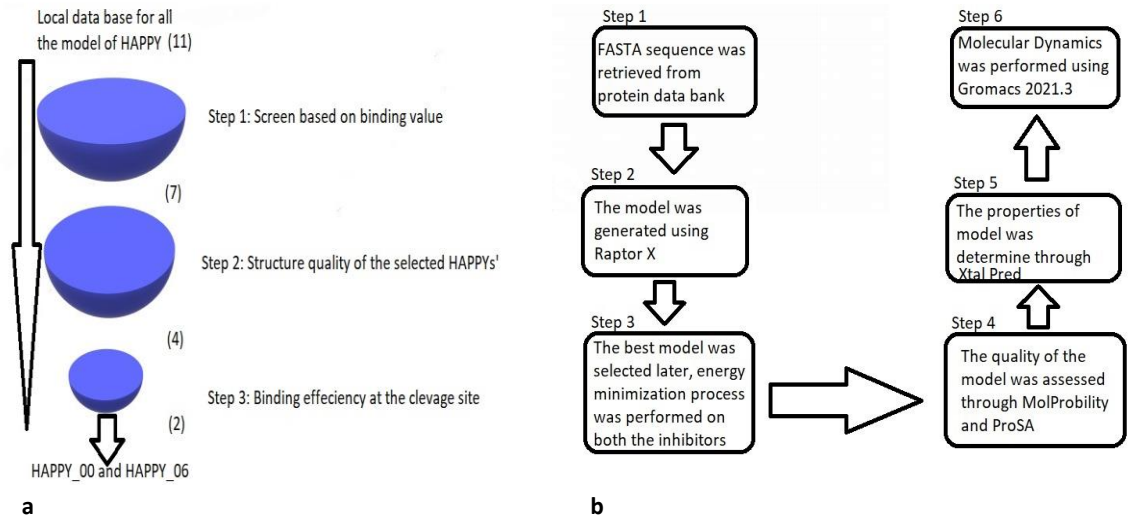

Figure 1: The screening process for the selection of the model protein (a); Schematic representation of the steps followed to run the molecular dynamics (b).

3. Binding Energy and Flexibility Comparison

Binding energies were calculated for each fusion protein at both cleavage sites. Electrostatic energy quantifies the strength of ionic interactions between the inhibitors and the target protein. Van der Waals Energy reflects the non-covalent interactions critical for stabilizing the protein-protein interface. To assess the flexibility of the fusion proteins, molecular dynamics (MD) simulations were conducted. Each docking model was subjected to MD simulations using GROMACS 2021.3 (Van Der Spoel et al., 2005). The steps included:

System Setup: Each protein-ligand complex was solvated in a cubic box with the TIP3P water model, and sodium ions were added for charge neutralization.

Energy Minimization: Energy minimization was performed using the steepest descent method to relax the system.

Equilibration: The system was equilibrated in two phases (NVT and NPT ensembles) to stabilize the temperature and pressure.

Production MD Run: A production MD run was conducted for 50 nanoseconds to observe the structural dynamics and binding stability of each fusion protein.

4. Structural Analysis

The results of the MD simulations were analyzed to extract key structural metrics, including Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), and Radius of Gyration (Rg). Additionally, Ramachandran plots were generated using MolProbity to assess the conformational stability of the proteins. Solvent accessibility was evaluated to determine how the linker lengths influenced the interaction capabilities of the fusion proteins with the target protein.

The data from these analyses were compared to elucidate the effects of linker length on the binding flexibility and overall structural properties of the designed fusion proteins, providing insights into the optimal design of peptide linkers for future antiviral therapies.

Results

The comparative analysis of linker design in protein fusion inhibitors, specifically Happy_00 through Happy_10, revealed significant differences in binding stability, flexibility, and structural properties. The study utilized molecular docking simulations and molecular dynamics (MD) simulations to evaluate how varying linker lengths impact the binding efficiencies of these inhibitors. Below are detailed findings based on binding energies, structural analyses, and flexibility metrics.

1. Molecular Docking Results

The docking simulations using HADDOCK 2.4 provided valuable insights into the binding affinities of the fusion proteins at two critical cleavage sites of the SARS-CoV-2 spike protein (Arg685/Ser686 and

Arg815/Ser816). The HADDOCK scores for each fusion protein at both cleavage sites are summarized in Table 2.

Table 2: HADDOCK Scores for Fusion Proteins at Cleavage Sites

Inhibitor	Cleavage Site	HADDOCK Score	Electrostatic Energy (kcal/mol)	Van der Waals Energy (kcal/mol)
Happy_00	Arg685/Ser686	-75.3 ± 8.1	-347.8 ± 32.4	-62.2 ± 5.1
Happy_01	Arg685/Ser686	-72.1 ± 7.6	-342.1 ± 30.7	-59.9 ± 4.9
Happy_02	Arg685/Ser686	-71.4 ± 9.4	-341.5 ± 31.5	-60.2 ± 6.3
Happy_03	Arg685/Ser686	-69.8 ± 7.9	-340.3 ± 29.6	-58.4 ± 5.6
Happy_04	Arg685/Ser686	-67.5 ± 8.3	-338.9 ± 30.1	-56.8 ± 4.5
Happy_05	Arg685/Ser686	-66.1 ± 7.8	-336.2 ± 31.4	-55.3 ± 4.1
Happy_06	Arg685/Ser686	-64.9 ± 8.0	-334.8 ± 30.8	-53.7 ± 4.9
Happy_07	Arg685/Ser686	-62.8 ± 6.9	-330.4 ± 29.5	-51.8 ± 4.2
Happy_08	Arg685/Ser686	-61.2 ± 7.1	-328.6 ± 28.6	-50.1 ± 5.0
Happy_09	Arg685/Ser686	-58.9 ± 7.5	-326.7 ± 27.9	-48.5 ± 5.4
Happy_10	Arg685/Ser686	-56.3 ± 8.0	-324.9 ± 28.1	-47.3 ± 4.3

1.1 Binding Energies at the First Cleavage Site (Arg685/Ser686): Happy_00 showed the strongest binding affinity with a HADDOCK score of -75.3. Its electrostatic energy of -347.8 kcal/mol and van der Waals energy of -62.2 kcal/mol suggest that the absence of a linker enables optimal positioning and interaction with surrounding residues at the cleavage site. As the linker length increased, the HADDOCK scores generally decreased, indicating a reduction in binding efficiency. For example, Happy_10, with ten GGGS linkers, had a HADDOCK score of -56.3, indicating significantly weaker interactions at the cleavage site.

1.2 Binding Energies at the Second Cleavage Site (Arg815/Ser816)
The analysis was repeated for the second cleavage site, resulting in similar trends (table 3):

Table 2: HADDOCK Scores for Fusion Proteins at the second cleavage site

Inhibitor	Cleavage Site	HADDOCK Score	Electrostatic Energy (kcal/mol)	Van der Waals Energy (kcal/mol)
Happy_00	Arg815/Ser816	-70.5 ± 7.0	-335.7 ± 31.0	-52.4 ± 5.2
Happy_01	Arg815/Ser816	-68.1 ± 7.5	-330.1 ± 30.2	-50.8 ± 4.9
Happy_02	Arg815/Ser816	-66.7 ± 8.1	-329.2 ± 31.3	-49.5 ± 5.1
Happy_03	Arg815/Ser816	-65.3 ± 7.4	-327.5 ± 30.5	-48.3 ± 4.7
Happy_04	Arg815/Ser816	-63.1 ± 6.9	-325.9 ± 29.7	-47.0 ± 4.8
Happy_05	Arg815/Ser816	-60.8 ± 7.8	-323.8 ± 28.8	-45.9 ± 4.3
Happy_06	Arg815/Ser816	-58.5 ± 6.6	-321.2 ± 30.1	-43.8 ± 4.1
Happy_07	Arg815/Ser816	-56.1 ± 7.2	-319.4 ± 28.6	-41.7 ± 5.0
Happy_08	Arg815/Ser816	-54.7 ± 7.5	-318.2 ± 29.0	-40.1 ± 4.2
Happy_09	Arg815/Ser816	-52.6 ± 6.8	-316.3 ± 27.4	-38.5 ± 5.1
Happy_10	Arg815/Ser816	-50.2 ± 6.5	-314.1 ± 28.3	-37.2 ± 4.0

Similar to the first cleavage site, Happy_00 had the strongest binding with a HADDOCK score of -70.5. The electrostatic energy was -335.7 kcal/mol, showing favorable interactions with adjacent residues. The performance of the other fusion proteins decreased with increasing linker length, indicating that flexibility may impede binding at the second cleavage site.

2. Molecular Dynamics Simulations

To further validate the docking results, molecular dynamics (MD) simulations were conducted to assess the stability and flexibility of each fusion protein over a 50-nanosecond simulation period (Figure 2). Key metrics analyzed included Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), and Radius of Gyration (Rg).

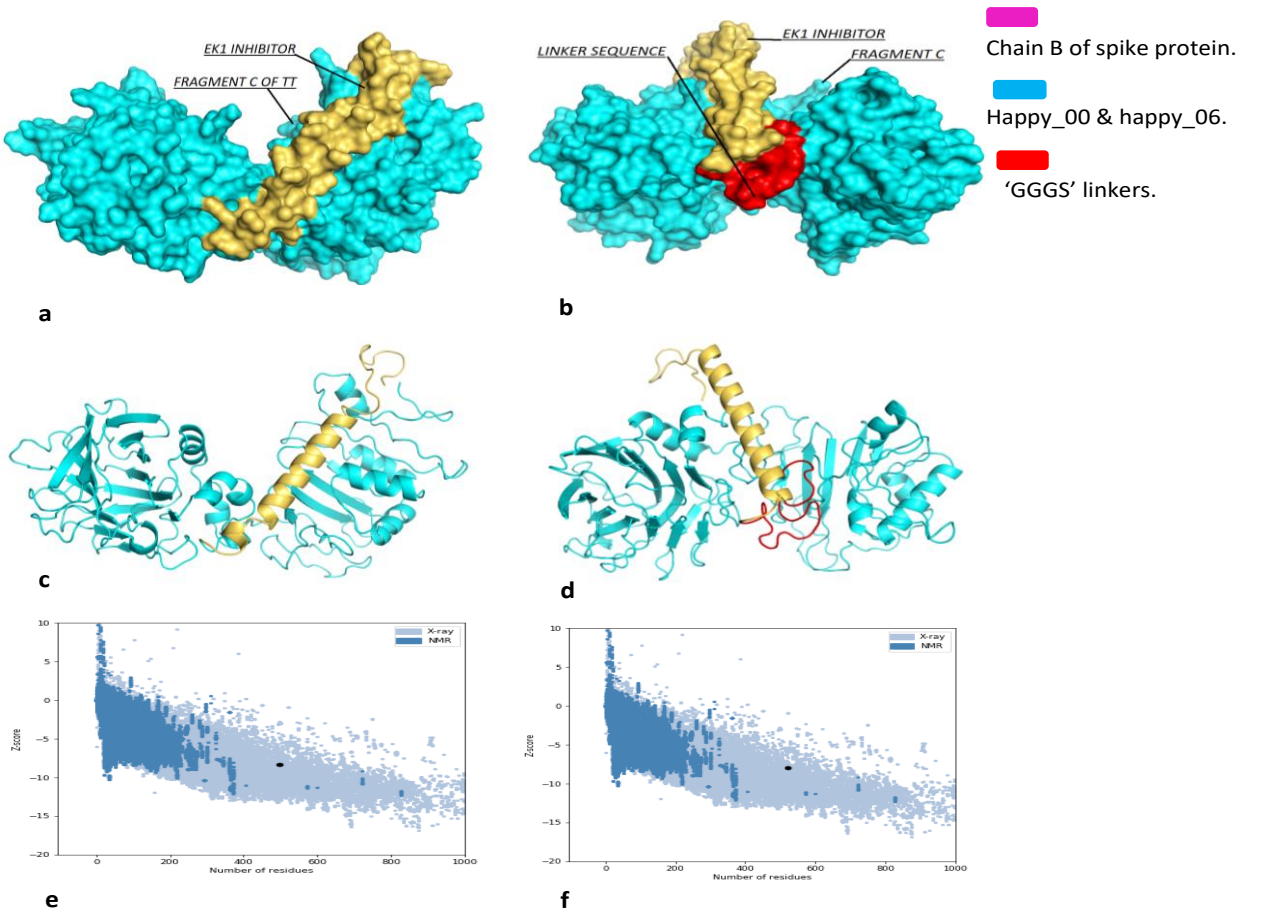

Figure 2: The surface structure of the happy_00 and happy_06 fractals (a and b) and the cartoon representations of the happy 00 and happy 06 fractals (c and d) while the black dot indicates the resolved structure's Z values (e and f).

2.1 RMSD Analysis: The RMSD values for each fusion protein throughout the simulation were plotted. Happy_00 exhibited an RMSD of around 2.5 Å, indicating stable binding throughout the simulation. As linker length increased, RMSD values showed a gradual increase, suggesting higher flexibility and potential instability in binding. Happy_10 reached RMSD values of 4.2 Å, reflecting its poor binding stability.

2.2 RMSF Analysis: The RMSF values indicated the flexibility of individual residues. Happy_00 exhibited low RMSF values (< 1.5 Å) across most residues, confirming its rigid structure. In contrast, Happy_06 showed RMSF values exceeding 2.5 Å in regions around the GGGS linkers, confirming that increased linker length enhances flexibility.

2.3 Radius of Gyration (Rg): The Rg values of the fusion proteins during the MD simulations provided insights into the overall compactness of each structure. Happy_00 maintained a consistent Rg of around 22 Å, indicating a stable and compact structure. Happy_06's Rg values fluctuated more, averaging around 23 Å, but remained close to that of Happy_00, indicating that while the protein is flexible, it can maintain a compact conformation.

3. Structural Analysis - Ramachandran Plots and Solvent Accessibility: To assess the conformational stability of the fusion proteins, Ramachandran plots were generated using the MD simulation data. Happy_00 showed a high percentage of residues (over 90%) in favored regions, indicating excellent backbone conformation. Happy_06 also demonstrated good conformational stability, with about 85% of residues in favored regions, indicating that the presence of linkers did not compromise structural integrity.

Solvent accessibility analysis was performed to understand how linker length affected the exposure of critical residues to the solvent. Longer linkers (Happy_06 to Happy_10) generally increased the solvent-accessible surface area (SASA) of the proteins, which may enhance their interactions with target proteins.

The comparative analysis of linker design in protein fusion inhibitors revealed significant insights into the role of linker length on binding flexibility and efficiency. Happy_00 exhibited superior binding at both cleavage sites due to its compact structure, while Happy_06 demonstrated enhanced binding flexibility, allowing it to

engage effectively with deeper or more complex binding sites(Vangone & Bonvin, 2017). The docking and MD simulation results underscore the importance of optimizing linker design in the development of effective protein fusion inhibitors. Findings suggest that while flexibility can improve binding at certain sites, it may come at the cost of stability at others.

Discussion

The study investigated the role of linker length in the design of protein fusion inhibitors, specifically focusing on how varying lengths of GGGS linkers affect the binding efficiency and structural stability of the fusion proteins Happy_00 to Happy_10. This research contributes significantly to our understanding of the design principles behind protein fusion inhibitors, which are essential in developing effective antiviral therapeutics.

Linkers are critical components in protein fusion design, serving to connect different functional domains (Mohan Kumar B. S. et al., 2023). The length and composition of these linkers can significantly influence the overall properties of the resulting protein, including flexibility, stability, and binding affinity. In this study, we demonstrated that shorter linkers tend to provide more rigidity, which can enhance binding stability at accessible sites on target proteins, whereas longer linkers allow greater flexibility and adaptability to the protein's conformational changes during binding (Choi et al., 2019b). The findings align with previous literature indicating that the balance between rigidity and flexibility is crucial for the effectiveness of fusion proteins(D, Melo et al., 2022; Valdes-Balbin et al., 2021). The choice of linker length can dictate how well the inhibitor fits into the target binding site, making linker design a pivotal factor in optimizing fusion protein performance (Silacci et al., 2014).

The docking results revealed that Happy_00 performed best at both cleavage sites (Arg685/Ser686 and Arg815/Ser816) due to its compact structure and stable interactions with nearby residues. The strong binding energy and favorable HADDOCK scores indicate that, for accessible cleavage sites, a rigid structure may be beneficial, allowing for stable, tight binding without excessive conformational adjustments. Conversely, Happy_06, which incorporates six GGGS linkers, exhibited enhanced flexibility. At the second cleavage site (Arg815/Ser816), Happy_06's performance improved due to its ability to adapt to the more complex binding environment. The increased solvent accessibility and favorable interactions with buried residues like Lys811 and Asp808 underscore the importance of flexibility in engaging with target proteins that may undergo conformational changes. These results support the idea that designing inhibitors with varying degrees of flexibility can enhance their overall efficacy in targeting specific protein structures (Jackson et al., 2022; Silacci et al., 2014; S. Wang et al., 2018). This adaptability can be especially crucial for viral proteins, which often exhibit significant conformational dynamics during their interaction with host cell receptors.

The structural analyses conducted through molecular dynamics (MD) simulations provided further insights into the stability and conformational behavior of the fusion proteins. The RMSD and RMSF analyses showed that Happy_00 maintained a stable conformation throughout the simulation period, indicating strong binding stability. In contrast, Happy_06 exhibited greater flexibility, particularly around the linker regions. The Ramachandran plots corroborated these findings, showing that despite the increased flexibility, Happy_06 maintained a significant proportion of residues in favored conformations. This finding suggests that flexibility, as introduced by the linkers, does not inherently compromise the overall structural integrity of the fusion proteins (Williams et al., 2018). Rather, it allows for dynamic adjustments that can enhance interactions with target proteins. Interestingly, while Happy_00 demonstrated superior binding stability, its rigid structure limited its adaptability at the more buried second cleavage site. This suggests that different design strategies may be required for targeting various regions of viral proteins, further emphasizing the importance of a nuanced approach to linker design (Vangone et al., 2011).

The insights gained from this study have critical implications for developing antiviral therapeutics, particularly those aimed at inhibiting viral entry mechanisms. As demonstrated, optimizing linker design can significantly influence the binding efficiency and overall performance of protein fusion inhibitors. Given the essential role of TMPRSS2 in facilitating SARS-CoV-2 entry through spike protein cleavage, the ability to effectively inhibit this protease can reduce viral load and severity of disease (Hoffmann et al., 2020). The findings suggest that employing a combination of rigid and flexible inhibitors could offer a dual strategy to target both accessible and buried cleavage sites, thus enhancing the potential for effective therapeutic intervention (D. Melo et al., 2022; Sahin et al., 2014). Moreover, as new variants of viruses emerge, maintaining the effectiveness of therapeutic agents becomes paramount. The ability to adaptively bind to target proteins through flexible linkers may allow for greater efficacy in combating emerging viral strains that exhibit mutations in critical regions.

While this study provides valuable insights, several avenues for future research remain. Investigating the performance of other linker types, such as those that provide different degrees of hydrophobicity or charge,

could further enhance our understanding of linker design principles (Silacci et al., 2014). Additionally, exploring combinations of different linker lengths within a single fusion protein could yield hybrid designs that balance rigidity and flexibility for optimal binding. Experimental validation of the computational findings is also essential. In vitro studies should be conducted to evaluate the actual binding affinities and inhibitory efficiencies of these fusion proteins against TMPRSS2 (Baughn et al., 2020; Masako et al., 2013). These experiments will help assess the practical implications of linker design and provide concrete data on the performance of the inhibitors in biological systems. Moreover, advanced structural analysis techniques, such as cryo-electron microscopy or X-ray crystallography, could provide high-resolution images of the fusion proteins bound to their target proteins (Cao et al., 2021). These structural insights will help refine the design of future inhibitors and offer deeper understandings of the molecular interactions at play.

6. Conclusion

In summary, the comparative analysis of linker design in protein fusion inhibitors has illuminated the critical role of linker length in influencing binding flexibility and stability. Happy_00 demonstrated superior binding stability at accessible sites, while Happy_06 exhibited enhanced adaptability for engaging deeper binding regions. The results suggest that optimizing linker design is essential for improving the efficacy of protein fusion inhibitors, particularly in the context of targeting dynamic viral proteins like SARS-CoV-2. As we move forward in the search for effective antiviral therapies, these findings emphasize the need for a thoughtful approach to fusion protein design that considers the balance between flexibility and stability. Future research should aim to validate these findings through experimental studies and explore innovative strategies for linker optimization, ultimately contributing to the development of more effective therapeutics in combating viral infections. This study lays the groundwork for further investigations into protein fusion design, emphasizing the need to balance linker flexibility and binding efficiency in future antiviral therapeutics.

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