Synthetic KL4 peptide as new carrier of siRNA therapeutics for pulmonary delivery

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Summary

Small interfering RNA (siRNA) has great potential for the treatment of various respiratory diseases through RNA interference (RNAi), but their clinical application is hindered by the lack of a safe and effective pulmonary delivery system. KL4 peptide is a synthetic amphipathic peptide that was previously developed to mimic the function of pulmonary surfactant protein B (SP-B). Its potential as siRNA carrier for pulmonary delivery was examined in this study. The cationic KL4 peptide was able to bind with siRNA to form complexes at 15:1 ratio (peptide to siRNA weight ratio) or above. It also mediated efficient gene silencing on lung epithelial cells, with 20:1 ratio as the optimal ratio for siRNA transfection. Furthermore, the KL4/siRNA complexes were not toxic at concentrations used for transfection *in vitro*. The study shows that KL4 peptide appears to be a promising candidate for siRNA delivery. Further investigation on animal study and work on aerosol formulation are required to develop KL4 peptide as siRNA carrier for clinical application.

Introduction

Small interfering RNA (siRNA) holds great promise as therapeutics to treat many diseases including respiratory diseases, by inhibiting the expression of the disease-causing gene(s) through a post-transcriptional gene silencing mechanism, called RNA interference (RNAi) [1]. One of the major barriers of siRNA therapeutics development is the lack of a safe and effective delivery system suitable for clinical application [2]. siRNA is a negatively charged, hydrophilic macromolecule, it is incapable of crossing the biological membrane unassisted. Moreover, it is extremely susceptible to enzymatic degradation. Therefore, a carrier is often required to facilitate the cellular uptake of nucleic acids as well as to protect the nucleic acids from nuclease degradation.

For the treatment of respiratory diseases, the most direct way to deliver nucleic acid therapeutics is by pulmonary delivery, which is non-invasive and easily accepted by patients. It avoids the interaction with serum and the rapid nuclease degradation that occurs in the bloodstream. In addition, it can minimize systemic exposure and thereby reducing systemic adverse effects [3]. Cationic polymers and lipids are commonly used for transfection of nucleic acids. However, they are often associated with toxicity problem. A safe and efficient nucleic acid delivery system for pulmonary delivery remains highly sought after.

KL4 peptide is a 21-residue amphipathic peptide containing repeating KLLLL sequences. This synthetic peptide was designed to mimic the overall ratio of cationic to hydrophobic amino acids in native surfactant protein B (SP-B) [4, 5]. KL4 peptide exhibits SP-B-like surface activity. It is one of the active components in Surfaxin, a FDA approved intratracheal suspension of pulmonary surfactant indicated for the prevention of respiratory distress syndrome in premature infants. In this study, we investigated the potential of KL4 peptide as siRNA carrier for pulmonary delivery. Due to its cationic nature, it is anticipated that the KL4 peptide can form complexes with siRNA and promote cellular uptake. The siRNA binding affinity of KL4 peptide was assessed by gel retardation assay. Its transfection efficiency and cytotoxicity were also evaluated on human lung epithelial cells.

Experimental Methods

Materials and cell culture – KL4 peptide (KLLLLKLLLLKLLLLKLLLLK) was purchased from ChinaPeptides (Shanghai, China) as 90% purity grade and used as provided. siRNAs (SilencerSelect GAPDH positive control siRNA and SilencerSelect negative control siRNA) were purchased from Ambion (Austin, TX, USA). GelRed™ nucleic acid stain was purchased from Biotium (Hayward, CA, USA). A549 cells (human lung adenocarcinoma epithelial cells) were obtained from ATCC (Manassas, VA, USA). The cells were maintained at 5% CO_2, 37° C in DMEM supplemented in 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. The cells were sub-cultured twice weekly. All other reagents were obtained from Sigma Aldrich (Saint Louis, MO, USA) and of analytical grade or better.

Gel retardation assay – KL4/siRNA complexes were prepared at various ratios (from 2.5:1 to 30:1 of peptide to siRNA weight ratio) with 0.4 µg siRNA in 10 µl TAE buffer. The samples containing gel loading buffer were loaded into a 2% w/v agarose gel stained with GelRed™. Gel electrophoresis was run in TAE buffer at 100 V for 20 min and the gel was visualised under the UV illumination.

siRNA transfection study – A549 cells were transfected with KL4/siRNA complexes containing 100 nM of GAPDH siRNA or negative control siRNA per well in 24-well plates. The complexes were prepared in Opti-MEM I reduced serum medium. After 4 h of incubation at 37 °C, the cells were washed with PBS and fresh DMEM supplemented with 10% FBS were added to the cells. After 72 h, the GAPDH expression was detected by Western blot. The cells were washed and lysed. The cell extracts containing 20 µg of protein were loaded into a 10% SDS-polyacrylamide gel and electrophoresis was run at 120 V for 90 min.

After the proteins were resolved, they were transferred into a nitrocellulose membrane which was blocked in 5% non-fat dry milk for 1.5 h with shaking. The membrane was washed and incubated with primary antibody overnight at 4 °C on shaking. After rinsing, the membrane was incubated with horseradish peroxide conjugated secondary antibody for 2 h at room temperature. The bound secondary antibodies were detected with ECL™ Western blotting detection reagents. Densitometry was performed to analyse the protein expression on the Western blot. The experiments were conducted in three independent experiments.

Cytotoxicity study – The cytotoxicity of the KL4 peptide was assessed by MTT (3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. A549 cells were seeded in 24-well plates and the KL4/siRNA complexes were prepared as described above. After 4 h of incubation at 37 °C, the cells washed with PBS and fresh DMEM supplemented with 10% FBS were added to the cells. MTT assay was carried out at 4 h and 24 h post-transfection. MTT solution (0.8 mg/ml in PBS) was added into each well. The samples were incubated for 4 h at 37 °C. The MTT solution was removed and isopropanol was added to dissolve the insoluble formazan crystals for at least 15 min. The samples were examined by measuring the absorbance at 595 nm using UV/Vis spectrophotometer. The cell viability was calculated as percentage of the absorbance from cells treated with KL4/siRNA complexes against that obtained from cells treated with OptiMEM-reduced serum medium only. The experiments were conducted in three independent experiments.

Results

KL4 peptide was evaluated for its binding affinity to siRNA by gel retardation assay. The disappearance of siRNA band in the gel image indicated that binding occurred between siRNA and KL4 peptide (Figure 1). The gel image shows that as the peptide to siRNA ratio increased, the siRNA band became fainter. siRNA was only partially bound to KL4 peptide at 5:1 and 10:1 ratios (peptide to siRNA weight ratio). At ratios 15:1 and above, the siRNA was completely bound to KL4 peptide as the siRNA band disappeared.

Figure 1 - Gel retardation assay of siRNA binding. KL4/siRNA complexes were prepared at various was ratios (2.5:1 to 30:1 peptide to siRNA weight ratio). Sample containing siRNA only was used as control.

To investigate the siRNA delivery efficiency of KL4 peptide on lung epithelial cells, A549 cells were transfected with KL4/siRNA complexes prepared at different ratios, using siRNA targeting glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and negative control. The cellular expression of GAPDH after transfection was evaluated by Western Blot and analysed by densitometry (Figure 2). At ratio 5:1 and 10:1, only moderate GAPDH knockdown of around 20% was observed. The most efficient gene silencing effect was observed at 20:1 ratio, with over 40% of GAPDH knockdown was detected. The result was comparable to Lipofectamine™2000, a lipid-based commercial transfection agent. As the ratios further increased, the gene silencing effect gradually subsided.

The cytotoxicity of KL4 peptide was examined by MTT assay on A549 cells at 4 h and 24 h post-transfection, and the cell viability was calculated (Figure 3). There was no sign of cytotoxicity with KL4/siRNA complexes prepared at ratio up to 30:1. The cell viability was maintained over 90% for all the samples tested. Since the transfection efficiency of the complexes reduced at ratio above 20:1, the cytotoxicity of complexes formed at ratio above 30:1 was not investigated in this study.

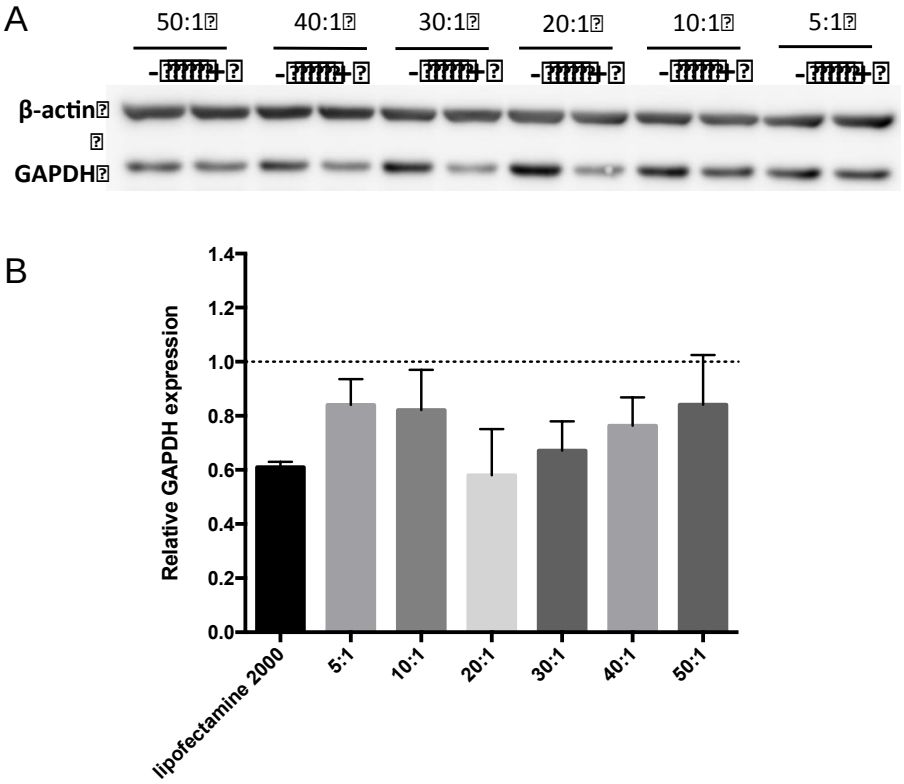

Figure 2 - GAPDH siRNA transfection on A549 cells analysed by Western blotting. (A) Cells were transfected with KL4 /siRNA complexes prepared at various ratios (5:1 to 50:1 peptide to siRNA weight ratio) using GAPDH siRNA (+) or negative control siRNA (-). Protein analysis was carried out at 72 h post-transfection. β-actin served as internal control for equal total protein loading. (B) The bands were analysed by densitometry with the density of GAPDH band normalised to that of the β-actin band of the corresponding sample. Lipofectamine™2000 (a lipid-based commercial transfection agent) was used as a control for comparison.

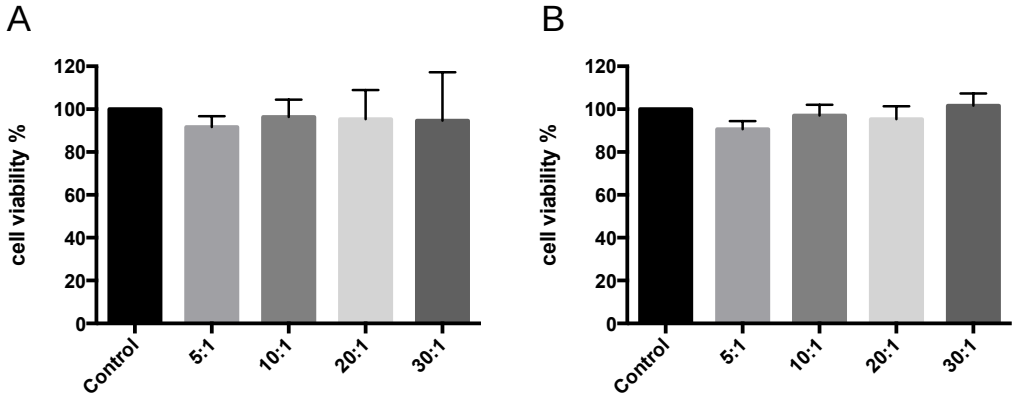

Figure 3 - Cytotoxicity of KL4 peptide was determined by MTT assay. A549 cells were transfected with KL4/siRNA complexes prepared at various ratios (5:1 to 30:1 peptide to siRNA weight ratio). The cell viability was evaluated at (A) 4 h and (B) 24 h post-transfection.

Discussion

In this study, synthetic KL4 peptide was investigated as siRNA carrier for pulmonary delivery. KL4 peptide was originally designed to mimic SP-B to reduce alveolar surface tension. It is currently used clinically as one of the active components in pulmonary surfactant for intratracheal administration. Due to its cationic amphipathic nature, it is hypothesized that the KL4 peptide can bind with siRNA to form complexes through electrostatic interaction and promote cellular uptake of siRNA. First, the siRNA binding affinity of KL4 peptide was evaluated. It was found that complete binding with siRNA was achieved at 15:1 peptide to siRNA ratio.

The siRNA transfection efficiency was assessed on A549 cells, which are human lung epithelial cell line. The gene silencing effect was relatively low at ratios 10:1 or below. This was expected as the gel retardation assay revealed that binding with siRNA was not complete at these ratios. At high peptide to siRNA ratio (above 30:1), the gene silencing effect was also poor. This was probably due to the strong binding between KL4 peptide and siRNA at high ratios, leading to incomplete release of siRNA following cellular uptake. As a result, free siRNA was not readily available in the cytoplasm to initiate RNAi. The optimal ratio for siRNA transfection was found to be 20:1.

To develop a delivery system for clinical application, the delivery agent must be non-toxic. KL4 peptide is clinically approved to be used for pulmonary administration. However, its current application is to lower the alveolar surface tension at the cell surface. It is not expected to be absorbed into the body. Hence, it is important to examine the cytotoxicity of the complexes formed between KL4 and siRNA. The MTT assay showed that KL4 peptide was not toxic after it bound and formed complexes with siRNA. Complexes with ratio above 30:1 was not tested as their transfection efficiency was not satisfactory.

Conclusions

Overall, we demonstrated that KL4 peptide could mediate gene silencing effect of siRNA in lung epithelial cells. The peptide was not cytotoxic at concentrations used for transfection. Our result shows that KL4 peptide appears to be promising candidate as new siRNA carrier for pulmonary delivery. Further studies will be carried out to examine the siRNA transfection efficiency and safety profile of KL4 peptide in animal model following pulmonary administration. In addition, the formulation of KL4 peptide/ siRNA delivery system as powder aerosol will be investigated.

References

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