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Development of an indolicidin-derived peptide by reducing membrane perturbation to decrease cytotoxicity and maintain gene delivery ability

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ABSTRACT

Indolicidin (IL) is a cationic antimicrobial peptide and our previous study has demonstrated its potential as a cell penetrating peptide (CPP) to promote gene delivery. However, the cytotoxicity of IL arisen from its membrane perturbation capacity may restrict its clinical application. To promote gene delivery safety and efficiency, an almost mirror-symmetric IL derivative, SAP10 (RRWKFFPWRR-$CONH_2$), was designed in this study. All-atom molecular dynamics (MD) simulations were performed to understand the association between SAP10 and model lipid bilayers. By comparison with IL, SAP10 with high positively charged density resisted its deep insertion into lipid bilayers, which thus reduced its perturbation to lipid bilayers and improved biocompatibility. Consequently, we further mixed SAP10, polyethylenimine (PEI) and DNA to form the ternary nanocomplexes for gene delivery investigation. Both IL and SAP10 weakened the interaction between to DNA and PEI, which may be beneficial to promote the dissociation of internalized DNA from the carrier molecules. *In vitro* experiments demonstrated that the SAP10-associated ternary nanocomplexes highly promoted the transfection efficiency to various cells with low cytotoxicity. The effect of the SAP10 on promoting gene delivery was mainly contributed by the adsorbed peptides on the nanoparticles rather than the free ones. In particular, the dose of SAP10 could be increased to broaden the administration window, which ensured its safety on transfection. Therefore, our results suggested the argument that the designed SAP10 is a safe and an efficient peptide to promote PEI-mediated gene delivery.

1. Introduction

For gene delivery, an appropriate carrier is extremely important because anionic nucleic acid cannot directly penetrate cell membrane. To overcome the cell membrane barrier, cell-penetrating peptides (CPPs) is considered as potential carriers because of their membrane penetration capability [1,2]. Depending on their structures, there are several models to explain the translocation mechanism of CPPs. For example, amphipathic peptides, such as transportan, may insert into the membrane and adopt α-helical conformation to induce pore formation [3]. Cationic peptides, such as penetratin, have strong electrostatic interaction with phospholipids to induce their translocation to the hydrophobic cavity of lipid bilayer and form inverted micelles, which then is released in the reverse process [4]. Arginine-rich peptides, such as TAT, their guanidinium groups can interact with anionic phospholipids to form lipophilic ion pairs, which can diffuse along the membrane potential inside the cells [5]. Therefore, the transport of CPPs is mono-directional that the internalized contents can remain inside the cell [6,7]. Although the delivery mechanism of CPPs is still under debate, their translocation obviously relative to the perturbation of cell membrane, which probably elicits potential cytotoxicity [8–10].

Indolicidin (IL) is a well-known cationic antimicrobial peptide derived from bovine neutrophils. Because IL is rich in cationic arginine/lysine and hydrophobic tryptophan (ILPWKWPWWPWRR-$CONH_2$), it can interact to anionic cellular membrane and exhibit as an extended structure in lipid bilayer [11]. Indolicidin has been broadly applied as an alternative of antibiotics due to its perturbation which eventually causes membrane disruption [12]. Its Trp 8 & Trp 9 in the *C*-terminal domain have been proved to

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perturb head-groups and hydrophobic core of mammalian-mimic membrane significantly [13]. Such membrane penetration ability implies that IL is potential to carry cargo as CPPs [14]. Therefore, we have demonstrated that IL can be an adjuvant to facilitate polyethylenimine (PEI)-mediated gene delivery [15]. Although the transfection efficiency was highly improved, we also found that the cytotoxicity of IL limits its application. Similar toxicity of IL has also been reported to its treatment to erythrocytes and T lymphocytes [11,16]. The hemolytic activity is likely elicited by the aggregation of IL in aqueous environment [17]. As IL inserts to hydrophobic environment, such as the interior cavity of lipid bilayer, the aggregation is greatly promoted which thus destroys membrane structure. In addition, the membrane leakage induced by amphipathic peptides seems to correlate with their hydrophobic moments [18]. The cytotoxicity of IL also has been proved to be relative to its hydrophobic tryptophan residues [19]. These results all indicated that decreasing hydrophobicity of IL should be able to reduce it membrane perturbation and cytotoxicity.

In our previous study, we have designed an IL derivative, IL-K7F89 (ILPWKWKFFPWRR-$CONH_2$). Compared to the IL, IL-K7F89 is a potential IL analogue with not only low hemolytic activity but also high antibacterial capability [13]. These results reveal that the amino acids changed in the C-terminal domain of IL may significantly reduce its disruption of erythrocytes. Therefore, we developed a mirror-symmetric peptide, SAP10, which was derived from C-terminal of IL and replaced partial of tryptophan by phenylalanine. Both molecular dynamics (MD) simulation and small-angle X-ray scattering (SAXS) analysis were performed to investigate the interaction between SAP10 and lipid bilayer. For gene delivery application, IL or SAP10 peptides were mixed with DNA and PEI to form ternary particles, and their transfection efficiency as well as biocompatibility were compared to determine whether the modified SAP10 peptide can reduce cytotoxicity and maintain transfection capability.

2. Experimental section

2.1. Materials

The indolicidin (ILPWKWPWWPWRR-$CONH_2$), SAP10 (RRWKFFPWRR-$CONH_2$), and R9 (RRRRRRRRR-$CONH_2$) peptides were obtained from MDBio, Inc. (Taipei, Taiwan) with purity higher than 95%. Tetradecane, 2-methylbutane, ethidium bromide (EtBr), 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal), ortho-nitrophenyl-β-D-galactopyranoside (ONPG), 4′,6-diamidino-2-phenylindole (DAPI), alginate with molecular weight of 80–100 kDa, and polyethylenimine (PEI) with molecular weight of 750 kDa were purchased from Sigma-Aldrich (St. Louis, MO, USA). N,N-dimethyl formamide (DMF) was bought from Acros (Geel, Belgium). Dioleoylphosphatidylethanolamine (DOPE) dissolved in chloroform was obtained from Avanti (Alabaster, AL, USA); the DOPE stock solution was stored at −20 °C before the sample preparation.

2.2. Membrane perturbation by molecular dynamics (MD) simulation

All-atom MD simulations were applied to study the membrane perturbation of peptides. IL and SAP10 peptides were inputted into the MD simulations in the presence of zwitterionic 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) lipid bilayers which were used to mimic the membrane of erythrocytes [20]. The modeling systems contain a peptide, 98 lipids (49 lipids for each leaflet), 5834 waters, and the counterions Na^+ and Cl^- were added to neutralize the system and yielded a salt concentration of 0.15 M for each system. Because both IL and SAP10 exhibited random in phosphate buffered saline (PBS) environment, these two peptides with extended structure were put 5 Å above the surface of membrane bilayers [21]. All initial configurations of these systems were constructed using the VMD program and the CHARMM-GUI [22]. MD simulations were performed using the CHARMM36 all-atom force field [23] with the backbone phi/psi dihedral angle cross-term correction [24]. The water molecules were simulated using the TIP3 model [25]. MD simulations were run with an NPT ensemble at 310 K and 1 bar under three-dimensional periodic boundary conditions by parallel MD NAMD 2.7b3 software [26]. Long-range electrostatic interactions were determined by the particle-mesh Ewald technique. Cutoffs at 12.0 and 13.5 Å were applied to calculate the pair-wise interactions and generate the neighboring list of pairs, respectively. The non-bonded neighboring list was updated every 10 time steps. The force switching function was applied to smooth the non-bonded electrostatics and van der Waals potential energy whenever the internuclear distance of two atoms was between 9 and 12.0 Å [27]. An integration time step of 2 fs was used by constraining the hydrogen atom-involved covalent bond lengths via the SHAKE algorithm [28]. Before performing the production simulations, each system was minimized using a conjugate gradient algorithm to avoid bad contacts of the initial configuration. After 100,000 minimization steps, an energy tolerance of 0.0001 kcal/mol was reached for each modeling system. Then a 0.1-ns slow heating simulation was performed until the system temperature achieved 310 K. In addition, the trajectories were recorded every 5 ps. MD simulations were performed for each system for a 80 ns duration and the last 20 ns trajectory was used for statistical analysis.

The order parameters of peptide-membrane interaction were calculated to measure of the ordering of lipid molecules. It indicated possible structural deformations/perturbation of a lipid bilayer. The order parameter of the membrane's acyl chains was defined by the function $< S_{CC} >= \frac{1}{2}\langle 3\cos^2(\theta_i) - 1\rangle$, where θ_i was the angle between the directions of the carbon atom of the acyl SN1 chain at positions i and i–1 and the membrane normal. The symbol <> denoted the average over time.

2.3. Hemolysis analysis

Hemolytic activity of peptides was evaluated through measuring the release of hemoglobin from human red blood cells (RBCs). These RBCs were separated by centrifuging whole blood collected from a healthy donor at 3000 rpm for 10 min at 4 °C. Through removing the supernatant and washed by PBS, 1% (v/v) of RBCs in PBS was gently mixed with peptide in concentrations of 0–64 μM. After incubating at 37 °C for 1 h, the samples were centrifuged for 5 min at 1000 rpm, and their supernatants were analyzed spectrophotometrically at a wavelength of 540 nm (Synergy H1m, Biotek, Winooski, VT, USA). The absorbance results by mixing RBCs with PBS only and PBS with 1 wt% Triton-X100 were defined as 0 and 100% hemolysis, respectively.

2.4. Small-angle X-ray scattering (SAXS)

To prepare the SAXS sample, DOPE films were used to mimic the mammalian cells [29]. A specific amount of the DOPE stock solution with the DOPE dry weight of 8 mg was dispensed into a Teflon-capped glass vial. Chloroform was evaporated by a gentle flow of argon gas, followed by an overnight incubation in vacuum. The obtained dry film of DOPE was mixed with tetradecane in 2-methylbutane under vigorous vortex to relieve the packing frustration of the lipid hydrocarbon chains and thereby make the spontaneous curvature measurable [30]. 2-methylbutane was evaporated following the same procedures as for removing chloroform; the concentration of tetradecane in the dry film of