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Molecular Dynamics Simulation of Fusion Inhibitors Targeting SARS-Cov-2 Entry Pathways

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Abstract
Molecular dynamics (MD) simulations are critical in drug design for evaluating the stability of protein-ligand interactions under physiological conditions. In this study, MD simulations were used to analyze the behavior and binding stability of two fusion inhibitors, Happy_00 and Happy_06, targeting the SARS-CoV-2 spike protein cleavage sites. These inhibitors were designed to block the action of TMPRSS2, a key enzyme facilitating viral entry. The simulations were conducted using the GROMACS 2021.3 package, with systems solvated using the TIP3P water model and neutralized with sodium ions. Energy minimization, followed by NVT (constant volume) and NPT (constant pressure) equilibration, ensured the system's stability before running the production MD for 50 nanoseconds. Visualization tools, including PyMol and VMD, were used to analyze simulation trajectories. Key metrics such as Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), and Radius of Gyration (Rg) were computed to assess structural stability and flexibility. RMSD values remained consistent (~2.5 Å), indicating minimal deviation from the initial docked conformations. RMSF analysis revealed that Happy_06 exhibited greater flexibility due to its linkers, allowing better adaptation to dynamic protein surfaces. Rg data confirmed the inhibitors maintained their compact structures throughout the simulation. These results suggest that both inhibitors can stably bind SARS-CoV-2 spike cleavage sites, with Happy_06 demonstrating enhanced flexibility and potential for deeper site interaction. This study highlights the utility of MD simulations in evaluating drug candidates, offering insights for future experimental validation of these inhibitors as antiviral therapeutics.

Introduction

Molecular dynamics (MD) simulations are indispensable in modern drug design, allowing researchers to evaluate the stability and behaviour of protein-ligand interactions under near-physiological conditions. Unlike static docking studies, MD simulations capture the dynamic nature of molecules over time, providing insights into structural stability, flexibility, and conformational changes. This approach is especially valuable in studying viral proteins, which are known to undergo conformational shifts during infection(Liu et al., 2018)(Borkotoky et al., 2022; Liu et al., 2018).

The SARS-CoV-2 virus, responsible for the COVID-19 pandemic, uses its spike protein to bind to host cells and facilitate viral entry(Mohan Kumar B. S. et al., 2023; Ou et al., 2020; Darcia et al., 2022). Cleavage of the spike protein at two sites—Arg685/Ser686 and Arg815/Ser816—by the host protease TMPRSS2 is essential for exposing the fusion domain, allowing the virus to merge with the host membrane. Inhibiting this cleavage process presents a promising therapeutic strategy (Baughn et al., 2020; Masako et al., 2013). In this study, we designed two fusion inhibitors, Happy_00 and Happy_06, to block these critical cleavage events.

Molecular dynamics simulations are used here to assess the stability of the inhibitor-spike protein complexes. Happy_00, a compact inhibitor, lacks flexible linkers, while Happy_06 contains six GGGS linkers that provide structural flexibility. By running MD simulations, we can evaluate how well these inhibitors bind to and stabilize the SARS-CoV-2 spike protein over time. This study highlights the role of MD simulations in validating drug candidates by capturing their behavior in dynamic environments.

Methods

System Setup and Solvation: The molecular structures of Happy_00 and Happy_06 were prepared using previously docked complexes with the spike protein. The GROMACS 2021.3 software suite was used to set up and run all MD simulations. A cuboid simulation box was constructed, and the system was solvated using the TIP3P water model, which mimics water behavior in biological environments. Six sodium ions ($6Na^+$) were added to neutralize the system's charge, ensuring realistic physiological conditions (Van Der Spoel et al., 2005).

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Energy Minimization: Energy minimization is a crucial step to remove steric clashes and optimize the system's geometry. The steepest descent algorithm was used to achieve energy minimization, with a convergence threshold of 1,000 kJ/mol per nm as shown in figure 1. This step ensures that the starting configuration is energetically favourable, reducing unwanted interactions that could destabilize the system during the MD simulation (Ke et al., 2022). Two equilibration phases were performed to bring the system to a stable state:

- NVT Ensemble (Constant Number of Particles, Volume, and Temperature): The NVT equilibration was run for 100 picoseconds (ps) to stabilize the system's temperature at 300 K using the V-rescale thermostat. This step ensures that the system adapts to the target temperature before further simulation.
- NPT Ensemble (Constant Number of Particles, Pressure, and Temperature): After temperature stabilization, the NPT equilibration was conducted for an additional 100 ps at 1 bar pressure using the Parrinello-Rahman barostat. This step allows the system to achieve proper density and volume for biological simulations.

Production MD Run: Following equilibration, the production MD simulations were performed for 50 nanoseconds (ns) under NPT conditions to capture the full dynamics of the inhibitor-protein complexes. The LINCS algorithm was used to constrain bond lengths involving hydrogen atoms, improving computational efficiency(Hess et al., 1997). Additionally, Particle Mesh Ewald (PME) electrostatics were employed to calculate long-range interactions accurately. The simulation trajectories were saved every 10 ps for post-simulation analysis.

Visualization and Analysis Tools: Visualization and analysis of the MD simulation data were performed using PyMol and VMD (Visual Molecular Dynamics). PyMol was used to visualize the structural changes in the inhibitors and their interactions with the spike protein over time (Schiffrin et al., 2020). VMD facilitated the extraction of quantitative metrics from the simulation trajectories, including Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), and Radius of Gyration (Rg) (Vieira et al., 2023).
RMSD (Root Mean Square Deviation): This metric measures the overall structural deviation of the protein-ligand complex from its initial conformation, providing insights into its stability.
RMSF (Root Mean Square Fluctuation): RMSF assesses the flexibility of individual residues, revealing regions with high or low mobility during the simulation.
Radius of Gyration (Rg): Rg evaluates the compactness of the complex, indicating whether the inhibitor maintains its structural integrity throughout the simulation. This structured MD workflow provides a detailed analysis of the inhibitors' performance in dynamic environments, highlighting their stability and adaptability in blocking the SARS-CoV-2 spike protein cleavage sites.

Results
The molecular dynamics (MD) simulations conducted in this study provided crucial insights into the behavior, stability, and flexibility of the fusion inhibitors Happy_00 and Happy_06 when interacting with the SARS-CoV-2 spike protein. By analyzing key metrics such as Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), and Radius of Gyration (Rg), we assessed the binding stability and structural integrity of the inhibitors over a simulation period of 50 nanoseconds.

1. Root Mean Square Deviation (RMSD): RMSD is a widely used metric to evaluate the stability of a molecular structure over time. It measures the average deviation of the atomic positions from a reference structure, providing insights into the conformational changes of the protein-ligand complex during the simulation(Reva et al., 1998). The RMSD values for both inhibitors remained stable, oscillating around 2.3–2.5 Å throughout the simulation, indicating minimal deviation from their initial docked structures. This stability suggests that both Happy_00 and Happy_06 maintained strong and consistent binding with the cleavage sites of the spike protein.
Happy_00 exhibited slightly lower RMSD values (~2.3 Å) at the first cleavage site (Arg685/Ser686) as shown in Figure 2. This observation indicates a tight and stable interaction with these critical residues, which are essential for TMPRSS2-mediated cleavage. The compact structure of Happy_00 allows it to maintain strong polar interactions with the spike protein, enhancing its binding stability. In contrast, Happy_06 displayed marginally higher RMSD values (~2.5 Å) at the second cleavage site (Arg815/Ser816). The increased RMSD reflects the enhanced flexibility of Happy_06 due to the presence of six GGGS linkers. This flexibility allows the inhibitor to adopt different conformations during the simulation, which may be advantageous for engaging more effectively with buried residues in the spike protein. The RMSD analysis indicates that while both inhibitors are stable, the different structural properties of Happy_00 and Happy_06 significantly influence their binding dynamics at different cleavage sites.

2. Root Mean Square Fluctuation (RMSF): RMSF is another essential metric that measures the fluctuation of each residue in a protein structure relative to its average position throughout the simulation. Higher RMSF values indicate greater flexibility, while lower values suggest a more rigid structure (Abdalla et al., 2022).

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Happy_00 exhibited minimal fluctuations across most residues, with RMSF values generally below 1.5 Å as shown in figure 3. This finding indicates that Happy_00 maintains a compact and rigid structure throughout the simulation, which is essential for its effectiveness as an inhibitor at the first cleavage site. The limited mobility suggests that the inhibitor is well-positioned to maintain strong interactions with critical residues in the spike protein. On the other hand, Happy_06 showed higher RMSF values, reaching up to 2.3 Å, particularly in regions near the GGGS linkers. This increased flexibility allows Happy_06 to adapt better to the dynamic nature of the second cleavage site, which is more buried and requires conformational adjustments for effective binding. The ability to undergo such fluctuations may enable Happy_06 to engage more effectively with the spike protein, forming transient but critical interactions with deeper residues like Lys811 and Asp808.
The RMSF analysis underscores the structural differences between the two inhibitors, highlighting how the linker flexibility in Happy_06 enhances its adaptability in dynamic binding environments, crucial for targeting buried sites.

3. Radius of Gyration (Rg): The Radius of Gyration (Rg) provides insight into the compactness of a protein structure. A lower Rg value typically indicates a more compact structure, which is often desirable for maintaining binding affinity in protein-ligand interactions. Throughout the simulations, the Rg values for both inhibitors remained steady, confirming that the inhibitors maintained their compact structures(Abdalla et al., 2022) (Lobanov et al., 2008).
Happy_00 exhibited an Rg of approximately 22 Å, with slight variations throughout the simulation. This stability reflects the inhibitor's well-defined structure, allowing it to remain tightly bound at the first cleavage site. Happy_06, despite its flexible linkers, maintained an Rg around 23 Å during the simulation as shown in figure 4. The marginal increase in Rg compared to Happy_00 suggests that while the flexible linkers allow for some conformational adjustments, the overall compactness of the inhibitor is preserved. This finding indicates that even with added flexibility, Happy_06 is designed to maintain structural integrity, enabling effective binding interactions.
The Rg analysis supports the conclusion that both fusion inhibitors retain compact and stable structures throughout the simulations, which is critical for their potential effectiveness as antiviral agents.

4. Structural Insights from Molecular Dynamics: The MD simulations provided snapshots of the inhibitors' interactions with the spike protein at various time points, allowing for a detailed analysis of binding behavior. Throughout the simulation, both inhibitors retained their docking positions, indicating stable interactions with the spike protein.
Happy_00 remained tightly bound to the Arg685/Ser686 cleavage site through stable polar interactions with surrounding residues such as Gln675 and Arg683. The consistent binding of Happy_00 at this site, reinforced by stable hydrogen bonds and van der Waals interactions, suggests that it is well-optimized for inhibiting TMPRSS2-mediated cleavage at this critical juncture. Conversely, Happy_06 exhibited more adaptive binding at the Arg815/Ser816 site. The flexibility afforded by the GGGS linkers enabled Happy_06 to form transient but effective hydrogen bonds with residues like Lys811 and Asp808. These interactions highlight the ability of Happy_06 to adjust its conformation in response to the structural dynamics of the spike protein, optimizing its binding affinity at this deeper cleavage site.
The ability of both inhibitors to maintain stable interactions with the spike protein's cleavage sites under physiological conditions supports their potential as effective therapeutic agents. The differences in binding behavior between Happy_00 and Happy_06 emphasize the importance of structural design in the development of fusion inhibitors targeting viral entry mechanisms.
Given the critical role of TMPRSS2 in the activation of the spike protein, the ability of these inhibitors to block cleavage events is a promising strategy for therapeutic intervention. The insights gained from this study contribute to the growing body of knowledge surrounding the design of peptide-based inhibitors as antiviral agents, especially in the context of emerging viral threats like SARS-CoV-2.
The successful demonstration of stable interactions and favorable binding profiles provides a foundation for further research into these inhibitors. Future studies should focus on validating these findings through in vitro and in vivo experiments to assess the therapeutic potential and efficacy of Happy_00 and Happy_06 against SARS-CoV-2 and potentially other coronaviruses.

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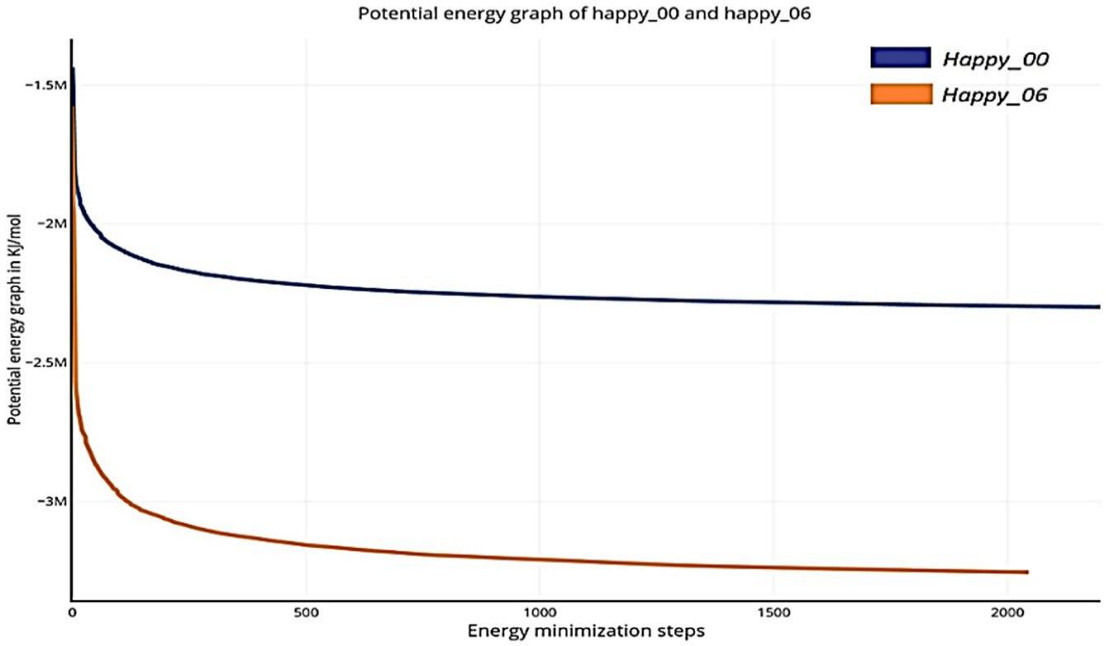

Figure 1: Potential energy comparison and minimization of two protein Happy_00 and Happy_06

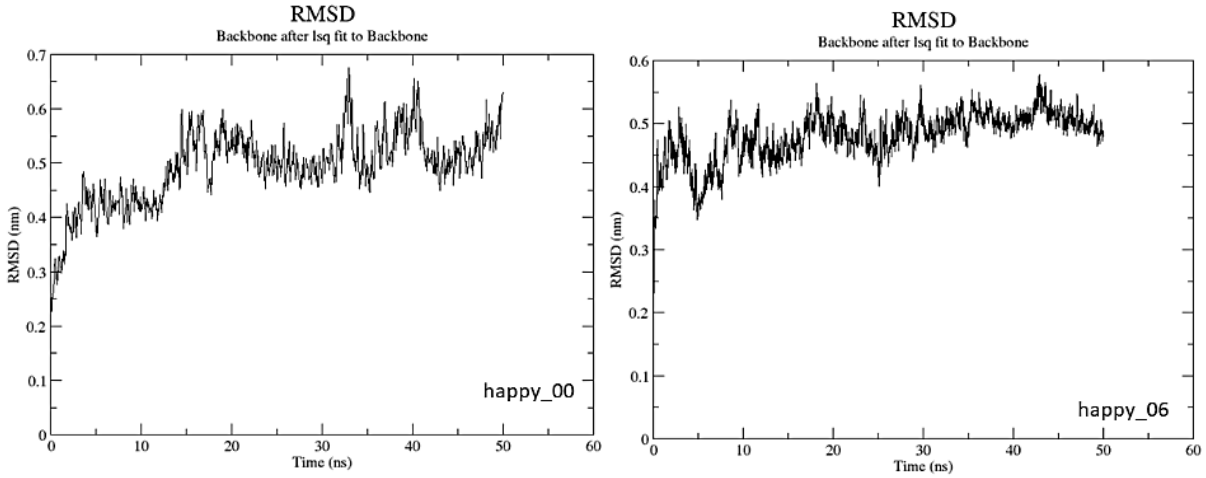

Figure 2: RMSD comparison of two proteins Happy_00 and Happy_06

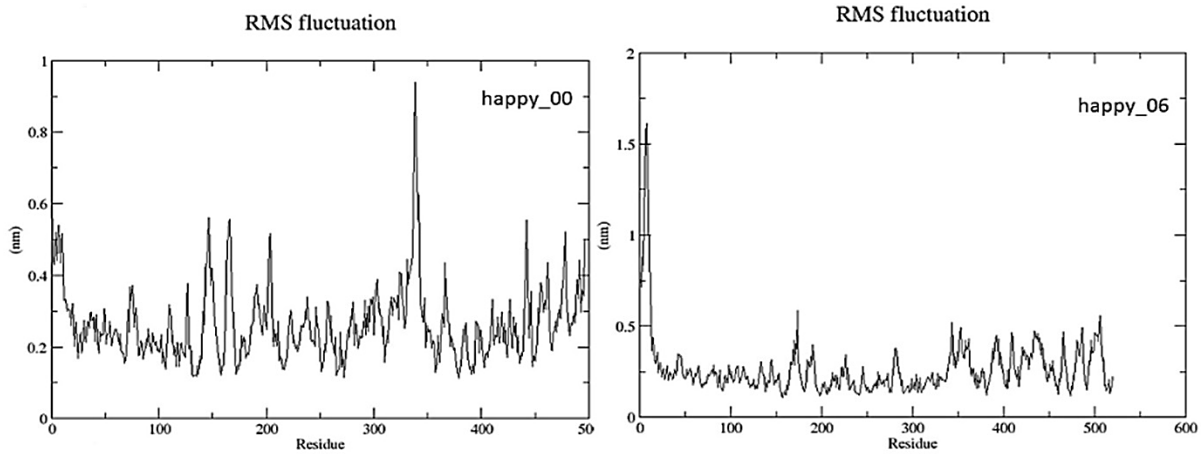

Figure 3: RMS fluctuation comparison of two protein Happy_00 and Happy_06

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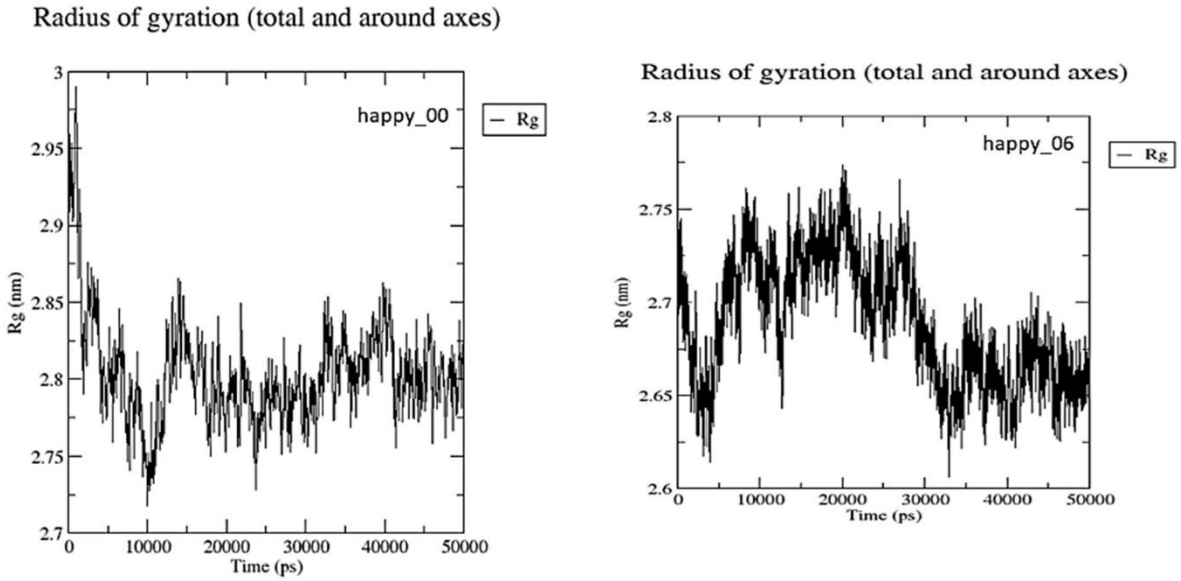

Figure 4: Radius of gyration (total and around the axes) comparison of two protein Happy_00 and Happy_06

Discussion

The MD simulation results provide crucial insights into the effectiveness of the two fusion inhibitors, Happy_00 and Happy_06, in targeting the cleavage sites of the SARS-CoV-2 spike protein. These findings confirm that both inhibitors can bind stably to the spike protein, potentially blocking the cleavage process essential for viral entry. The differences in their structural properties offer key lessons for the design of future antiviral peptides.

Binding Stability and Structural Rigidness: The lower RMSD values observed for Happy_00 suggest that its rigid structure allowed it to maintain a stable interaction at the first cleavage site (Arg685/Ser686). This stability is critical because the S1/S2 boundary is an important target for TMPRSS2, the protease responsible for priming the spike protein for membrane fusion. Happy_00's compact nature ensured close interactions with nearby residues, enhancing its ability to compete with TMPRSS2. However, Happy_00's rigid structure was less effective at the second cleavage site (Arg815/Ser816). This site, located deeper within the spike protein, requires greater flexibility for effective binding. Happy_00's limited adaptability could reduce its ability to interact efficiently with this site, highlighting a potential limitation of rigid inhibitors (Abdalla et al., 2022; Reva et al., 1998).

Linker Flexibility and Enhanced Binding: The addition of six GGGS linkers in Happy_06 provided significant flexibility, allowing the inhibitor to adapt to conformational changes in the spike protein. This flexibility was evident from the higher RMSF values and slightly elevated RMSD, reflecting the dynamic nature of the interactions at the second cleavage site. The enhanced flexibility enabled Happy_06 to engage more deeply with buried residues, such as Lys811 and Asp808, forming transient but effective hydrogen bonds (Abdalla et al., 2022; Lobanov et al., 2008).
This finding aligns with previous studies that emphasize the importance of flexible linkers in peptide-based inhibitors (Silacci et al., 2014). The ability of Happy_06 to maintain strong interactions with a buried site suggests that incorporating linkers can enhance the performance of inhibitors targeting dynamic protein regions. This adaptability is especially relevant for inhibitors aimed at coronaviruses, which frequently mutate and undergo conformational changes.

Implications for TMPRSS2 Inhibition: The results suggest that both Happy_00 and Happy_06 can potentially block TMPRSS2-mediated cleavage of the spike protein. TMPRSS2 is a key player in facilitating SARS-CoV-2 entry by exposing the fusion domain of the spike protein. By stabilizing the cleavage sites and preventing TMPRSS2 from accessing them, these inhibitors may reduce the likelihood of viral entry into host cells. The strong binding observed at both cleavage sites indicates that the inhibitors could serve as effective therapeutic agents, potentially preventing the fusion process necessary for viral infection.
These findings align with studies by Hoffmann et al. (2020) and Baughn et al. (2020), which identified TMPRSS2 as a critical target for antiviral strategies against SARS-CoV-2. By targeting the cleavage sites directly, the Happy inhibitors offer an alternative approach to TMPRSS2 inhibition, potentially providing broader protection against viral entry(Baughn et al., 2020) (Hoffmann, Kleine-Weber, & Pöhlmann, 2020) (Hoffmann, Kleine-Weber, Schroeder, et al., 2020).

Design Implications for Future Inhibitors: The contrasting behaviors of Happy_00 and Happy_06 highlight the trade-offs between structural stability and flexibility in inhibitor design. While rigid inhibitors like Happy_00 can provide stable

binding at accessible sites, flexible inhibitors like Happy_06 are better suited for engaging buried or dynamic targets. The use of GGGS linkers in Happy_06 underscores the importance of optimizing linker length and composition to balance stability with adaptability.
In future designs, combining rigid and flexible regions within a single inhibitor could further enhance performance. Additionally, incorporating strategies to prevent degradation by host proteases could improve the stability and longevity of these inhibitors in clinical applications.

Limitations and Future Directions: Although the in-silico results are promising, several limitations must be acknowledged. MD simulations are inherently approximations, and experimental validation is required to confirm the efficacy of these inhibitors. Future studies should evaluate the inhibitors in cell-based assays to assess their ability to block viral entry. Moreover, testing against emerging variants of SARS-CoV-2 is essential to determine whether these inhibitors retain their effectiveness against mutated spike proteins.
Further optimization of the inhibitors could involve modifying the linker sequences or incorporating additional domains to enhance binding. Additionally, exploring different simulation conditions, such as varying temperature and ionic strength, could provide more comprehensive insights into the inhibitors' behaviour under diverse physiological environments.

Conclusion

The MD simulations provide strong evidence that the fusion inhibitors Happy_00 and Happy_06 can effectively bind the SARS-CoV-2 spike protein, potentially blocking the cleavage required for viral entry. Happy_00 demonstrated stability and strong binding at accessible sites, while Happy_06's flexibility allowed it to engage deeper, more challenging targets. These results underscore the value of MD simulations in evaluating drug candidates and highlight the potential of fusion inhibitors as antiviral therapeutics. Experimental validation and further optimization are recommended to confirm these findings and enhance the inhibitors' clinical relevance.

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