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Palindromic Peptide Foldamers : A Strategy for Structural Stability and Cellular Uptake

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Mid-sized peptide therapeutics have gained significant attention for their potential to overcome the limitations of small molecules and biologics. However, their clinical application is often hindered by poor stability and low cellular permeability. In this study, we designed a palindromic peptide foldamer composed of L-leucine and L-arginine residues to investigate its structural and functional properties. CD spectroscopy confirmed that the designed peptide adopts a stable α-helical conformation, even under denaturing conditions. Cellular uptake studies using LC-MS/MS and flow cytometry indicated efficient intracellular delivery, suggesting that the peptide's amphiphilic structure enhances membrane permeability. These findings provide valuable insights into the rational design of structurally stable and functionally enhanced peptide therapeutics.

Introduction

In recent years, research on mid-sized peptides has advanced rapidly, establishing them as a new focus in drug development. In particular, peptide drugs are expected to have a broad spectrum of applications, including protein-protein interaction (PPI) inhibitors, treatments for infectious diseases, vaccines, and as carriers for drug delivery systems (DDS).[1,2,3,4] The design of highly functional peptides incorporating non-natural amino acids or side-chain modifications is gaining attention due to their unique therapeutic potential beyond conventional small molecules and antibody therapies.[5,6,7] Their chemical synthesis enables cost-effective production, thereby driving development in academia and industry. Next-generation peptide drugs feature enhanced metabolic stability, plasma stability, and cell membrane permeability.[8,9,10,11] These improvements stem from the introduction of non-natural amino acids and molecular modifications. While naturally occurring peptides are rapidly degraded by hydrolytic enzymes in vivo, peptides with non-natural amino acids evade enzymatic recognition, increasing their stability and prolonging their effects. Among these modifications, commonly utilized examples include L-amino acid derivatives, D-amino acids, N-alkylated amino acids, β-amino acids, and α,α-disubstituted amino acids.[12,13,14] Studies have shown that these modifications stabilize the steric structure of peptides, improve their resistance to enzymatic degradation, and enhance drug efficacy duration.

Many mid-sized peptides exhibit cyclic or stable helical structures, and controlling these steric structures is a key approach in developing highly functional mid-sized drugs.[15,16] Such peptides are referred to as 'peptide foldamers' (foldamers),[17] and a variety of foldamers incorporating non-natural α,α-disubstituted amino acids, β-amino acids, and side-chain cross-links have been reported.[18,19] These foldamers have gained significant attention in drug discovery for applications such as PPI inhibitors and DDS carriers.[20,21,22] Stabilizing α-helices via side-chain cross-linking (stapling) has been shown to enhance peptide membrane permeability, and several studies have reported the application of peptide foldamers and stapled peptides as cell-penetrating peptides (CPPs).[23,24] Our studies have demonstrated that cationic and amphiphilic CPP foldamers containing α,α-disubstituted amino acids form stable helical structures and exhibit high cell membrane permeability.[25,26] CPP stapling has been clearly demonstrated to stabilize the helical structure and enhance membrane permeability. These studies suggest that peptide structural modifications have a significant impact on their functionality, providing important insights into future peptide drug development. Thus, these studies have demonstrated that stapling techniques, which stabilize α-helices through side-chain cross-linking, enhance both membrane permeability and bioactivity. However, such modifications are not universally effective across all peptide structures, highlighting the need for alternative design strategies.

In this study, we designed a novel palindromic peptide foldamer consisting exclusively of L-leucine (L) and L-arginine (R) residues and systematically investigated its structural stability, functional properties, and impact on cell membrane

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permeability (**Figure 1**). The palindromic nature of the sequence reduces terminal fraying, a common issue in linear peptides, and promotes the formation of a stable α-helical conformation. Additionally, we investigated N- and C-terminal stapling to enhance the peptide's structural integrity and improve its proteolytic stability and cellular uptake. Using a combination of circular dichroism (CD) spectroscopy, proteolytic stability assays, fluorescence analysis, and flow cytometry, we systematically evaluated the effects of structural modifications on peptide function. Our results revealed that the designed peptide foldamers exhibited intrinsic helicity and exceptional stability under denaturing conditions, making them promising candidates for therapeutic applications. Furthermore, cellular uptake studies demonstrated that the amphiphilic design facilitates efficient intracellular delivery. By elucidating the interplay between sequence symmetry, secondary structure stabilization, and membrane permeability, this study offers valuable insights into the rational design of next-generation peptide therapeutics with improved bioavailability and functionality.

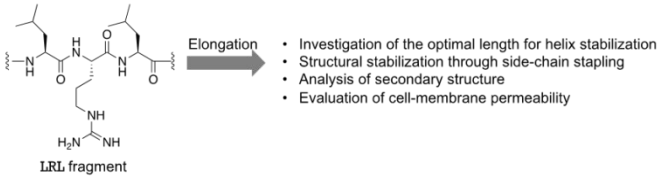

Figure 1. Palindromic peptide foldamers consisting exclusively of L-leucine (L) and L-arginine (R) residues.

Result and Discussion

The LRL motif was selected based on its structural and functional properties; L-leucine (L) was chosen as a hydrophobic amino acid to stabilize the α-helix, while L-arginine (R) was incorporated as a cationic residue to facilitate membrane permeability evaluation. To determine the optimal number of LRL repeat motifs required to form a stable α-helical structure, we first conducted molecular dynamics (MD) simulations. Additionally, to systematically evaluate the stabilizing effect of N-terminal and C-terminal stapling, we introduced the LAL [alanine (A)] sequence at the center of the peptide, ensuring a palindromic design. Based on these principles, we designed three peptide variants (peptides **A**, **B**, and **C**), in which the LAL sequence was centrally positioned, flanked by one to three LRL repeats on both the N- and C-terminal sides. The results of the MD simulations (see supporting information for detail methods and conditions), shown in **Figure 2**, revealed that peptides **A** and **B** exhibited significant conformational fluctuations, particularly compromising the consistency of the helical structure. In contrast, peptide **C**, which contained an increased number of LRL repeats at both termini, displayed reduced structural fluctuations, suggesting enhanced stability of the α -helical conformation. These findings demonstrated that increasing the number of LRL repeats contributes to structural stabilization. Based on these computational simulations, we designed a palindromic peptide, Ac-LRLLRLL-K(FAM)-LLRLLRL-NH2, composed solely of natural amino acids. This sequence was further optimized to maintain symmetry around a fluorescent-labelled lysine [K(FAM)], replacing alanine. The design was guided by the hypothesis that the LRL repeat motif would promote α-helical formation through hydrophobic interactions between leucine residues. The symmetrical architecture was expected to minimize terminal fraying, thereby enhancing structural robustness and resistance to denaturation. Additionally, the amphiphilic nature of the sequence was anticipated to facilitate membrane permeability, making it a promising candidate for intracellular delivery applications. We further explored N- and C-terminal stapling strategies to enhance structural stability and enzymatic resistance. That is, the N-terminal and C-terminal stapled peptides **Nst-1** and **Cst-1** were designed, respectively (**Table 1**). The designed peptides were synthesized using the Fmoc-based solid-phase peptide synthesis method, followed by on-resin side-chain stapling via Grubbs-catalyzed olefin metathesis (for **Nst-1** and **Cst-1**). The peptides were cleaved from the resin, purified via reversed-phase high-performance liquid chromatography (RP-HPLC), and characterized by liquid chromatography–mass spectrometry (LC-MS) to confirm their identity and purity.

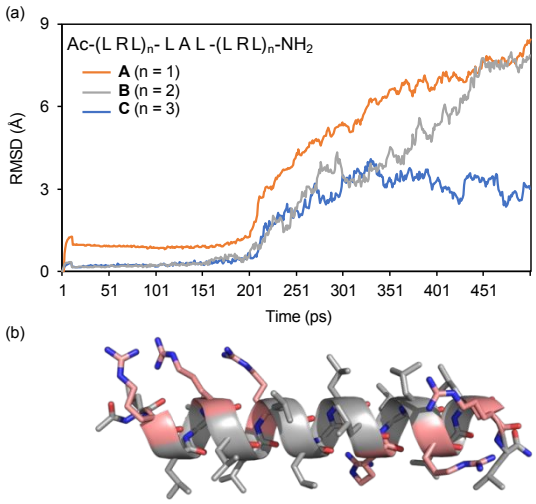

Figure 2. (a) The structural flexibility of peptides **A**, **B**, and **C** was calculated using molecular dynamics simulation. The flexibility is expressed as root mean square deviation. (b) The calculated structure of peptide **C** (gray: L and A, pink: R).

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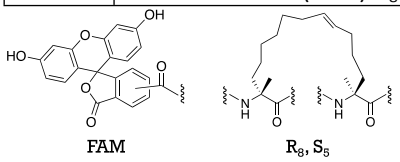
Table 1. Peptide sequence in this study.

Peptide	Sequence
1	Ac-LRLLRLLRLL-K(FAM)-LLRLLRLLRL-NH_2
Nst-1	Ac-LR-R_8-LRLLRLL-S_5-K(FAM)-LLRLLRLLRL-NH_2
Cst-1	Ac-LRLLRLLRLL-K(FAM)-R_8-LRLLRL-S_5-RL-NH_2



FAM R_8, S_5

Secondary structural analysis of synthesized peptides was performed using CD spectra. All peptides adopted a stable α-helical conformation in HEPES (pH 7.5, 25 °C) (**Figure 3a**). Notably, peptide **1**, composed solely of natural amino acid sequences, also retained a stable α-helical conformation, with no appreciable difference in helical stability between **Nst-1** and **Cst-1**, which feature stapled N- or C-termini. Furthermore, in the CD spectra recorded in HEPES (pH 7.5, 65 °C) (**Figure 3b**), all peptides retained their α-helical conformation. These findings indicate that the designed LRL repeat sequence is intrinsically thermostable. Moreover, all peptides retained their α-helical conformation even in the presence of 1 M guanidine hydrochloride, a known denaturant of secondary structure (**Figure 3c**). These findings demonstrate that LRL repeat-containing peptides exhibit high structural stability and function as peptide foldamers, maintaining a robust α-helical structure under varying environmental conditions.

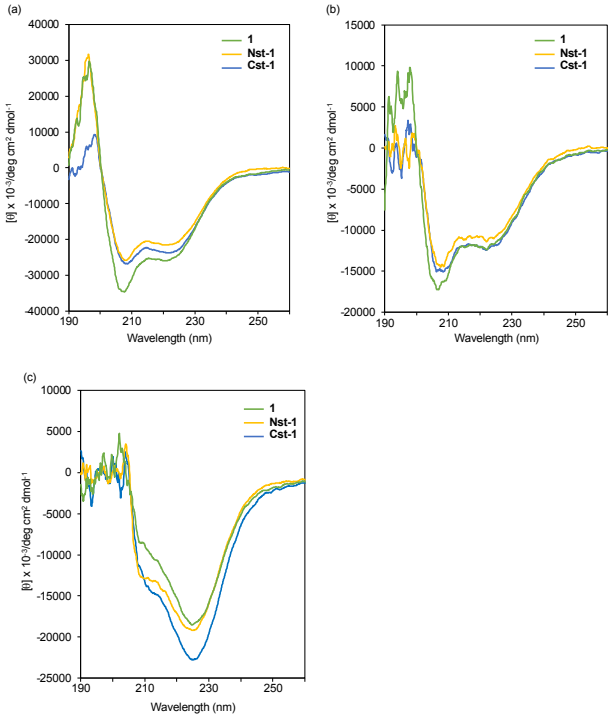


Figure 3. CD spectra in the 190–260 nm region of peptides **1**, **Nst-1**, and **Cst-1** (a) in 10 mM HEPES buffer (pH 7.5) at 25 °C, (b) in 10 mM HEPES buffer (pH 7.5) at 65 °C, (c) in 10 mM HEPES buffer containing 1M guanidine hydrochloride (pH 7.5) at 25 °C. Peptide concentration: 0.05 mM.

To evaluate the enzymatic stability of the designed peptides, we performed trypsin and proteinase K digestion assays. All three peptides—**1**, **Nst-1**, and **Cst-1**—exhibited considerable resistance to both trypsin and proteinase K digestion, retaining a significant portion of their intact forms (Figure S1). This suggests that the LRL repeat motif in the palindromic sequence intrinsically contributes to proteolytic stability. Furthermore, **Nst-1** and **Cst-1**, which feature N- and C-terminal stapling, respectively, demonstrated slightly higher resistance to proteinase K compared to peptide **1**, indicating that stapling modifications can further enhance stability against enzymatic degradation.

Prior to assessing the cellular permeability of the synthesized peptides **1**, **Nst-1**, and **Cst-1**, we characterized their fluorescence properties (**Figure S2**). A comparison of **1**, **Nst-1**, and **Cst-1** revealed that N-terminal stapling reduced fluorescence intensity, whereas C-terminal stapling had little effect. These results suggest that stapling affects the local environment of the fluorophore, resulting in structural alterations that modulate its photophysical properties. The ability of the designed peptides to penetrate cell membranes was evaluated using LC-MS/MS and flow cytometry. LC-MS/MS analysis revealed that all peptides demonstrated efficient cellular uptake, with intracellular concentration increasing over time (**Figure 4**). Notably, no significant differences in uptake efficiency were observed between **1**, **Nst-1**, and **Cst-1**, suggesting that the palindromic sequence itself enhances membrane permeability, independent of stapling modifications.

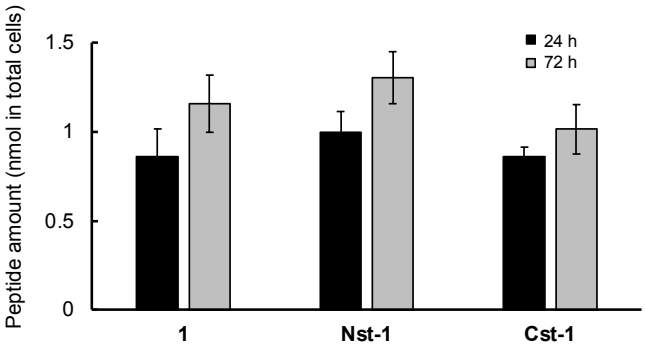


Figure 4. Evaluation of intracellular uptake efficiency of peptides (3 μM) in MCF-7 cells using LC-MS/MS after incubation 24 h and 72 h.

Flow cytometry further confirmed these findings. Under standard conditions, **Cst-1** exhibited a slightly higher fluorescence signal than **1**, but overall, no substantial differences were observed between the peptides, even after 72 hours of incubation (**Figure 5**). To investigate the uptake mechanism, cellular uptake assays were conducted at low temperatures. The fluorescence intensity of all peptides was significantly decreased at low temperatures, indicating that the primary uptake mechanism is endocytosis. These results underscore the fact that the amphiphilic design of the palindromic sequence is crucial for cellular uptake, and terminal stapling may provide benefits such as enhanced stability while minimizing the impact on uptake efficiency.

While both LC-MS/MS and flow cytometry confirmed efficient uptake of all three peptides, discrepancies in quantitative trends likely stem from differences in detection principles. Flow cytometry results may be influenced by fluorophore quenching, endosomal entrapment, and environmental pH effects, whereas LC-MS/MS reflects total intracellular peptide levels. These differences have been documented in previous studies[27,28] and highlight the importance of using complementary methods for accurate uptake assessment.

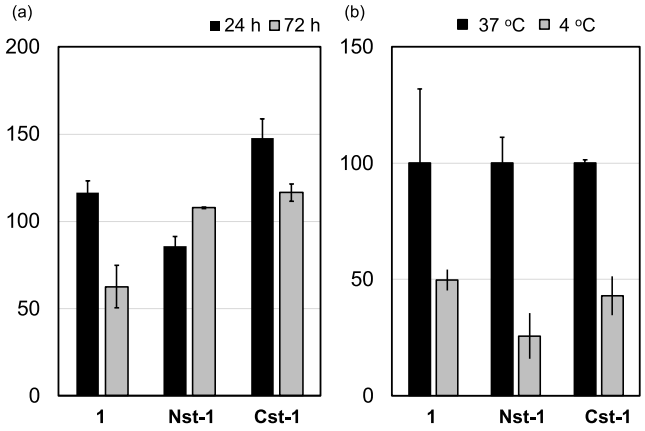


Figure 5. Evaluation of intracellular uptake of peptides (3 μM) in MCF-7 cells efficiency using flow cytometry (a) after incubation 24 h and 72 and (b) under conditions at 37 °C and 4 °C.

Conclusions

The findings of this study present a compelling case for the rational design of palindromic peptide foldamers as structurally stable and membrane-permeable therapeutic candidates. By leveraging the unique properties of L-leucine (L) and L-arginine (R) residues in a palindromic arrangement, the researchers successfully engineered a peptide backbone with intrinsic α-helicity, exceptional stability under denaturing conditions, and efficient cellular uptake. One of the most remarkable aspects of this study is the demonstration that palindromic sequence design can inherently confer stability and membrane permeability, independent of additional modifications. Traditional approaches to peptide drug development often rely on non-natural amino acids or extensive chemical modifications to enhance stability and permeability. However, this study challenges that paradigm by showing that an optimized sequence architecture alone can achieve these critical properties. The LRL motif, in particular, appears to be an effective structural element for α-helix stabilization and amphiphilicity, making it a promising scaffold for future peptide therapeutics. Another key insight from this study is the role of terminal stapling. While N- and C-terminal stapling were explored as potential strategies for further enhancing structural integrity and enzymatic resistance, the findings indicate that their effects on cellular uptake were minimal. This suggests that, although stapling can contribute to peptide stability, its role in enhancing permeability is not universally applicable and may depend on other factors, such as sequence composition and overall amphiphilic balance. This underscores the need for a more nuanced understanding of how sequence symmetry and targeted modifications interact to optimize peptide function.

This study successfully demonstrates that palindromic peptide foldamers can serve as a rational design strategy for developing stable, membrane-permeable therapeutic peptides. The intrinsic stability of the LRL repeat sequence, coupled with

the benefits of selective terminal modifications, presents a promising platform for further optimization. Future research should explore additional sequence variations, alternative stapling chemistries, and targeted intracellular delivery strategies to expand the functional versatility of these peptides. These insights will be instrumental in driving the development of peptide-based therapeutics with improved pharmacokinetic properties and clinical applicability.

Author contributions

Y.D. designed the research, and H.Y. and Y.D. wrote the paper. M.F., T.I., A.I., and K.S. performed the experiments and analyzed the results. All authors discussed the results and commented on the manuscript.

Conflicts of interest

There are no conflicts to declare.

Data availability

Additional experimental data supporting this article are included in the ESI.† Reasonable requests for additional information can be made to the corresponding authors.

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Data Availability Statement

Additional experimental data supporting this article are included in the ESI. † Reasonable requests for additional information can be made to the corresponding authors.