Modulation of Coiled-Coil Binding Strength and Fusogenicity through Peptide Stapling

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ABSTRACT: Peptide stapling is a technique which has been widely employed to constrain the conformation of peptides. One of the effects of such a constraint can be to modulate the interaction of the peptide with a binding partner. Here, a cysteine bis-alkylation stapling technique was applied to generate structurally isomeric peptide variants of a heterodimeric coiled-coil forming peptide. These stapled variants differed in the position and size of the formed macrocycle. C-terminal stapling showed the most significant changes in peptide structure and stability, with calorimetric binding analysis showing a significant reduction of binding entropy for stapled variants. This entropy reduction was dependent on cross-linker size and was accompanied by a change in binding enthalpy, illustrating the effects of preorganization. The stapled peptide, along with its binding partner, were subsequently employed as fusogens in a liposome model system. An increase in both lipid- and content-mixing was observed for one of the stapled peptide variants: this increased fusogenicity was attributed to increased coiled-coil binding but not to membrane affinity, an interaction theorized to be a primary driving force in this fusion system.

INTRODUCTION

Intramolecular cross-linking of peptides, commonly referred to as peptide stapling, is often employed to change or constrain the secondary structure of small peptides and to induce unstructured peptides to mimic complex protein folds and protein−protein interactions (PPIs).1−4 Stapling also contributes to an increased resistance to denaturation and proteolytic degradation, making it a useful technique for the modification of peptide-based therapeutics.5 Hydrocarbon stapling, a technique which is based on catalyzed olefin metathesis, has seen widespread application with multiple compounds being investigated in academic, preclinical, and clinical studies.6−10

Peptide stapling techniques can be broadly divided into two categories: single- and two-component strategies. Single-component strategies incorporate amino acids that can be cross-linked selectively, or protection strategies are chosen that allow selective cross-linking. Common single-component stapling strategies include disulfide bonding,11 lactam bridges,12,13 and olefin metathesis.10 Two-component staple adds a bifunctional cross-linker to bridge two amino-acid side chains; the most common techniques are based on cysteine cross-linking and triazole linkages.14−17 Two-component strategies are in principle more complex than single-component strategies, but they allow for a more flexible cross-linker design, as it does not need to be compatible with solid-phase peptide synthesis. Although most stapling techniques are used to increase or constrain peptide helicity, systems that compare different methods are often based around short peptide sequences, and although multiple comparisons have been made,18,19 the ideal cross-linking technique is still open to debate.20

The α-helix secondary structure motif has been mimicked using stapled peptides due to its common occurrence in proteins and therefore its potential as a PPI mimic.21 Coiled coils, which are protein-folding motifs comprising two or more α-helices, are intrinsically helical, and therefore, techniques commonly used for the stapling of helices should permit modulation of coiled-coil interactions. Indeed, Rao et al. have shown lactam bridges can be used to generate short, helical, cFos binding peptides,22 and Haney and Horne have used oxime cross-linking to generate stapled variants of the GCN4-p1 coiled-coil domain.23 More recently, Wu et al. used a bistriazole stapling technique to increase peptide binding to the polymerase α accessory factor ctf4,15 and Lathbridge and Mason showed that lactam-bridged heptapeptides can be used for the de novo design of a coiled-coil binding peptide.24

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Together, these studies provide methods for the cross-linking of coiled-coil or coiled-coil-binding peptides, but it is unclear which method would prove to be the most effective when applied to a different coiled-coil system. The size of the macrocycle formed varies significantly between the different cross-linking techniques, as do the polarity and hydrophobicity of the cross-linkers in question. Interactions of the asymmetric oxime moiety with different amino acid side chains resulted in different binding strengths when the cross-linker was reversed in Haney and Horne’s method. This necessitated the preparation and evaluation of both variants, and indicates oxime cross-linking effectiveness is dependent on amino acid composition.

Our lab has developed a model system for membrane fusion, inspired by naturally occurring SNARE (soluble NSF attachment protein receptors) proteins.25 This system consists of a pair of complementary peptides dubbed E and K, which form a heterodimeric coiled-coil that can be attached to lipid membranes via a PEG spacer and lipid anchor.26 Like SNARE proteins, this model system promotes the fusion of lipid membranes, and it can be facilely modified to study the process of membrane fusion via structure–activity relationships.27 It has recently been discovered that these two peptides play different roles in the fusion process.28 The interactions of the K peptide with lipid membranes have been hypothesized as an important factor in membrane fusion efficiency.29 Membrane interactions can occur simultaneously with the formation of the coiled-coil domain in a membrane fusion interface (as visualized in Scheme 1C); therefore, a fine balance between the two must be achieved. In addition, both membrane binding and coiled-coil formation depend on the peptides adopting a helical structure; we believe stapling should allow for the generation of peptides with varied helical structures, which will in turn affect coiled-coil formation and membrane binding interactions. Studying the effects of modulating the membrane interactions and coiled-coil binding affinity will generate insights into the importance of both factors in membrane fusion.

When attempting to modulate the behavior of the heterodimeric coiled coil used in our group,30 the choice of cross-linking technique was not obvious, due to the differences observed in previously employed cross-linking techniques (vide supra). The position of the cross-linker and the macrocycle size were deemed the most influential characteristics in the previously mentioned cross-linking strategies; therefore, we wanted to evaluate both of these criteria independently for our system. The most favorable candidates could then be used to test the effect of structural changes on coiled-coil-based membrane fusion. One stapling strategy that attracted our attention was developed by the DeGrado lab, and it is based on the alkylation of cysteine using dibromoxylene.31 The advantage of this system lies in the rigidity provided by the aromatic ring, allowing precise spacing between the two thiol moieties by selecting one of the three different structural isomers of dibromoxylene: ortho, meta, and para; Scheme 1. In the original study, meta-xylene showed the most promise as a cross-linker, and further investigations in the same group have therefore focused on this variant.32,33 Other recent investigations have also predominantly used the meta derivative,34,35 and when a comparison was made between the isomers, only short or unstructured peptides were used. This means the question of whether, for a helical or coiled-coil peptide, meta-xylene is indeed the best cross-linking moiety is unanswered. Therefore, to probe the effect of stapling on coiled-coil peptides, we elected to investigate dibromoxylene cross-linking of cysteines, employing all three structural isomers in order to elucidate the role of cross-linker size and its effect on structure and activity.

In this study, a library of nine stapled peptides was prepared by modifying peptide K via cysteine alkylation. These stapled K-peptide derivatives exhibited systematic variations in helicity and thermal stability, as observed by circular dichroism (CD) spectroscopy. The coiled-coil binding thermodynamics were studied using isothermal titration calorimetry (ITC), and it was discovered that increased coiled-coil binding is based on a preorganization effect. These observed changes in structure and binding dynamics were heavily dependent on the location of the staple and the choice of cross-linker. In lipid- and content-mixing experiments, a significant change in fusogenicity was measured for selected stapled peptides, which was attributed to the altered coiled-coil interactions.
RESULTS AND DISCUSSION

Stapled Peptide Design. The starting point for structural modification is one peptide of a three-heptad heterodimeric coiled-coil pair first reported by Litowski and Hodges. The two peptides are named after the abundance of either glutamic acid (Glu, E) or lysine (Lys, K), and each peptide contains a C-terminal glycine and either tyrosine or tryptophan as a fluorescent reporter, giving rise to E3GY and K3GW. To facilitate stapling, two amino acids in peptide K3GW were modified to cysteine, spaced i to i + 4 to best match a single α-helical turn. Amino acids that are involved in electrostatic (positions e and g) or hydrophobic (positions a or d) interactions were not varied to ensure the stapled peptides retained the same stabilizing coiled-coil interactions as the parent peptides. Three different variants were generated each with the cysteines and therefore the staple, in a different heptad, Table 1. Each of these positional variants was stapled with ortho-, meta-, and para-dibromomethylene, generating a library of nine stapled peptides. When referring to these stapled peptide variants, a notation which reflects the position and type of cross-linker is used, for example, K3GW-1M signifies the cross-linker is in the first heptad and the meta variant has been employed.

Secondary Structure Analysis. CD spectroscopy was employed to determine the secondary structure of the stapled peptide variants; the effects of both the stapling location and the size of the cross-linker can be clearly observed, Figures 1 and S1. Peptide stapling close to the C-terminus (K3GW-3 variants) showed the largest increase in α-helicity for all three xlenes, whereas modification in the second heptad (K3GW-2 variants) showed the lowest increase. Notably, when para-xylene was used as the cross-linker in the second heptad, the overall peptide helicity was reduced, Figure 1B, showing para-xylene is too large to form an ideal overall peptide helicity was reduced, Figure 1B, showing xylene was used as the cross-linker in the second heptad, the xylenes, whereas modi variants) showed the largest increase in helical turn. Amino acids that are involved in electrostatic interactions were not varied to ensure the stapled peptides retained the same stabilizing coiled-coil interactions as the parent peptides. Three different variants were generated each with the cysteines and therefore the staple, in a different heptad, Table 1. Each of these positional variants was stapled with ortho-, meta-, and para-dibromomethylene, generating a library of nine stapled peptides. When referring to these stapled peptide variants, a notation which reflects the position and type of cross-linker is used, for example, K3GW-1M signifies the cross-linker is in the first heptad and the meta variant has been employed.

Table 1. Sequences of the Coiled-Coil Parent Peptides and Cysteine-Containing Variants

| peptide  | sequence | g | abcdefg | abcdefg | abcdefg | a |
|----------|----------|---|---------|---------|---------|---|
| E3GY     | E        | IAALKE | IAALKE  | IAALKE  | Y  |
| K3GW     | K        | IAALKE | IAALKE  | IAALKE  | W  |
| K3GW-1   | K        | IAALKE | IAALKE  | IAALKE  | W  |
| K3GW-2   | K        | IAALKE | IAALKE  | IAALKE  | W  |
| K3GW-3   | K        | IAALKE | IAALKE  | IAALKE  | W  |

In contrast to the stapled peptides in isolation, meta-xylene-modified peptides show the most α-helical structure as a coiled coil. ortho-Xylene stapled peptides had the largest increase in $T_m$ for all three positions (Figure 2), and the trends in coiled-coil stability are similar to those observed for the single peptides, with an average increase in $T_m$ of 4.9 °C for the stapled peptides (Table S1) and 4.8 °C for their coiled coils (Table S2). meta-Xylene was previously shown to have the largest increase in helicity in small unstructured peptides, but in the E/K system ortho-xylene stapled variants yielded the highest single-peptide helicity and largest increase in $T_m$ for both the peptides and their respective coiled-coils. Because it is possible that stapling affects coiled-coil interactions without changing peptide helicity as observed via the thermal unfolding experiments, the effect of peptide stapling on coiled-coil binding was further investigated using isothermal titration calorimetry (ITC).

Binding Thermodynamics of Stapled Coiled-Coils. Direct determination of the dissociation constant ($K_d$) and enthalpy of binding ($\Delta H_b$) and therefore calculation of the free energy ($\Delta G_b$) and entropy of binding ($\Delta S_b$) is possible using ITC (Figure S3), allowing investigation of peptide interactions independent of peptide structure. The results shown in Figure 3 and Table S3 show that, in general, coiled-coil binding of peptides K3GW and E3GY is strongly enthalpically favored but entropically unfavored. The effect of enthalpy can be explained via the formation of amide hydrogen bonds and electrostatic interactions upon folding of the peptide. When the C-terminally stapled variants of peptide K3GW are analyzed, the $K_d$ is decreased from 73 to 22 and 24 nM for the 30 and 3M variants, respectively, and to 51 nM for the 3P variant. A large decrease in $\Delta S_b$ was observed and was directly related to the size of the implemented staple. ortho-Xylene stapling at the C-terminus reduced the effect of entropy upon binding from 37 to 24 kJ/mol, a reduction of 35%. At the same time, an increase in the $\Delta H_b$ from −77 to −68 kJ/mol was observed, counteracting the observed entropic effects and leading to the conclusion that the mechanism of peptide stapling relies on a preorganization effect: through conformational restriction, the peptide is preorganized in a helical conformation which reduces the entropic effects of binding, but some of the energy that is gained upon formation of an α-helix is also lost. Although the $K_d$ for the C-terminal ortho- and meta-xylene stapled peptides is comparable, the $\Delta S_b$ is more favorable for the ortho variant, explaining the large differences in $T_m$ observed for these two peptides. At all three stapling positions, the ortho variants show a reduced effect of entropy upon binding compared to the meta variants, which is likely caused by the smaller size of the ortho cross-linker. A smaller cross-linker restricts the maximum distance between the two helical turns and therefore limits the number of possible conformations that the peptide can assume.

Recently, Miles et al. screened hydrocarbon-stapled peptides as protein–protein interaction (PPI) mimics against Bcl-xL/Mcl-1 and observed similar changes in the $\Delta H_b$ and $\Delta S_b$ for their stapled peptides; however, they observed an overall increase in $\Delta G_b$. Binding kinetics determined via a surface plasmon resonance (SPR) assay showed that the binding of their PPI mimic could best be explained via an induced fit mechanism, where the PPI can interact via multiple binding modes. Restricting the potential conformations of the peptide through the introduction of a staple reduced the number of possible binding modes and therefore increased the overall $K_d$. 

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of the system. The E/K peptides used in this paper are designed and experimentally confirmed to form heterodimeric coiled-coils exclusively.40 As there is only one binding mode, the observed changes in structure and stability, as determined via CD, show a direct correlation with the binding thermodynamics in ITC: C-terminal stapling using ortho- and meta-xylene is the most effective way to increase the binding strength of coiled-coil peptides.

Membrane Interactions of Peptide K₃GW Are Perturbed by Peptide Stapling. The effectiveness of E/K-based membrane fusion is partially attributed to the membrane interactions of peptide K, which are theorized to induce membrane curvature and therefore accelerate the transition from membrane docking to hemifusion.41 The interactions of peptide K with lipid membranes are based on a lysine snorkeling mechanism, which describes the hydrophobic amino acids in the "a" and "d" position inserting in a lipid membrane, helped by the favorable electrostatic interactions between lysines and the phosphate groups of the lipid membrane.42 This is a reversible process that can only happen when the peptide folds into an amphipathic helix and all the hydrophobic amino acids are positioned on the same face. Peptide stapling, which changes the overall peptide conformation, is therefore theorized to have an effect on membrane binding. The membrane partition coefficient ($K_p$) of the stapled K variants was assayed via tryptophan fluorescence titration experiments, and the results are shown in Figure 4. Membrane binding was either comparable to that of unmodified K₃GW or was increased up to a factor of 2 and did not show any correlation to the location of the staple or to the overall helicity of the peptide (Figure S4). The difference in partition coefficient between K₃GW-3O and K₃GW-3M is striking, as the value is almost half for the ortho variant despite the helicity of the two being very similar. This shows that the

Figure 1. CD spectra of stapled peptides. (A) CD spectra of C-terminal stapled peptides, (B) heat map of the percentage of peptide helicity for all stapled variants and, in brackets, the change in helicity compared to K₃GW as a control, (C) CD spectra of the C-terminal stapled peptides mixed with peptide E₃GY to form a coiled coil, and (D) heat map of average peptide helicity of all stapled peptides when combined with peptide E₃GY and, in brackets, the change in helicity compared to the coiled-coil formed with K₃GW as a control. Total peptide concentration is 50 μM, and spectra were recorded at 20 °C in pH 7.4 PBS buffer.

Figure 2. Change in peptide (left) and coiled-coil (right) melting temperature for stapled peptides in comparison to K₃GW as determined via CD spectroscopy. Total peptide concentration is 50 μM C in pH 7.4 PBS buffer, and spectra were recorded from 5 to 95 °C and are shown in Figure S2.
addition of a hydrophobic cross-linking moiety between the “b” and “f” position does not increase the membrane affinity of amphiphilic α-helical peptides in a structure-dependent manner and leads to the hypothesis that peptide K₃GW does not bind to liposomal membranes as a highly structured α-helix.

CD experiments were performed with the C-terminal stapled peptides in the presence of liposomes, and this data showed a reduced ellipticity at 222 nm and a high 208/222 nm ratio (see Figure S6). This indicates that the peptides are less α-helical in the presence of liposomes, which supports this hypothesis. If partitioning from the aqueous phase into the membrane is assumed to require partial unfolding of the peptide helix, the difference in binding strength between the ortho and meta variants can also be explained by the smaller size of the ortho cross-linker, which restricts the ability of the peptide to unfold.

Lipid- and Content-Mixing Is Increased for C-Terminal Stapled Peptides. Complete fusion of two lipid-membrane-enclosed spaces will result in homogeneous mixing of the lipids in the inner and outer leaflets, as well as mixing of the inner contents. In a liposomal system, this process can be studied via the incorporation of chromophores into the lipid bilayer or on the inside of the liposomes.

Fusion of these liposomes with nonlabeled liposomes will result in a fluorescence change which can be quantified to compare the peptide fusogenicity. Lipopeptides were prepared which contained cholesterol and a polyethylene glycol (PEG₄) spacer at the N-terminus, facilitating membrane anchoring. Stapled peptides K₃GW-3O and K₃GW-3M were selected for fusion studies because these gave rise to the largest structural and thermodynamic changes. Moreover, their binding strength is comparable, but their partition coefficient differs by a factor of 2; therefore, by testing both and comparing them to unmodified K₃GW, the effect of both coiled-coil binding strength and membrane binding on fusogenicity can be determined. The lipopeptides were prepared using a novel on-resin stapling technique enabled by the use of 4-methoxytrityl (Mtt) protected cysteine; full details are available in the Experimental Section. These peptides were tested for fusogenicity together with the lipidated variant of E₃GY (structures can be found in Scheme S1).

Lipid mixing was quantified using a Förster resonance energy transfer (FRET) pair incorporated in the lipid membrane, and the results are shown in Figure 5A. The amount of lipid mixing observed was comparable for K₃GW and K₃GW-3M at 1% peptide concentration, while the K₃GW-3O variant showed increased lipid mixing 6 min after the start of the experiment. This indicates that docking of the liposomes occurs at the same speed but more lipid mixing occurs for the K₃GW-3O variant. As the absolute amount of lipid mixing was low, the same experiment was also performed with 2% of the lipopeptides, which doubled the amount of lipid mixing observed while retaining the same trends (Figure S7).

Content mixing experiments when performed properly are the best measure for complete fusion of two lipid membranes. The membrane-impermeable sulfonhodamine B (SrhB) dye was employed as a fluorescent reporter and showed significant increases in fusion for both stapled peptide variants, Figure 5B, with the K₃GW-3M variant doubling the amount of content mixing compared to K₃GW (from 17.1% to 36.2%). The K₃GW-3O variant produced an even larger increase; up to 93% content mixing was observed after 60 min, with an average of 79.5%.

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**Figure 3.** Thermodynamic binding parameters of K₃GW and its stapled derivatives in complex with E₃GY, determined via ITC. Error bars show the fitting error to a single-binding site model for both (A) the dissociation constant and (B) the binding enthalpy (ΔH). Entropy (−TΔS) is calculated from these parameters, and no error bars are drawn.

**Figure 4.** Partition coefficient of peptides with liposomal membranes. Partition was measured via a tryptophan fluorescence titration at 20 °C in pH 7.4 PBS buffer. Error bars represent the error in the fitting of K_p. Values and fitting of the titration data can be found in Table S4 and Figure S5.
This is surprising, since there was no observed difference between K3GW and the K3GW-3M variant during lipid mixing experiments. An immediate difference between the three peptides is observed at the start of the experiment, which is not the case for lipid mixing, raising the concern that the stapled peptide variants might be destabilizing the liposomes and causing leakage of SrB across the lipid membranes. Plain liposomes and liposomes modified with 1% lipidated E3GY were tested for leakage but did not show significant differences (Figure S8), indicating that the stapled peptides do not destabilize the liposomal membranes.

**Insights into the Mechanism of Coiled-Coil Based Membrane Fusion.** Membrane fusion occurs in multiple stages, starting with the docking of two membranes to create a membrane fusion interface, followed by hemifusion which results in the mixing of the outer lipid leaflets, and proceeding via the formation of a fusion pore to complete fusion of the two liposomes, meaning their contents are exchanged.27 Both lipid- and content-mixing experiments showed increased fusogenicity for the lipidated K3GW-3O peptide, with increased content mixing also observed for the K3GW-3M variant. Differences in the lipid-mixing amount are obvious after 6 min, indicating that the rates of initial docking and outer leaflet mixing are comparable for the three peptides. Because complete fusion of the liposomes, as judged by content mixing, is increased significantly for the K3GW-3O variant, the observed difference in lipid mixing is most likely caused by an increased mixing of the inner leaflet lipids. The increased coiled-coil binding strength observed via ITC, could explain the increase in fusion except for the fact that K3GW-3O and K3GW-3M are dissimilar in their fusogenicity, yet they have a comparable Kd.

The K3GW-3O and K3GW-3M stapled peptides differ in their effects of entropy on coiled-coil binding and the strength of their membrane interactions, which are both increased for K3GW-3M. The Kd of coiled-coil formation is dependent on the association and dissociation rate constants, which show different behavior in temperature dependent stopped-flow experiments of coiled-coil peptides.44 The dissociation rate was shown to be more dependent on temperature and therefore had a much larger entropic component then the rate of association. The stapled peptide variants tested have a decreased entropic binding component and should therefore also show a lower rate of dissociation. At a membrane fusion interface, dissociation of the coiled-coil is most likely followed either by another peptide binding event or by the insertion of peptide K into the lipid membrane. A decrease in the dissociation rate should therefore result in an increase in the rate of fusion, although the total amount of fusion observed is not expected to change.

For SNARE-mediated membrane fusion, it is known that multiple protein complexes are required to drive fusion of a single vesicle, and the likelihood of fusion occurring is dependent on the number of protein complexes at the fusion interface.45,46 This cooperativity is likely also necessary for our coiled-coil based system, and any interactions that influence the amount of coiled-coil that can be coassembled around a fusion interface will influence the amount of fusion observed. In this case, both K3GW-3O and K3GW-3M show increased binding and lowered binding entropy and therefore increased fusion via a lower dissociation rate. For K3GW-3M, this difference is less significant and is likely to be partially counteracted by the increased membrane affinity of the peptide. This is a competitive interaction in the formation of the coiled-coil complex, and an interaction which can provide a pathway for dissipation of the free peptide after dissociation of the coiled coil.41 In this manner, the total number of peptide complexes that are formed around a membrane fusion interface is reduced, and no increase in membrane fusion is observed. This reasoning can also be applied to homomeric peptide interactions, which could provide a pathway for dissipation of the lipopeptide away from the fusion interface. CD titration was performed with K3GW and the K3GW-3O and K3GW-3M analogues to test for homodimerization (Figure S9 and Table S5), but the dimerization constant was found to be comparable for all variants and weak enough that this should not be
considered an important part of the fusion mechanism. This mechanistic understanding derived from the observed differences between the two stapled peptide variants will require further confirmation in different systems and experiments.

CONCLUSIONS

We have employed a cysteine bisalkylation stapling technique to generate a series of nine structurally isomeric α-helical peptides that can form a heterodimeric coiled-coil when mixed with their binding partner. CD and ITC experiments showed that both stapling location and choice of staple affected the properties of the resulting peptides and coiled-coil complexes, with the largest increase in structure, binding, and stability observed for peptides stapled close to the C-terminus with ortho-xylene. Binding strength is increased via a preorganization mechanism, which consists of a large reduction of the unfavorably entropic binding component, combined with a negative change in binding enthalpy. ortho- and meta-Xylene cross-linkers resulted in similar coiled-coil binding strengths, although ortho-xylene reduced the effect of entropy the most. This effect was true for all three stapling sites and is due to the smaller size of the ortho-xylene cross-linker. Although there may be some dependence on amino acid composition, we conclude that ortho-xylene is the best cross-linker to stabilize helical peptides, despite meta-xylene being more widely employed to date.

The effect of stapling on peptide-membrane partitioning was determined and showed a 2-fold difference between stapled peptide variants, although no direct correlation to location or stapling type could be made. Lipopeptides of K3GW-3 and K3GW-3O were prepared via a novel on-resin stapling method. These peptides were tested in lipid- and content-mixing experiments, and large increases in fusogenicity for the K3GW-3O variant were observed. K,GW-3O also showed significantly increased content mixing, but it exhibited a similar amount of lipid mixing to the parent peptide. We theorize that these differences in fusogenicity can be explained via reduced dissociation; increasing coiled-coil interactions without increasing lipid membrane interactions allows accumulation of more coiled-coil pairs at the fusion interface and therefore increases membrane fusion.

EXPERIMENTAL SECTION

Tentagel resin was purchased from Rapp Polymere. Dimethylformamide (DMF), piperidine, pyridine, acetic anhydride, trifluoroacetic acid (TFA), and acetonitrile (MeCN) were supplied from Biosolve. N,N-Diisopropylethylamine (DIPEA) and Oxyma were purchased from Carl Roth. Dichloromethane (DCM) and diethyl ether were supplied by Honeywell. HBTU and all protected amino acids except Fmoc-Cys(Trt)–OH were purchased from Novabiochem. All other chemicals were purchased from Sigma Aldrich. Ultrapure water was obtained from a Milli-Q water purification system. Peptide concentration was established via absorption at 280 nm, determined using a CARY-300 UV–vis spectrophotometer.

Peptide Synthesis and Purification. All peptides were synthesized on solid phase using a CEM liberty blue automated, microwave-assisted, peptide synthesizer. Peptides were prepared on a 0.1 mmol scale using Tentagel HL RAM resin with a loading of 0.39 mmol/g. Fmoc deprotection was performed using 20% piperidine in DMF at 90 °C for 60 s. Amide coupling was achieved using 5 equiv of protected amino acid, 5 equiv of DIC as the activator, and 5 equiv of Oxyma as the activator base, heated at 95 °C for 240 s. Acetylation of the peptide N-terminus after automated synthesis was performed using an excess of acetic anhydride and pyridine in DMF.

Lipidated peptides were made on resin via the coupling of 2.5 equiv of N3-PEG4-COOH (see Supporting Information methods for synthesis details), with 2.5 equiv of HBTU, and 5 equiv of DIPEA in DMF for 2 h at room temperature. After washing the resin with DMF, the azide was reduced using 10 equiv of PMe3 (1 M in toluene), with 4:1 dioxane/water as solvent for 2.5 h. After the reaction was finished, the resin was washed thoroughly with 4:1 dioxane/water, MeOH, and DMF. Lipidation was achieved using 2 equiv of cholesterol succinate, 2 equiv of HBTU, and 4 equiv of DIPEA in 1:1 DMF/DCM, and this lipidation step was performed twice to achieve complete conversion. After the final coupling, the resin was washed with DMF, MeOH, and DCM and dried under vacuum, and the peptide was cleaved using a 92.5:2.5:2.5:2.5 mixture of TFA/TIPS/EDDT/water for 1 h, after which the peptide was precipitated in cold diethyl ether, collected via centrifugation, and lyophilized.

All peptides were purified by HPLC on a Shimadzu system consisting of two KC-20A pumps and an SPD-20A or SPD-M20A detector equipped with a Kinetix Evo C18 column. Eluents consisted of 0.1% TFA in water (A) and 0.1% TFA in MeCN (B), with all peptides eluted using a gradient of 20–90% B over 35 min, with a flow rate of 12 mL/min. Collected fractions were checked for purity via LCMS, with the pure fractions being pooled and lyophilized. LC/MS spectra were recorded using a Thermo Scientific TSQ quantum access MAX mass detector connected to a Ultimate 3000 liquid chromatography system fitted with a 50 × 4.6 mm Phenomenex Gemini 3 μm C18 column. LC/MS spectra of the purified peptides can be found in the Supporting Information.

Peptide Stapling. Intramolecular cross-linking was achieved by dissolving the peptide in a 1:1 mixture of MeCN/H2O containing 10 mM NH4HCO3 up to a peptide concentration of 500 μM. TCEP, 1 equiv, was added as a 10 mM stock solution, and the reaction was stirred for 1 h, followed by addition of 1.2 equiv of the dibromomethylene cross-linker (50 mM in DMF) and reacted for 3 h. The reaction was quenched by the addition of 5% acetic acid and purified using preparative HPLC. For the lipidated peptides, the cross-linking was performed on the solid phase. In short, cysteines protected with Mtt were incorporated into the peptide, and after automated synthesis these protecting groups were removed by incubating the resin with 2% TFA, 3% TIS in DCM for 2 min, followed by washing the resin with DCM twice. This was repeated until no more color appeared when a small amount of the resin was mixed with TFA. Cross-linking was achieved by addition of 1.5 equiv of the cross-linker and 2.5 equiv of DIPEA in 1:1 DMF/TFE and incubating this reaction for 3 h. On-resin stapling was usually performed before lipolipidation.

Circular Dichroism Measurements. CD spectra were recorded on a JASCO J-815 CD spectrometer fitted with a Peltier temperature controller. Unless otherwise specified, samples were measured at 20 °C in a quartz cuvette with a 2 mm path length. Spectra were recorded from 190 to 260 at 1 nm intervals, with a bandwidth of 1 nm, with the final spectrum consisting of the average of 5 sequentially recorded...
spectra. The mean residue molar ellipticity ($\theta$, deg cm$^2$ dmol$^{-1}$) was calculated according to eq 1:

$$[\theta] = \left(100 \times \frac{[\theta]_{obs}}{c.n.l}\right)$$  

(1)

with $[\theta]_{obs}$ representing the observed ellipticity in mdeg, $c$ being the peptide concentration in mM, $n$ being the number of peptide bonds, and $l$ being the path length of the cuvette in cm. The fraction of the $\alpha$-helical peptide could be calculated from the mean residue ellipticity using eq 2:

$$F_{\text{helix}} = \frac{[\theta]_{222} - [\theta]_0}{(\theta)_{\max} - [\theta]_0}$$  

(2)

with the maximum theoretical mean residue ellipticity, $(\theta)_{\max}$ defined as $(\theta)_{\max} = (\theta)_{\omega}(n-x)/n$ for a helix with $n$ residues and $x$ being a number of amino acids assumed not to participate in helix formation (in this case 3). $(\theta)_{\omega}$ is defined as the theoretical helicity of an infinite $\alpha$-helix and is temperature dependent, defined via $(\theta)_{\omega} = (-44,000 + 250T)$, with $T$ being the temperature in °C. The minimal expected absorbance at 222 nm for a random coil is defined in $(\theta)_0$, which is also temperature dependent via the relationship $(\theta)_0 = 2220 - 53T$.

**Tryptophan Fluorescence Titration.** Fluorescence was measured in 96-well plates using a TECAN Infinite M1000 Pro microplate reader. Liposomes of the composition 2:1:1 DOPC/DOPA/cholesterol were prepared at a 10 mM concentration via extrusion in PBS buffer, using an Avanti mini extruder with 100 nm polycarbonate membranes. Titration series of liposomes in PBS buffer were prepared with concentrations between 25 and 3750 μM, with the peptide concentration held constant at 2.5 μM. Samples were prepared in 96-well plates, and after 60 min of incubation a fluorescence spectrum was taken between 300 and 450 nm. The maximum fluorescence of each sample was plotted as a fold increase of the fluorescence of the peptide without liposomes present and fitted against eq 3 to determine the partition constant:

$$F = 1 + (F_{\text{max}} - 1)(K_pX)/(55.3 + K_pX)$$  

(3)

where the normalized fluorescence, $F$, is dependent on the maximum fluorescence when all peptide is bound to the membrane $F_{\text{max}}$, the molar partition coefficient $K_p$, the lipid concentration $X$, and the concentration of water which is assumed to be constant at 55.3 M. Experimental data representing three separate experiments was fitted to eq 3 using the least-squares method to yield the partition coefficient and the standard error of fitting.

**Isothermal Titration Calorimetry.** ITC measurements were performed on a Malvern MicroCal PEAQ-ITC automated calorimeter. In a standard experiment, the measurement cell contained 200 μL of 10 μM peptide K and the syringe was filled with E$_2$G4 at 100 μM concentration, with both peptides dissolved in PBS. The syringe content was added in 21 injections of 1.9 μL at 120s intervals, except the first injection which was 0.5 μL. The reference power was set at 2.0 μcal/s, and experiments were performed at 25 °C. The data was analyzed with the Microcal PEAQ-ITC analysis software and fitted to a single binding site model to generate the thermodynamic binding parameters. The experiment was repeated on three separate occasions, and the experimental results with the lowest reduced $\chi^2$ value are represented in this paper.

**Lipid and Content Mixing Experiments.** Liposomes with the lipid composition 2:1:1 DOPC/DOPA/cholesterol were used at a 500 μM concentration, where 1% of the lipids was substituted with the respective lipopeptide. Lipid films were prepared via evaporation of lipid and lipopeptide stock solutions in 1:1 CHCl$_3$/MeOH under a stream of nitrogen, followed by high vacuum for at least 2 h. The lipid films were rehydrated via vortex mixing with PBS buffer and sonication for 5 min at 55 °C in a Branson 2510 bath sonicator. The liposomes were checked for size and polydispersity (PDI) via dynamic light scattering (Malvern Zetasizer Nano S) and then sonicated for a second time if the PDI was larger than 0.25. Lipid mixing was assayed via the incorporation of 0.5% DOPA-NBD (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)) and 0.5% DOPE-LR (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)) in the lipid membranes of the CPE-containing liposomes. A volume of 100 μL of fluorescent CPE-containing liposomes was mixed with 100 μL of nonfluorescent CPE-decorated liposomes, and the emission of NBD at 530 nm was followed over time. Each experiment included a positive control consisting of liposomes at a 500 μM concentration and 0.25% of both DOPE-LR and DOPA-NBD, and a negative control where the fluorescent liposomes were combined with liposomes without CPE. The standard deviation was calculated on the average of four separate measurement samples, and the experiment was repeated at least three times.

Content mixing was assayed via the incorporation of 10 mM sulforhodamine B in the hydration buffer of CPE-decorated liposomes. After sonication, the unincorporated rhodamine was removed using an Illustera NAP-25 size-exclusion column. For each experiment, 100 μL of sulforhodamine-containing CPE-liposomes was mixed with 100 μL of CPE-containing liposomes, and the fluorescence of sulforhodamine followed over time at 585 nm. The value was normalized via referencing a positive control consisting of liposomes containing 5 mM sulforhodamine B prepared in the same manner and a negative control where the fluorescent CPE liposomes were combined with plain liposomes. The standard deviation was calculated on the average of four separate measurement samples, and the experiment was repeated at least three times.

Change in fluorescence was measured in 96-well plates using a TECAN Infinite M1000 Pro microplate reader. The percentage of lipid and content mixing was calculated using the following formula (eq 4):

$$\% \text{ mixing} = \frac{F_t - F_0}{F_{\text{max}} - F_0}$$  

(4)

where $F_t$ is the fluorescence at time $t$ and $F_0$ and $F_{\text{max}}$ are the fluorescence of the negative and positive controls at the same time point, respectively. Processing of fluorescence data and one-way ANOVA analysis were performed in GraphPad Prism 8.1.1.
mixing; details of the fitting procedure for homodimer formation; synthesis protocols; NMR spectra; LC/MS spectra (PDF)

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