Oligomer Formation Propensities of Dimeric Bundle Peptides Correlate with Cell Penetration Abilities

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Supporting Information

ABSTRACT: LK-3, an amphipathic dimeric peptide linked by two disulfide bonds, and related isomeric bundles were synthesized, and their cell penetrating abilities were investigated. The measurements using size exclusion chromatography and dynamic light scattering show that LK-3 and its isomers form cell penetrating oligomers. Calculations, performed for various types of peptide isomers, elucidate a strong correlation between the amphipathic character of dimers and cell penetration ability. The results suggest that the amphipathicities of LK-3 and related bundle dimers are responsible for their oligomerization propensities which in turn determine their cell penetrating abilities. The observations made in this study provide detailed information about the mechanism of cell uptake of LK-3 and suggest a plausible insight of the early stage of nanoparticle formation of the cell penetrating amphipathic peptides.

INTRODUCTION

The properties of cell penetrating peptides (CPPs) have recently gained increasing attention, since TAT, penetratin were identified as cell penetrating domains. Mechanistic studies have been carried out for several classes of CPPs to elucidate their cell penetration pathways. However, most studies are focused on arginine-rich CPPs including oligoarginines, in spite of the fact that many CPPs are characterized as cationic and amphipathic. Its detailed mechanism also remains controversial because the mechanism is altered with CPP, cell type, CPP concentration, and the cargo types. Consequently, the mechanistic studies of amphipathic CPPs have become important not only for understanding commonalities of CPPs but also for developing improved CPPs.

The cell penetration process involves interactions between the CPPs and membranes. To date, only a few studies have been conducted to determine how nanoparticle formation of peptides affects interactions of peptides with membranes and the extent of cell penetration. Especially for amphipathic peptides, the interactions at hydrophobic and hydrophilic interfaces are key properties governing peptide folding and particle formation. For example, DeGrado and Lear demonstrated that the short amphipathic model peptide, LKα, aggregates at apolar-water interfaces. Consequently, this peptide can be used as a reference system with which to investigate the link between nanoparticle formation of peptides and their cell penetration.

Recently, we discovered that LK-3, a bis-disulfide bridged dimer derivative of LKα, is translocated into cells at nanomolar concentrations. This finding stimulated us to explore the mechanistic underpinnings of cell penetration of these bundle peptides. Motivated by the others’ earlier studies, we synthesized the amphipathic peptide LK-3 and related isomeric bundles, investigated their cell penetrating abilities, and elucidated the link between nanoparticle formation of peptides and their cell penetration. The results suggest that the amphipathicities of LK-3 and related bundle dimers are responsible for their oligomerization propensities which in turn determine their cell penetrating abilities. The observations made in this study provide detailed information about the mechanism of cell uptake of LK-3 and suggest a plausible insight of the early stage of nanoparticle formation of the cell penetrating amphipathic peptides.
observations of LKet14,15 and our results of cell penetrability of LK-3, here in this report, we carried out the current investigation aimed at elucidating the details of structural characteristics leading the interactions between helical bundles that promote nanosized oligomer formation (Figure 1). While crucial in the early stage of nanoparticle formation, such a species is practically not detectable in the low-concentration regime (e.g., low nM). Thus, we posit that the dimeric bundle peptide is the precursor of the ensuing aggregation process. In the MD simulation of LK-3 in an aqueous environment, a stable structure was obtained and used as the precursor (Figure S2, almost the same structure previously reported without disulfide bonds15).

In the current study, by synthesizing isomers of the dimeric bundle LK-3, we studied how the extents of nanoparticle formation and cell penetrability are affected by various factors such as the lengths of dimer bundles, positions of two disulfide bonds, variations of hydrophobic residues, and antiparallel versus parallel alignments. Furthermore, the tentative structural details of the dimers and nanosized oligomers were explored using molecular modeling and simulations. The findings from this investigation not only offer important mechanistic insight into the cell penetrability of CPPs but also provide strategies to develop improved CPPs.

RESULTS AND DISCUSSION

Variation of Length of Dimers, Disulfide Positions, Hydrophobic Residues, and Antiparallel and Parallel Alignments of Dimer Bundles. First, we prepared peptides composed of Leu and Lys repeats of varying length and two Cys residues (Figure S3a). Fluorescence dye-labeled peptide
monomers were oxidized to produce bis-disulfide-linked dimers, which were probed on HeLa cells by using flow cytometry experiments. The results show that a 16 amino acid long peptide generates the dimeric bundles that are the most efficient in cell penetration (Figure S3b).

We next synthesized dimers arising from bis-Cys isomeric peptides to explore the cell penetration properties associated with variations in hydrophobic patches. The difference in the cell penetrating abilities of the isomeric peptides was substantial (Figure 2a,b). Specifically, LK-3 [same as LK-3 (5, 12), where numbers in the parentheses refer to Cys positions in the precursor peptide monomers] and LK-3 (7, 14) were found to display the highest cell penetrating abilities, followed by LK-3 (4, 11), and then LK-3 (1, 8) and LK-3 (8, 15). To assess if there is any correlation between the cell penetrating abilities and the hydrophobicities of dimers which are modulated by the position of disulfide bonds, we measured reverse-phase HPLC retention times of the dimers. The results show that LK-3 and LK-3 (7, 14) have respective retention times of 27.1 and 35.8 min, and that those of LK-3 (4, 11), LK-3 (1, 8), and LK-3 (8, 15) are 19.9, 18.8, and 21.7 min, respectively (Figure S4a). Thus, a rough correlation exists between the cell penetrating ability of a dimeric peptide and its hydrophobicity. The more hydrophobic peptides tend to penetrate cells more efficiently (Figure S4a).

Figure 3. Synthesis and cell penetration activities of antiparallel and parallel aligned dimeric peptides. (a) Schematic representation: (i) Cleavage from resin under acidic condition. (ii) Air oxidation. (iii) Iodine oxidation. (b) HPLC traces of LK-3 (black), parallel dimer (gray), and antiparallel dimer (dotted black). (c) Circular dichroism spectra of 20 μM of peptides in 10 mM sodium phosphate (pH 7.4). α-Helicities of LK-3 (black), parallel dimer (gray), and antiparallel dimer (dotted black) were calculated as 88%, 51%, and 87%, respectively. (d) Results of flow cytometry analysis of cell penetration into HeLa cells after 24 h incubations. LK-3 (black), parallel dimer (gray), and antiparallel dimer (dotted black). (Representative histograms are shown in Figure S7.)

Stimulated by these results, the dependence of cell penetrating ability on the nature of residues in hydrophobic patches was evaluated by using dimeric bundle peptides containing modified hydrophobic residues. For this purpose, Leu residues were replaced by Ile, Phe, Val, and cyclohexyl (Cha) moieties while keeping the same positions of the disulfides (Figure 2c,d). The cell penetrating ability of Cha containing dimer is lower than that of the Leu analogue, LK-3, while the Cha containing dimer is the most hydrophobic as judged by its C18 reverse-phase HPLC retention time (ChaK-3 > IK-3 > LK-3 > FK-3 > VK-3; Figure S4b). These results indicate that intrinsic hydrophobicities of the dimers, even in cases where they have identical locations of hydrophobic faces, do not govern cell penetration propensities.

Antiparallel and parallel alignments of the chains in dimer bundles, which differ in intramolecular hydrophobicity, could have an impact on cell penetration. To explore this issue,
parallel and antiparallel peptides retaining the sequences of LK-3 and its isomers were separately synthesized in high yields (Figure 3a and Figure S5) using Acm and Trt selectively protected Cys residues. The orientation of LK-3 made by the oxidation of LK-2 was determined as antiparallel alignment by comparing the HPLC retention times, CD spectra, and cell penetrating abilities of separately synthesized parallel LK-3 and antiparallel LK-3 (Figure 3b−d). Finally, flow cytometry analysis demonstrates that the antiparallel dimer penetrates into HeLa cells more efficiently than its parallel counterpart (Figure 3d, and representative fluorescent histogram plots are shown in Figure S6).

Molecular Dynamic Calculations and Investigation of the Origin of Cell Penetrability of Dimer Bundles. The question about the molecular origin of the enhanced cell penetrability of the antiparallel dimer was addressed by performing MD simulations on each of six different bisdisulfide bridged dimers (Figure 4a). Depending on the dimer type, the ensembles of conformations exhibit distinct spatial arrangement of the Leu (hydrophobic, magenta) and Lys (polar, cyan) patches. Especially significant is the observation that the antiparallel and parallel LK-3 display major differences in the orientations of the polar and hydrophobic patches. Specifically, the polar and hydrophobic patches are oriented asymmetrically on two different sides of the dimer structure in antiparallel LK-3, (Figure 4a) while in parallel LK-3, the polar residues are located at the center of the dimer structure and are surrounded by the hydrophobic residues. The spatial arrangement of Leu and Lys patches of LK-3 (7, 14) is even more asymmetric than that of LK-3. It is worth noting that the two dimers, LK-3 and LK-3 (7, 14), demonstrate the highest cell penetrabilities of all dimers tested in this study.

To express the amphipathicity of peptide dimers more quantitatively, we determined the extent of asymmetry in the spatial arrangement of the hydrophobic and polar patches by calculating the size of an average vector between all the Leu and Lys residues shown in eq 1 (see the details of calculation in experimental section).

\[
|\tilde{\text{d}}_{\text{LK}}^{\text{1}}| = \left( \frac{1}{N_{\text{Lys}} N_{\text{Leu}}} \sum_{i=1}^{N_{\text{Lys}}} \sum_{j=1}^{N_{\text{Leu}}} \text{d}_{ij}(t) \right)^{1/2}
\]

The value \(|\tilde{\text{d}}_{\text{LK}}^{\text{1}}|\) quantifies an average “dipole moment” between Leu and Lys residues, which characterizes the amphipathicity of a given ensemble of conformations. Thus, \(|\tilde{\text{d}}_{\text{LK}}^{\text{1}}|\) increases when Leu and Lys residues are more asymmetrically arranged over the structure. Analysis of the calculated \(|\tilde{\text{d}}_{\text{LK}}^{\text{1}}|\) values demonstrates that the amphipathicities of dimer peptides increase in the following order: parallel LK-3 \(\approx\) LK-3 (1, 8) < LK-3 (4, 11) \(\approx\) LK-3 (8, 15) < LK-3 < LK-3 (7, 14). Remarkably, the amphipathicities represented by \(|\tilde{\text{d}}_{\text{LK}}^{\text{1}}|\) values for the six dimers display a good correlation with their cell penetrabilities (CP50 values) (Figure 4b).

Oligomer Formation of Dimer Bundles. The foregoing correlation stimulated an investigation of the higher-order structure of the dimer peptides. It was noted earlier that
hydrophobic effects driven by exposed hydrophobic patches are a major driving force for protein aggregation and folding. The results of our previous studies suggested that isomers with better cell penetrating ability are also those that display a tendency to form nanosized particles. To see if the antiparallel dimers, which have higher cell penetrating abilities, also form nanosized particles, we used dynamic light scattering to determine their sizes (Figure 5a). The results show that only the antiparallel dimers form nanometer-sized particles (3.6 ± 1.2 nm), which corresponds to the molecular weight in the range 15 ± 9 kDa. The octamer formation was further confirmed by molar mass calculations using MALS analysis (Figure S8). In contrast, the parallel dimers only produce hundred-nanometer-sized (530 ± 50 nm) aggregates. We also observed that monomeric LK-1 and LK-2 peptides form larger aggregates with average sizes of 1.4 ± 0.5 and 1.2 ± 0.1 μm, respectively. Because the cell penetrating ability of the large aggregate forming peptides is significantly lower than that of the oligomeric peptides, the high cell penetration abilities of the antiparallel dimer bundles may well be related to oligomer formation.

To confirm the propensity for oligomer formation, we investigated the dimeric bundle peptides by using size exclusion chromatography (bio SEC-5, 5 μm, 150 Å, 7.8 × 300 mm column) of LK-3, and the isomers. LK-3 (1, 8) (black), LK-3 (4, 11) (blue), LK-3 (red), LK-3 (7, 14) (green), LK-3 (8, 15) (gray), and parallel LK-3 (dotted gray). The peak in the chromatogram of the antiparallel dimeric bundle peptide, LK-3, occurs in the oligomeric range. In contrast, the parallel dimer and monomeric peptide, LK-1, display indistinct peak patterns over wide ranges due to the dominance of hydrophobic interactions with the stationary phase. We also investigated the positional isomers having varying hydrophobic patches. Again, the antiparallel dimers LK-3 (4, 11), LK-3, and LK-3 (7, 14), which have similar cell penetrating abilities, display oligomer-sized peaks. These isomers also have higher amphipathicity propensities (Figure 4b). In contrast, oligomeric peaks were not detected in the chromatograms of LK-3 (1, 8) and LK-3 (8, 15), which have low cell penetrating abilities.

Mechanism Study of Cell Penetration of Dimer Bundles. The cell penetration mechanism(s) adopted by antiparallel and parallel LK-3 was (were) explored using the endocytosis inhibitors methyl-β-cyclodextrin (MβCD), N-ethyl-isopropyl amiloride (EIPA), and sodium chlorate (NaClO₃) (Figure 5c). Cholesterol depletion by MβCD is known to inhibit caveolin-mediated endocytosis, lipid raft, and pinocytosis. In addition, EIPA inhibits Na⁺/H⁺ exchange decreasing macropinocytosis. Sodium chlorate inhibits proteoglycan-dependent cell uptake. The results show that cell uptake of the antiparallel LK-3 is inhibited 43% by EIPA, 58% by MβCD, and 61% by NaClO₃. In contrast, cell uptake of the parallel LK-3 is inhibited 73% by EIPA and 53% by NaClO₃ and not at all by MβCD. Thus, both antiparallel and parallel LK-3 penetrate into cells via proteoglycan interactions. However, cell uptake of antiparallel LK-3 is affected by both macropinocytosis and cholesterol-dependent endocytosis. In contrast, cell penetration by parallel LK-3 is more greatly dependent on macropinocytosis, a process by which larger particles (0.2−5 μm) are translocated into cells. It is known that the rate of cell uptake is accelerated by forming nanoparticles. Indeed, antiparallel LK-3 is translocated into cells within 1 h, whereas the parallel isomer at the same concentration requires at least 24 h which involves the formation of large aggregates and slow internalization.

Molecular Model Conformation of Oligomer Formed by LK-3. To gain an insight into oligomer conformations, we assembled four units of antiparallel LK-3 dimer using molecular
molecular modeling (Figure 6) based on the DLS (Figure 5a) and SEC-MALS analysis (Figure S8). M-ZDOCK\textsuperscript{31} was used to assemble LK-3 into an octamer, and a structure that has the smallest solvent-accessible surface area (SASA) was selected as the candidate for the octamer. The octamer displays structures with D2 symmetry in which hydrophobic residues (gray) occupy the interior of the structure, surrounded by the charged residues (blue) (Figure 6, top right). The subsequent RMSD as a function of time. After a 10 ns increase, RMSD was maintained for ~1 μs simulation time, which indicates that the oligomer is quite stable. It is notable that the amphipathic trait of the antiparallel dimer leads to the formation of an oligomer and quite stable. It is notable that the amphipathic trait of the residues (blue) (Figure 6, top right). The subsequent occupancy of the interior of the structure, surrounded by the charged residues (blue) (Figure 6, top right). The subsequent peptide Dimer Formation. An air oxidation process was performed. Normally, 1 mM peptide Cys containing monomers were incubated for 48 h under 0.1 M ammonium bicarbonate. The reaction completion was monitored by HPLC trace and mass detection. For iodine oxidation, Acm-protected Cys containing peptide was diluted with 10% AcOH in water, and the concentrations were measured using a Direct Detect spectrometer (Millipore). Aminohexanoic acid (Ahx) was used as a linker between S-TAMRA and the N-terminus to reduce the fluorescence effect in cell penetration experiments. However, cell penetration of S-TAMRA-LK-3 was shown to be almost identical with that of S-TAMRA-Ahx-LK-3.

Peptide Purification. All peptides were purified with using a preparative RP-HPLC (Waters). A preparative C18 column (XBridge Prep C18 OBD, 5 μm, 19 × 150 mm) was used as the stationary phase. For the mobile phase, buffer A (water with 0.1% v/v TFA) and buffer B (acetonitrile with 0.1% v/v TFA) were used as a gradient.

Circular Dichroism (CD). CD spectra were measured as previously described. Briefly, using a Chirascan plus Circular Dichroism detector (Aplied Photophysics) with 0.05 cm path-length cell, CD spectra were scanned from 190 to 260 nm with 0.2 s integration, 1 nm step resolution, and 1 nm bandwidth at 20 °C. Three scans were performed and averaged. Peptide solutions were prepared in 10 M sodium phosphate (pH 7.4) at 20 μM concentration. α-Helicities were calculated using CDNN secondary structure analysis software (version 2.1, authored by Gerald Böhm at the Institute for Biotechnology, Martin Luther University, Halle-Wittenberg, Germany) by analyzing the averaged CD spectra ranging from 200 to 260 nm.

Dynamic Light Scattering (DLS). The hydrodynamic diameter of peptide nanoparticles was determined by DLS.

In summary, we investigated the cell penetrating abilities of dimeric bundle peptides. A systematic approach was employed to synthesize selected peptide derivatives. Among these peptides, LK-3, which forms an antiparallel helical bundle that exposes a leucine-rich, hydrophobic patch, is found to be the most efficient in nanosized oligomer formation. The proposal that hydrophobic interactions between dimeric bundle peptides promote formation of stable oligomers was supported by experimental results and MD simulations. Moreover, we have shown that nanosized oligomerization of dimer bundles is a key factor for the enhanced cell penetration. The investigation has also led to insight into the mechanisms operating in cell penetration of oligomers of amphipathic dimeric bundle peptides. These findings could pave the way for the use of nanosized oligomer assemblies as cell penetrating motifs, whose mechanism is quite different from that of the conventional “arginine-rich” cell penetrating peptides. Amphipathic monomeric stapled peptides were also shown to have good cell penetrability by others.\textsuperscript{20} However, the readily reducible disulfide bonds in reducing condition may provide an opportunity to regulate the conformational changes of peptides between the outside of cells and the reducing cytosol. In future studies, we plan to create peptides that lead to oligomers with improved stabilities and to determine the detailed structures of the nanosized oligomers.

\section*{EXPERIMENTAL SECTION}

No unexpected or unusually high safety hazards were encountered. In the following, we describe the experimental and computational procedures. A more detail description can be found in the Supporting Information.

Peptide Synthesis. All peptides were synthesized by using the 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS) method using rink amide MBHA resin (Novabiochem) on an SPS microwave peptide synthesizer (Discover, CEM) as previously described.\textsuperscript{32} The 5-carboxytertamethylrhodamine (5-TAMRA) fluorescence dye was conjugated to the N-terminal amino group using O-(benzotriazol-1-y1)-N,N,N′,N′-tetramethyl-uronium hexafluorophosphate (HBTU) or O-(1H-6-chlorobenzotriazole-1-y1)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) activation. HPLC chromatograms and MALDI-MS data are shown in Figure S1. All peptides were dissolved in distilled water, and the concentrations were measured using a Direct Detect spectrometer (Milipore). Aminohexanoic acid (Ahx) was used as a linker between S-TAMRA and the N-terminus to reduce the fluorescence effect in cell penetration experiments. However, cell penetration of 5-TAMRA-LK-3 was shown to be almost identical with that of 5-TAMRA-Ahx-LK-3.

Peptide Dimer Formation. An air oxidation process was performed. Normally, 1 mM peptide Cys containing monomers were incubated for 48 h under 0.1 M ammonium bicarbonate. The reaction completion was monitored by HPLC trace and mass detection. For iodine oxidation, Acm-protected Cys containing peptide was diluted with 10% AcOH in water solution. The solution was bubbled using nitrogen flow with gentle stirring during dropwise addition of 5 mM iodine in methanol until a brown color remained.\textsuperscript{33}
Peptide solutions of 100 μM in 150 mM sodium phosphate (pH 7.4) were incubated for 20 min at room temperature before measurements. Measurements were acquired with a Zetasizer Nano-Zs instrument (Malvern Instruments, Worcs-tershire, U.K.). Three scans of measurement were averaged (mean ± SD).

**Size Exclusion Chromatography (SEC).** HPLC traces were overlaid using a size exclusion column (Bio SEC-S, 5 μm, 150 Å, 7.8 × 300 mm column) as a stationary phase at HPLC (Agilent HPLC 1260 series instrument) system. Sodium phosphate buffer (150 mM, pH 7.4) was used as a mobile phase. The flow rate was 1.0 mL/min. Chromatography data shown in Figure Sb was obtained by injection of 5 nmol of peptides. A smaller amount of LK-3 up to 0.2 nmol was confirmed to show the same SEC traces. However, the lower concentration of peptides could not be detected because of the molar extinction coefficient.

**SEC Coupled with Multi Angle Light Scattering (SEC-MALS) Analysis.** LK-3 solution (10 mg/mL, 100 μL) was analyzed using a MALS system [Wytaye DAWN Helecos II (18 Angles) and Wyatt Optilab T-Rex (RI)] coupled with HPLC (Shimadzu). A size exclusion column (TSK-gel-G2000SWXL, 5 μm, 125 Å, 7.8 × 300 mm column) was used as a stationary phase. Sodium phosphate buffer (150 mM, pH 7.4) was used as a mobile phase at 0.5 mL/min rate. Astra 6.0 software was used for analysis of molar mass moments (g/mol) and polydispersity. See details in the Supporting Information.

**Cell Culture.** The human cervical cancer cell line HeLa (ATCC) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS. The experimental procedure used followed previously published methods. Briefly, cells were incubated with 5-TAMRA-labeled peptides for 2 h. The suspended cells detached by trypsin treatment were analyzed by using a flow cytometer (BD Accuri C6, BD Biosciences). Each histogram data was obtained by distribution of the fluorescence intensities of 10 000 cells.

**Flow Cytometry Analysis.** HeLa cells were seeded in 24-well plates at a density of 5 × 10⁴ cells well per well in DMEM containing 10% FBS. The experimental procedure used followed previously published methods. Briefly, cells were incubated with 5-TAMRA label, each sample in fresh complete growth medium for 24 h. The suspended cells detached by trypsin treatment were analyzed by using a flow cytometer (BD Accuri C6, BD Biosciences). Each histogram data was obtained by distribution of the fluorescence intensities of 10 000 cells. Cellular uptake of peptides was analyzed by BD Accuri C6 software and determined by percentage of fluorescence positive cells treated with the TAMRA-labeled peptides. For a mechanistic study, HeLa cells were preincubated in 24-well plates with various endocytosis inhibitors [10 mM NaN₃ and 6 mM 2-deoxy-D-glucose in DMEM without glucose to deplete ATP, 15 μg/mL 5-(N-ethyl-N-isopropyl)amiloride (EIPA) to inhibit macropercincotysis, 5 mM methyl-β-cyclodextrin (M/CD) to deplete cholesterol, and 80 mM sodium chloride (NaClO₃) to disrupt proteoglycan synthesis; all inhibitors are obtained from Sigma] for 30 min, and incubated further with 100 nM of 5-TAMRA-labeled peptides for 2 h.

**Molecular Modeling and MD Simulations. Preparation of the Systems.** The initial helix peptide structures were obtained from PEP-FOLD. The first model, representative of the most populated cluster, was used for making dimer conformation. On the basis of this model, two helical peptides were manually aligned and linked by the disulfide bonds. The dimer structures were constructed by CHARMM-GUL. To adjust the bis-disulfide dimer LK-3 in the explicit solvent environment, we carried out molecular dynamics (MD) simulation of the solvated dimer using GROMACS software (version 5.1.2) with charmm36 force field. Instead of charged termini (COO⁻ and NH⁺), we used modified peptide termini (acylated N-terminus and amidated C-terminus), based on the synthesized peptide sequences.

**Details of MD Simulations.** The initial box sizes of dimer and octamer systems were set to ~5 × 5 × 5 nm² and ~7 × 7 × 7 nm³, respectively, with 150 mM NaCl concentration. All the MD simulations were carried out on the basis of the following protocol. The total energy of the solvated system was minimized using the steepest descents algorithm with a tolerance value of 2000 kJ/(mol nm). The system was subjected to position-restrained MD simulations for 100 ps in NVT ensemble at constant volume and temperature at 300 K, followed by 100 ps of equilibration under NPT (P = 1 bar) ensemble. Production runs of each dimer and octamer systems were performed for 500 ns and ~1 μs, respectively, in the NPT ensemble at constant temperature and pressure achieved with a V-rescale thermostat and Parrinello–Rahman barostat. Both the short-range van der Waals and electrostatic interactions were truncated at the distance cutoff value of 12 Å, and the long-range electrostatic potential was calculated by Particle Mesh Ewald (PME) method.

**Calculation of Amphipaticity.** To calculate the extent of asymmetry in the spatial arrangement as shown in eq 1 of the main text, we averaged the size of vectors between all the Leu and Lys residues in the structure, where N₁ᵢₑᵤ = Nᵢₑᵤ = 12, dᵢₑᵤ(t) is the vector connecting the Cᵢ atom of the ith Leu and the side chain nitrogen of the ith Lys in a dimer structure generated at time t, and (⋯)ᵢ denotes the average over the ensemble of 600 dimer structures stored during the last 400 ns of the total 500 ns simulations.

**Building the Octamer Structure.** We have performed a protein–protein docking simulation using M-ZDOCK (http://zdock.umassmed.edu/m-zdock/) software to find the octamer structure of LK-2. The proper dimer structure after simulating the 500 ns MD was used for the docking as an input. The amphipatic trait of antiparallel dimer leads to an assembly of tetrameric oligomer in the following way. First, the antiparallel dimers are assembled to an oligomer structure such that the core of oligomer is mainly formed by the hydrophobic residues of the dimers, and its periphery is surrounded by hydrophilic residues. Second, a tetrameric structure is assembled from four dimers in such a way that structural motifs resembling a snap fastener is formed via tail-to-head binding between a lip (L7) and a groove (CS−C12) (Figure 6, middle). To be more specific, an L7 lip of dimer A (green) fits into the CS−C12 groove of dimer B (blue), A → B. Finally, a cyclic tetramer is formed via the tail-to-head binding of D → A (→ B → C → D). Then, the octamer conformation which represents the third scored cluster was selected among the resulting 10 clusters. The smallest solvent-accessible surface area (SASA) was observed in the structure. Finally, another ~1 μs MD simulation of octamer conformation was carried out to further refine the structure and to validate the stability of the octamer.
HPLC chromatograms and MS spectra of the purified peptides; MD calculation of LK-3; cell penetration of different length of dimers; plots of hydrophobicity and cell penetrability; synthesis and determination of antiparallel and parallel orientation of dimeric peptides; fluorescent histograms of flow cytometry analysis of cell penetration; and SEC and SEC-MALS of LK-3 (PDF)

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**Notes**
The authors declare no competing financial interest.

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