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Stearylated Antimicrobial Peptide Melittin and Its Retro Isomer for Efficient Gene Transfection

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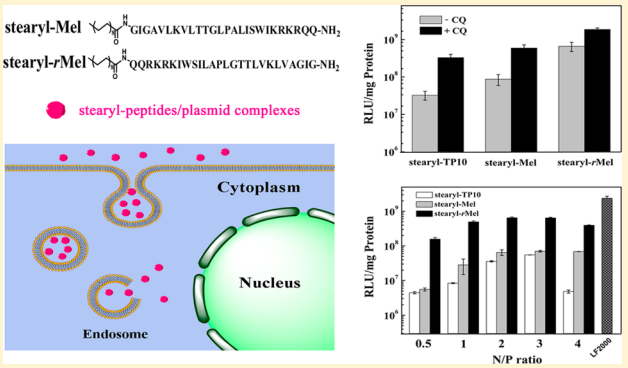
ABSTRACT: A crucial bottleneck in nonviral vector-mediated gene delivery is poor endosomal escape. Here, we constructed novel gene vectors by coupling the stearyl moiety to the N-terminus of the antimicrobial peptide melittin (stearyl-Mel) and its retro isomer (stearyl-*r*Mel) due to their high membrane-lytic activity. As expected, stearyl-Mel showed obvious increases in endosome-lytic activity and transfection efficiency compared with the reported stearyl-TP10. More gratifyingly, the transfection efficiency of stearyl-*r*Mel was around 10-fold greater than that of stearyl-Mel and almost reached the transfection levels of Lipofectamine 2000 due to the enhanced endosome-lytic activity. Furthermore, the stearyl-*r*Mel/p53 plasmid complex exhibited higher p53 expression and antitumor activity than stearyl-Mel, confirming the fact that stearyl-*r*Mel displayed higher transfection efficiency. Taken together, the combination of the stearyl moiety with retro melittin provides a novel framework for the development of excellent nonviral gene vectors.



INTRODUCTION

Gene therapy has gained significant attention over the past two decades as a potential method for the treatment of some severe diseases such as cancer, genetic disorders, and viral infections. However, naked therapeutic DNA is unable to efficiently translocate across the cellular lipid bilayer due to its large size, strong negative charge, and hydrophilicity. Thus, successful gene therapy relies heavily on vectors that can deliver therapeutic DNA into target cells. Initial research concentrated on the development of viral vectors due to their high efficiency in DNA delivery. However, fundamental drawbacks, such as toxicity, immunogenicity, and size limitations of the gene, have paved the way for other potential gene delivery vectors.[1,2]

Nonviral vectors, such as peptide-, polymer-, dendrimer- and lipid-based systems, serve as an alternative to viral vectors because of their advantage in inducing relatively low toxicity and nearly no immune responses.[2,3] One such group of nonviral vectors that is increasingly utilized for gene delivery is the cell-penetrating peptides (CPPs).[4−6] However, the relative efficiency of unmodified CPPs in promoting DNA delivery has generally been poor.[5,6] Recently, stearylation has proven to be a successful methodology to increase the transfection efficiency of CPPs.[7−15] The increased transfection efficiency is attributed not only to the enhanced compaction of DNA but also to the increased endosomal escape conferred by the stearyl moiety.[5,6] However, chloroquine (CQ), a lysosomotropic agent, was still able to significantly enhance the transfection efficiency of stearylated CPPs, indicating that a great amount of plasmids was still entrapped in endosomes.[5,6] Therefore, poor endosomal escape may be a critical bottleneck for stearylated CPPs to efficiently deliver DNA into the cytoplasm.

An effective strategy to facilitate endosomal escape of nonviral vectors is the coupling of membrane-destabilizing peptides.[16−18] Melittin (Mel), a classical cationic antimicrobial peptide with strong membrane-lytic activity, has been used as an efficient endosome-lytic agent to enhance gene transfer.[19−21] Consequently, Mel has been covalently attached to cationic lipids, PEI, and polyacridine to increase their gene transfection efficiency.[21−26] Therefore, in this study, we constructed a novel type of nonviral gene vector by introducing the stearyl moiety to the N-terminus of Mel (stearyl-Mel). Simultaneously, stearylation of retro Mel (stearyl-*r*Mel) with the reverse sequence was also constructed because *r*Mel was reported to display similar membrane-lytic activity and decreased toxicity.[27] Peptide sequences are shown in Table 1. We anticipate that these antimicrobial peptide-based nonviral gene vectors will display stronger transfection efficiency than general CPPs.

MATERIALS AND METHODS

Synthesis of Peptides. All peptides were synthesized on a MBHA resin using the standard Fmoc-chemistry-based strategy. Stearic acid was coupled as Fmoc amino acids. The

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Table 1. Sequences of Melittin and Its Analogues

peptide	sequence
Mel	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2
stearyl-Mel	C_{18}-GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2
*r*Mel	QQRKRKIWSILAPLGTTLVKLVAGIG-NH_2
stearyl-*r*Mel	C_{18}-QQRKRKIWSILAPLGTTLVKLVAGIG-NH_2

final cleavage was performed using standard protocol (95% trifluoroacetic acid/2.5% triisopropylsilane/2.5% water) for 3 h at room temperature. All crude peptides were purified and analyzed by reversed phase high performance liquid chromatography (RP-HPLC) on a C18 column and then characterized by electrospray ionization mass spectrometry (ESI-MS).

Cell Culture and Amplification of Plasmid DNA. The CHO cell line was grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% FBS (HyClone). Cos-7 and U251 cell lines were grown in DMEM (Gibco BRL) supplemented with 10% FBS (HyClone). All cell lines were cultured in a 5% CO_2 humidified atmosphere at 37 °C.

Plasmids were transformed in *Escherichia coli* DH5α and were amplified in LB medium at 37 °C overnight at 180 rpm. The plasmids were purified by an EndoFree Plasmid kit (TIANGEN). Then the purified plasmids were dissolved in distilled water and stored at −20 °C.

Complexes Formation. Plasmids (0.5 μg) were mixed with peptides at various N/P ratios (peptides amino groups: DNA phosphate) in 50 μL of medium (1/10 of the final treatment volume). Complexes were formed for 30 min at 37 °C and then were diluted to a final volume of 500 μL. All complexes were used immediately after their preparation.

Condensation of Complexes. DNA condensation was evaluated using the ethidium bromide (EtBr) exclusion assay. Briefly, peptide/pGL3 plasmid complexes were formed as described above. After 1 h of incubation, 135 μL milli-Q water was added to each sample and transferred into a black 96-well plate. Thereafter, 15 μL of EtBr solution was added to give a final EtBr concentration of 400 nmol/L. After 10 min, the 96-well plate was shaken orbitally for 30 s and the fluorescence intensity measured. Results were given as relative fluorescence and a value of 100% is attributed to the fluorescence of naked DNA with EtBr.

Particle Size and ζ-Potential Measurements. The peptide/pGL3 plasmid complexes were prepared at N/P ratios ranging from 1 to 4, as described above. The complexes were diluted in phosphate buffered saline (PBS) to 1 mL volume, and then, the size and ζ-potential were measured by Nano-ZS ZEN3600 (Malvern Instruments), respectively at 25 °C.

Transmission Electron Microscopy (TEM). The morphologies of peptide/pDNA complexes at an N/P ratio of 2 were observed by TEM. The peptide/pGL3 plasmid complexes were prepared as described above. The TEM samples were prepared by dropping the peptide/pGL3 plasmid complex solution onto a copper grid and then staining by 0.2% (W/V) phosphotungstic acid solution before measurement.

In Vitro Transfection Assays. Cos-7 cells were seeded at 1×10^5 cells/well in a 24-well plate 24 h before treatment. Cells were treated with peptide/pGL3 plasmid complexes in serum-free or 10% serum containing DMEM at different N/P ratios for 4 h. Thereafter, the medium was replaced with 1 mL of 10% serum containing DMEM. After 24 h of incubation, cells were washed and lysed using 100 μL/well of Reporter Lysis Buffer for 15 min at room temperature. Luciferase activity was measured using a luciferase detection kit (Promega, Nacka, Sweden) and normalized to protein content. Protein concentrations were determined by using a BCA protein assay kit (Pierce). Lipofectamine 2000 (LF2000, Invitrogen) and stearyl-TP10 were taken as a positive control for measuring transfection efficiency.

In experiments with endocytosis inhibitors, Cos-7 cells were preincubated with an endocytosis inhibitor (chlorpromazine, (CPZ 10 μg/mL), amiloride (50 μM), or methyl-β-cyclodextrin (MβCD, 5 mM)) for 30 min and then incubated with peptide/pGL3 plasmid complexes at an N/P ratio of 2 for 4 h. In experiments with chloroquine, Cos-7 cells were incubated with the complexes at an N/P ratio of 2 and chloroquine (final concentration 100 μmol/L) for 4 h. Thereafter, the medium was replaced with fresh medium containing 10% FBS. The measuring method of the transfection efficiency was performed as described above.

EGFP Expression Assay. For Enhanced Green fluorescent protein (EGFP) gene expression analysis, CHO cells were cultured in a glass-bottom culture dish 24 h before treatment. Cells were treated with peptide/EGFP plasmid complexes in serum-free DMEM at an N/P ratio of 2 for 4 h. Thereafter, the medium was replaced with 1 mL of DMEM containing 10% FBS. After 24 h of incubation, images were taken with an inverted Zeiss LSM 710 confocal microscope. Differential interference contrast (DIC) images were taken along with both fluorescence channels.

Cytotoxicity Assays. The cytotoxicity of peptide/pGL3 plasmid complexes was determined by the MTT assay. Cos-7 cells were seeded at 1×10^4 cells/well in a 96-well plate 24 h before treatment. After being washed, 100 μL of serum-free medium that contained peptide/pGL3 plasmid complexes at different N/P ratios was added to the wells and incubated for 4 h. Thereafter, 100 μL of 10% serum containing medium was added, and the cytotoxicity of the relevant agents was determined by the MTT assay after 20 h.

Cellular Uptake Assays. CHO cells were cultured in a glass-bottomed culture dish 24 h before treatment. The cells were incubated with peptide/FITC-labeled pGL3 plasmid complexes at an N/P ratio of 2 for 4 h. Labeling of the pGL3 plasmid with the fluorescent probe FITC was performed using a Label IT Tracker kit (Mirus), as described by the manufacturer. Thereafter, the cells were washed three times with PBS. Images were taken with an inverted Zeiss LSM 710 confocal microscope.

To quantify the cellular uptake of peptide/pGL3 plasmid complexes, CHO cells were cultured in a 24-well plate 24 h before treatment. The cells were incubated with peptide/FITC-labeled pGL3 plasmid complexes at an N/P ratio of 2 for 4 h.

To study the cellular uptake mechanism of peptide/pGL3 plasmid complexes, CHO cells were preincubated with an endocytosis inhibitor (chlorpromazine (CPZ, 10 μg/mL), amiloride (50 μM), or methyl-β-cyclodextrin (MβCD, 5 mM)) for 30 min and then incubated with peptide/FITC-labeled pGL3 plasmid complexes at an N/P ratio of 2 for 4 h. Thereafter, the cells were washed twice with PBS and then incubated with 0.02% trypsin for 10 min. The cells were harvested and centrifuged at 1500 rpm for 5 min. The cell pellet was suspended and washed twice with PBS and finally resuspended in PBS for fluorescence analysis with a BD FACS Caliber Flow Cytometer using 488 nm laser excitation.

LDH (Lactate Dehydrogenase) Leakage Assay. The LDH leakage assay was measured using Cytotoxicity Detection

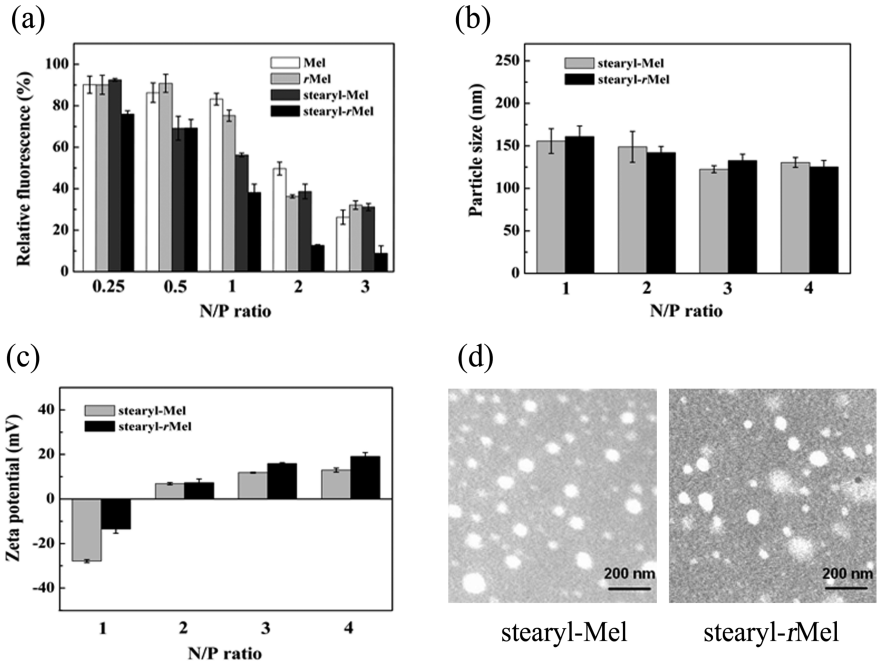

Figure 1. Characterization of the peptide/plasmid complexes. (a) Ability of peptides to form complexes with pGL3 plasmids using the ethidium bromide exclusion assay. (b) Particle size of the complexes. (c) ζ-potential of the complexes. (d) TEM micrographs of the complexes at an N/P ratio of 2.

Kit PLUS (Roche). Cos-7 cells were seeded at 1×10^4 cells/well in 96-well plate 24 h before treatment. After incubation with 100 μL of serum-free medium containing various concentrations of peptides for 1 h, 40 μL of medium was transferred to a 96-well plate and incubated for 15 min with 40 μL of reaction mixture, followed by 20 μL of stop solution. Fluorescence was measured at 490 nm. Untreated cells were defined as no leakage, and 100% leakage was defined as total LDH release by lysing cells in 0.2% Triton X-100.

Calcein Release Assay in Cells. CHO cells were cultured in a glass-bottomed culture dish 24 h before treatment. Then, the medium was removed and followed by treatment with 1 mL of phenol red free medium containing 0.3 mg/mL calcein with or without peptides for 30 min. Thereafter, cells were washed three times with PBS and then incubated with phenol red free medium for 1.5 h. Images were taken with an inverted Zeiss LSM 710 confocal microscope. To quantify the release of calcein from the endosome, CHO cells were treated with calcein and peptides as described in the above method, and then fluorescence was analyzed with a BD FACS Caliber Flow Cytometer using 488 nm laser excitation.

Antiproliferative Assay. The antiproliferative effects of peptide/p53 plasmid complexes were also determined by the MTT assay. U251 cells were seeded in 96-well plates at a density of 1×10^4 cells per well. After being washed, cells were treated with peptides alone, p53 plasmid alone, and peptide/p53 plasmid complexes in 100 μL of DMEM at different N/P ratios for 4 h. Thereafter, 100 μL of DMEM was added, and the cytotoxicity of the relevant agents was determined by the MTT assay after 96 h.

Expression of p53 Protein Assay. To determine the expression of p53, Western blot analysis was performed. Briefly, U251 cells were treated with peptide/p53 plasmid complexes as described above. After 48 h, cells were harvested and lysed, and protein concentrations were determined by using a BCA protein assay kit (Pierce). The total amount of 40 μg of protein from each sample was loaded and separated on a 10% SDS–PAGE gel. After electrophoresis, the samples were transferred onto a PVDF membrane. The membranes were probed with the primary antibody specific for p53 followed by incubation with the HRP-conjugated secondary antibody. The signal was detected by an enhanced chemoluminescence detection system.

RESULTS

Characterization of Peptide/Plasmid Complexes. To evaluate the ability of peptides to condense plasmid DNA, the EtBr exclusion assay was employed. As shown in Figure 1a, a clear increase in quenching was observed with stearyl-Mel and stearyl-*r*Mel compared with the unmodified peptides, indicating that N-terminal stearylation could significantly enhance the condensable ability of peptides. It is noteworthy that stearyl-*r*Mel had a more pronounced condensing effect than other peptides.

In order to get a view of the physicochemical properties of stearyl-peptides/plasmid complexes, we measured the particle sizes and ζ-potential of complexes. As shown in Figure 1b, overall particle sizes remained in the range of 120–160 nm, and the size of complex formed by stearyl-*r*Mel with the plasmid was similar to that of stearyl-Mel. Figure 1c shows that the ζ-potential of complexes increases with increasing N/P ratios. The ζ-potential of complexes formed by stearyl-*r*Mel was higher than that of stearyl-Mel.

In addition, TEM images of the complexes formed by stearyl-*r*Mel and stearyl-Mel with plasmids at an N/P ratio of 2 are shown in Figure 1d. The micrographs of complexes showed that most of the complexes displayed relatively smooth and spherical shapes with the size less than 100 nm, which was smaller than the particle size measured by the Nano-ZS ZEN3600 apparatus because the former one was observed in dry condition, and the latter one was measured in damp condition.[14] The morphology of complexes indicates that both

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stearyl-*r*Mel and stearyl-Mel could condense DNA to regular nanoparticles.

In Vitro Transfection of Peptide/Plasmid Complexes. To evaluate the transfection efficiency of different peptides, Cos-7 cells were transfected with complexes formed by different peptides with pGL3 plasmids encoding the luciferase reporter gene. As shown in Figure 2, the transfection efficiency

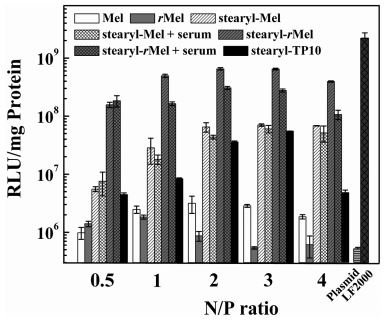

Figure 2. Transfection efficiency of peptides in Cos-7 cells.

of unmodified Mel and *r*Mel showed a slightly higher transfection efficiency than that of pGL3 plasmid alone. However, stearyl-Mel and stearyl-*r*Mel showed significantly higher transfection efficiency than unmodified peptides at various N/P ratios. Intriguingly, the transfection efficiency of stearyl-*r*Mel was around 10-fold greater than stearyl-Mel and almost reached the transfection level of LF2000.

Serum is an important factor that always hinders in vivo application of nonviral vectors. To obtain information on the possible application in vivo, transfection efficiency of stearyl-Mel and stearyl-*r*Mel in the presence of 10% serum was assessed. As shown in Figure 2, the presence of serum only slightly inhibited the transfection efficiency of stearyl-Mel and stearyl-*r*Mel. Even though the serum had more effect on the transfection efficiency of stearyl-*r*Mel compared with that of stearyl-Mel, the luciferase expression level of stearyl-*r*Mel was still obviously higher than that of stearyl-Mel.

In order to address the ability of stearylated peptides to transfect entire cell populations, CHO cells were transfected with EGFP expressing plasmid and analyzed by confocal microscopy. As shown in Figure 3, both of the complexes formed by stearyl-Mel and stearyl-*r*Mel with EGFP expressing plasmids at a ratio of 2 could enable the transfection of almost 100% of the cell population.

Cytotoxicity of Peptide/Plasmid Complexes. The cytotoxicity of peptide/plasmid complexes in the present study was determined by the MTT assay, and LF2000 was

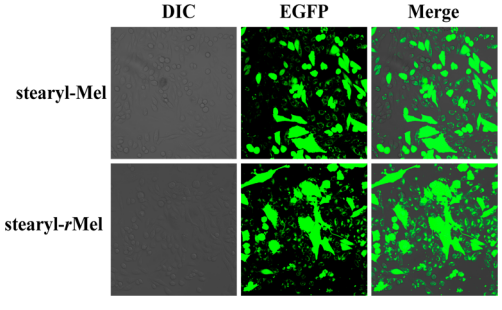

Figure 3. EGFP expression of stearyl-peptides/EGFP plasmid complexes at an N/P ratio of 2 in CHO cells.

used as the control. As shown in Figure 4, *r*Mel displayed obviously lower cytotoxicity than Mel. The cytotoxicity of

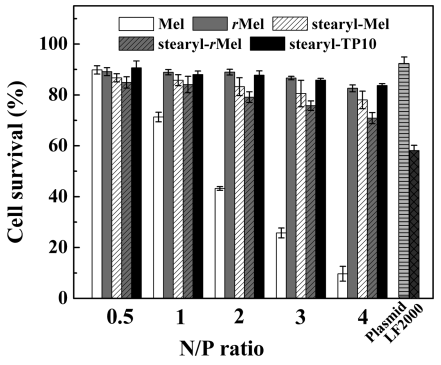

Figure 4. Cytotoxicity of peptide/pGL3 plasmid complexes at different N/P ratios. The cytotoxicity was determined by MTT assay after 24 h.

stearyl-Mel was significantly lower than that of Mel. In contrast, the stearyl moiety enhanced the cytotoxicity of stearyl-*r*Mel compared with that of *r*Mel, and the cytotoxicity of stearyl-*r*Mel was relatively higher than that of stearyl-Mel. Although stearyl-Mel and stearyl-*r*Mel showed slightly higher cytotoxicity than stearyl-TP10, their cytotoxicity was significantly lower than that of LF2000 after 24 h of treatment.

Cellular Uptake of Peptide/Plasmid Complexes. The uptake of the complexes formed by stearyl-Mel and stearyl-*r*Mel with FITC-labeled plasmids was assessed by confocal microscopy and FACS. Figure 5a shows that stearyl-Mel and stearyl-*r*Mel could deliver FITC-labled plasmids into nearly 100% of cells. In addition, the cellular uptake of the stearyl-*r*Mel/plasmid complex was almost equivalent to that of the stearyl-Mel/plasmid complex (Figure 5b).

Celluar Uptake Pathways of Peptide/Plasmid Complexes. To have a more precise insight into the uptake pathways of the complexes formed by stearyl-Mel and stearyl-*r*Mel with plasmids, we studied the specific endocytosis inhibitors (chlorpromazine (CPZ) for clathrin-mediated endocytosis, methyl-β-cyclodextrin (MβCD) for caveolin-mediated endocytosis, and amiloride for macropinocytosis) on the cellular uptake of complexes by FACS. As shown in Figure 6a, FACS analysis revealed that CPZ and MβCD decreased the cellular uptake of the stearyl-Mel/complex by 26 and 38%, respectively. The uptake efficiency of the stearyl-*r*Mel/complex in the presence of CPZ and MβCD was significantly lower than that of the stearyl-Mel/complex, decreasing by 56 and 72%, respectively. In contrast, amiloride had no effect on the uptake of the stearyl-Mel/complex and stearyl-*r*Mel/complex. This result suggested that these complexes internalized into cells by clathrin and caveolin rather than macropinocytosis. Furthermore, we also evaluated the effect of endocytosis inhibitors on the transfection efficiency of stearyl-Mel and stearyl-*r*Mel. As shown in Figure 6b, CPZ and MβCD significantly decreased the transfection efficiency of stearyl-Mel and stearyl-*r*Mel, whereas amiloride had no remarkable inhibitory effect on their transfection efficiency. This result agreed with the FACS analysis, confirming that the cellular uptake of the stearyl-Mel/plasmid complex and stearyl-*r*Mel/plasmid complex was mainly mediated by clathrin and caveolin.

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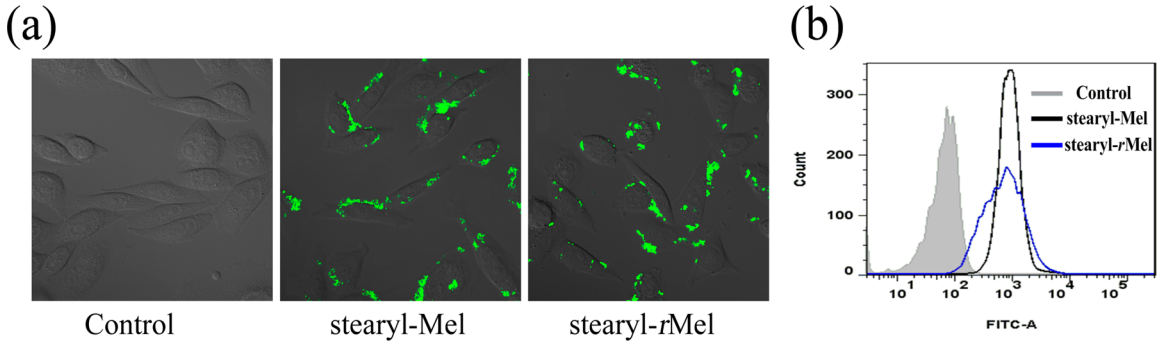

Figure 5. Uptake of stearyl-peptide/plasmid complexes. (a) Confocal images of CHO cells treated with the complexes formed by stearyl-Mel and stearyl-*r*Mel with FITC-labeled plasmids at an N/P ratio of 2 for 4 h. (b) Flow cytometry histogram of CHO cells treated with the complexes formed by stearyl-Mel and stearyl-*r*Mel with FITC-labeled plasmids at an N/P ratio of 2 for 4 h.

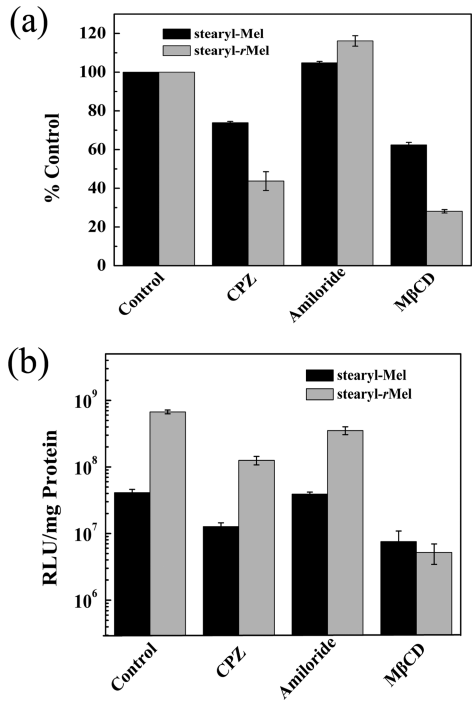

Figure 6. Uptake pathways of stearyl-peptide/plasmid complexes. (a) Cellular uptake of stearyl-peptides evaluated by FACS in the presence of specific endocytosis inhibitors. (b) Luciferase expression of stearyl-peptide/plasmid complexes at an N/P ratio of 2 in the presence of specific endocytosis inhibitors.

Endosome Lytic Activity of Stearyl-Mel and Stearyl-*r*Mel. Chloroquine (CQ) is an agent known to facilitate destabilization of endosomes and subsequent release of entrapped biomolecules. In order to estimate the degree of endosomal release conferred by stearyl-TP10, stearyl-Mel, and stearyl-*r*Mel, we investigated their transfection efficiency in the presence of chloroquine. As shown in Figure 7a, the transfection efficiency of stearyl-TP10, stearyl-Mel, and stearyl-*r*Mel in the presence of chloroquine were about 10-, 7-, and 3-fold higher than that in the absence of chloroquine, respectively. This result demonstrates that the stearyl-*r*Mel/plamsmid complex escaped from the endosome to a greater extent than both the stearyl-TP10/plamsmid complex and stearyl-Mel/plamsmid complex.

Lactate dehydrogenase (LDH) is an intracellular enzyme and is only released from the cells with damaged membranes. Therefore, we used the LDH leakage assay to evaluate the membrane lytic activity of stearyl-Mel and stearyl-*r*Mel in this study. As shown in Figure 7b, stearyl-*r*Mel significantly induced more LDH release than stearyl-Mel, indicating that stearyl-*r*Mel displayed stronger membrane lytic activity. This result suggested that stearyl-*r*Mel would exhibit stronger endosome-lytic activity than stearyl-Mel.

Calcein, a membrane-impermeable fluorophore, is readily taken up by cells via constitutive endocytosis and permanently sequestered into vesicles. Furthermore, calcein release from vesicles leads to a remarkable increase in the overall cell fluorescence. Therefore, calcein represents an ideal tracer to monitor the effects of membrane-lytic agents on internalized vesicles.[28] In this study, we used the calcein release assay to evaluate the endosome lytic activity of stearyl-Mel and stearyl-*r*Mel. As shown in Figure 7c, cells treated with calcein alone were restricted within intracellular vesicles and showed a punctate distribution of fluorescence, indicating that calcein was internalized into the endosomes. When the cells were treated with stearyl-Mel and stearyl-*r*Mel, calcein was released into the cytoplasm with increased green fluorescence. It was worth noting that stearyl-*r*Mel promoted more calcein release into the cytoplasm than stearyl-Mel, suggesting that stearyl-*r*Mel exhibited stronger endosome lytic activity than stearyl-Mel. Furthermore, the FACS data agreed with that derived from the confocal microscope assay (Figure 7d), confirming that stearyl-*r*Mel exhibited stronger endosome-lytic activity.

Antiproliferative Effect of Stearyl-Peptide/p53 Plasmid Complexes. To study the application of stearyl-Mel and stearyl-*r*Mel in gene therapy, we examined the antiproliferative effects of stearyl-peptide/p53 plasmid complexes in U251 cells. As shown in Figure 8a, the p53 plasmid alone displayed almost no growth inhibitory effect, whereas the complexes formed by stearyl-Mel and stearyl-*r*Mel with p53 plasmids significantly inhibited the growth of U251 cells. Note that the expression of p53 and the antiproliferative effect of stearyl-*r*Mel/p53 plasmid complex were significantly stronger than that of the stearyl-Mel/p53 plasmid complex (Figure 8).

DISCUSSION

Nucleic acids used in gene therapy share the common feature of low bioavailability due to their physicochemical properties. Searching for efficient delivery vectors for these molecules is a prerequisite for successful gene therapy. CPPs have gained more and more attention as attractive nonviral vectors for nucleic acids delivery due to their high internalization efficiency, low cytotoxicity, and flexible structural design.[4−6] Recently, a handful of papers have proved that stearylation is a

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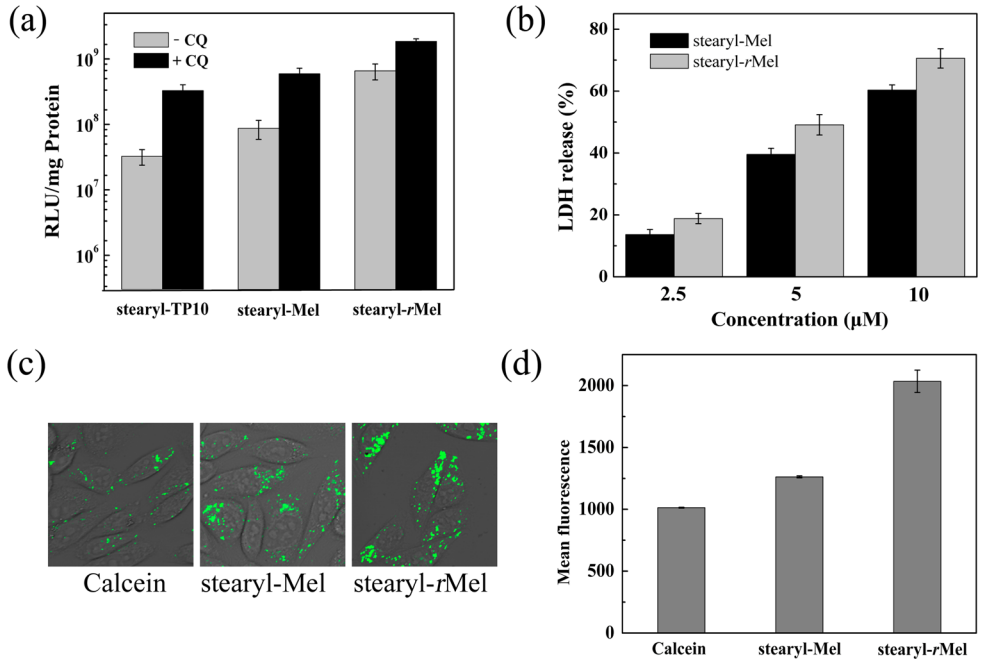

Figure 7. Endosome lytic activity of stearyl-peptides. (a) Effect of chloroquine (CQ) on the luciferase expression mediated by stearyl-peptides at an N/P ratio of 2. (b) LDH leakage in Cos-7 cells treated with stearyl-peptides. (c) Confocal images of CHO cells treated with stearyl-peptides in the presence of calcein. (d) Flow cytometry histograms of CHO cells treated with stearyl-peptides in the presence of calcein.

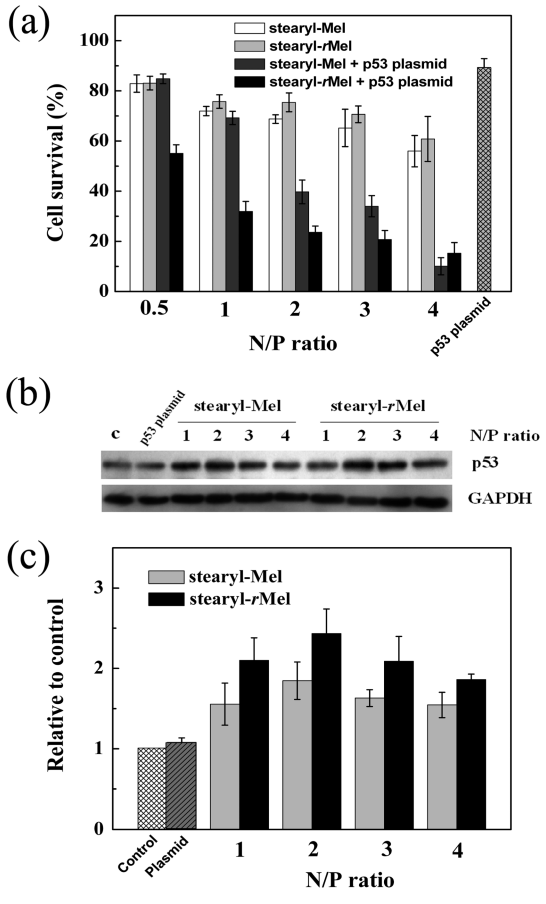

Figure 8. Antiproliferative activity and p53 protein expression of stearyl-peptide/p53 plasmid complexes in U251 cells. (a) Antiproliferative activity of the stearyl-peptides and the stearyl-peptides/p53 plasmid complexes for 96 h. (b) p53 protein expression using Western blotting. (c) Quantitative determination of the expressed p53 protein.

successful strategy to enhance the transfection efficiency of many CPPs.[7−15] In this study, we constructed novel gene delivery vectors by the conjugation of stearic acid to the N-terminus of Mel (stearyl-Mel) and its retro isomer (stearyl-*r*Mel). Although stearylation is not a universal method for all CPPs to enhance gene transfection efficiency, our results demonstrate that the stearyl moiety could significantly improve the transfection efficiency of stearyl-Mel and stearyl-*r*Mel. It is worth noting that both stearyl-Mel and stearyl-*r*Mel in terms of transfection efficiency were superior to stearyl-TP10, which has been reported to exhibit high transfection efficiency.[13] More surprisingly and gratifyingly, stearyl-*r*Mel resulted in around a 10-fold increase in luciferase expression level compared with that of stearyl-Mel and almost reached a transfection level similar to that obtained with the commercially available transfection agent Lipofectamine 2000 (LF2000). Furthermore, the cytotoxicity of stearyl-Mel and stearyl-*r*Mel was significantly lower than that of LF2000. In addition, the presence of serum only had a modest effect on the transfection efficiency of stearyl-Mel and stearyl-*r*Mel.

The formation of small and stable nanoparticles for stearyl-CPPs/plasmid complexes is undoubtedly an important feature to mediate high transfection efficiency.[5,6] The cationic nature enables CPPs to interact with the anionic plasmid DNA via electrostatic interactions. The N-terminal stearyl moiety renders the CPPs more hydrophobic and enables enhanced condensation of DNA, presumably facilitating more stable nanoparticle formation.[5,6] Ideally, the diameter of CPPs/plasmid particles should not exceed 100−300 nm for efficient uptake.[5] In this study, the particle sizes of stearyl-Mel and stearyl-*r*Mel with plasmid DNA at all N/P ratios were between 120−160 nm and fulfilled the size criteria required for an efficient transfection agent. As expected, both stearyl-Mel and stearyl-*r*Mel could deliver FITC-labeled plasmids into nearly 100% of cells. In addition, the cellular uptake of the stearyl-*r*Mel/plasmid complex was almost equivalent to the stearyl-

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*r*Mel/plasmid complex, demonstrating that the cellular uptake is not the factor contributing to the improved transfection efficiency of stearyl-*r*Mel.

Although the precise mechanism of internalization of CPPs remains controversial, there is a general consensus that most CPPs can internalize into cells via endocytosis. Endocytosis of CPPs may occur via several distinct pathways, such as clathrin-mediated endocytosis, caveolin-mediated endocytosis, and macropinocytosis.[5,6] It has been reported that stearylated CPPs carry plasmid DNA into cells via clathrin-mediated endocytosis or caveolin-mediated endocytosis.[8,15] To have more precise insight into the uptake pathways of the complexes formed by stearyl-Mel and stearyl-*r*Mel with plasmids, specific endocytosis inhibitors were used: chlorpromazine (CPZ), methyl-β-cyclodextrin (MβCD), and amiloride, which inhibit clathrin-mediated endocytosis, caveolin-mediated endocytosis, and macropinocytosis, respectively.[29,30] In this study, the FACS data showed that both MβCD and CPZ exhibited inhibitory effects on cellular uptake of the complexes, whereas amiloride had no inhibitory effect, indicating that the endocytosis of stearyl-Mel/plasmid complex and stearyl-*r*Mel/plasmid complex was mediated by clathrin and caveolin rather than macropinocytosis. Because the inhibitory effect of MβCD was relatively higher than that of CPZ, caveolin-mediated endocytosis may be the main uptake pathway for the stearyl-Mel/plasmid complex and stearyl-*r*Mel/plasmid complex. The result about the inhibitory effect of endocytosis inhibitors on the transfection efficiency of the stearyl-Mel/plasmid complex and stearyl-*r*Mel/plasmid complex supported the FACS data. Taken together, it can be concluded that endocytosis is the preferred uptake pathway of the stearyl-Mel/plasmid complex and stearyl-*r*Mel/plasmid complex. Correspondingly, endosomal entrapment will become the barrier for them as efficient vectors.

For the efficient delivery of cargoes into the cytoplasm, a series of novel chemically modified CPPs with endosome lytic properties have been designed.[28,31−35] Although stearylation has proven to be a successful method for increasing the endosomal escape of CPPs, coaddition of CQ can still significantly increase the transfection efficiency of many stearyl-CPPs/plasmid complexes, indicating there is still plenty of room for improvement.[12,13] In this study, the extent of increase in the transfection efficiency of stearyl-Mel in the presence of CQ was obviously lower than that of stearyl-TP10. More gratifyingly, CQ had a low effect on the transfection efficiency of stearyl-*r*Mel compared with that of stearyl-TP10 and stearyl-Mel, suggesting that stearyl-*r*Mel displayed stronger endosome lytic activity and could trigger more endosomal release of plasmids. Furthermore, the result derived from the LDH leakage assay, which showed that stearyl-*r*Mel exhibited obviously higher membrane lytic activity than stearyl-Mel, suggested that stearyl-*r*Mel would display stronger endosome lytic activity. To verify this suggestion, we used the calcein release assay to directly evaluate the endosome lytic activity of stearyl-Mel and stearyl-*r*Mel. Calcein, a membrane-impermeable fluorophore, represents an ideal tracer to monitor the effects of membrane lytic agents on internalized vesicles.[28] In this study, stearyl-*r*Mel could induce more calcein release into the cytoplasm than stearyl-Mel, confirming that stearyl-*r*Mel exhibited stronger endosome lytic activity. In short, the enhanced endosome lytic activity undoubtedly can contribute to the significant increase in the transfection efficiency of stearyl-*r*Mel compared with that of stearyl-Mel.

In order to study the application of stearyl-Mel and stearyl-*r*Mel in gene therapy, we evaluated the antitumor efficiency of the complexes formed by stearyl-Mel and stearyl-*r*Mel with p53 plasmids. Tumor suppressor protein p53 is a transcription factor involved in cell-cycle regulation, initiation of apoptotic cell death, and DNA repair.[36] Mutated p53 is observed in the majority of human tumors.[37] Malignant gliomas are the most common primary brain tumor type, and despite aggressive chemotherapy, the average survival rate for a patient can be less than 1 year.[38] Because p53 is frequently mutated or inactivated in human gliomas, *p*53 gene therapy by restoration of wild-type p53 function has been suggested as an effective treatment alternative for gliomas.[39] In this study, both stearyl-Mel and stearyl-*r*Mel could deliver sufficient p53 plasmids into human gliomas U251 cells, resulting in obvious p53 expression. Consequently, although stearyl-Mel and stearyl-*r*Mel displayed some cytotoxicity to U251 cells for 96 h, the stearyl-Mel/p53 plasmid complex and stearyl-*r*Mel/p53 plasmid complex showed significantly enhanced antiproliferative activity compared with that of peptides alone. It is worth noting that the complex formed by stearyl-*r*Mel with the p53 plasmid displayed stronger p53 expression and antitumor activity than stearyl-Mel. This result further confirmed that stearyl-*r*Mel displayed higher transfection efficiency than stearyl-Mel.

■ CONCLUSIONS

In this study, we constructed novel gene delivery vectors by coupling stearic aicd to the N-terminus of melittin (stearyl-Mel) and its retro isomer (stearyl-*r*Mel). Gratifyingly, stearyl-Mel and stearyl-*r*Mel exhibited significantly higher transfection efficiency than stearyl-TP10. It is worth noting that stearyl-*r*Mel showed around a 10-fold increase in transfection efficiency compared with that of stearyl-Mel due to the enhanced endosome-lytic activity, suggesting that stearyl-*r*Mel displays more potential as a gene vector. Nevertheless, the combination of the stearyl moiety with retro melittin would provide a novel framework for the development of an efficient transfection system.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Glover, D. J., Lipps, H. J., and Jans, D. A. (2005) Towards safe, non-viral therapeutic gene expression in humans. *Nat. Rev. Genet. 6*, 299−310.

(2) Mintzer, M. A., and Simanek, E. E. (2009) Non-viral vectors for gene delivery. *Chem. Rev. 109*, 259−302.

(3) Zhang, S., Zhao, Y., Zhao, B., and Wang, B. (2010) Hybrids of non-viral vectors for gene delivery. *Bioconjugate Chem. 21*, 1003−1009.
(4) Bolhassani, A. (2011) Potential efficacy of cell-penetrating peptides for nucleic acid and drug delivery in cancer. *Biochim. Biophys. Acta 1816*, 232−246.
(5) Hoyer, J., and Neundorf, I. (2012) Peptide vectors for the non-viral delivery of nucleic acids. *Acc. Chem. Res. 45*, 1048−1056.
(6) Nakase, I., Akita, H., Kogure, K., Gräslund, A., Langel, U., Harashima, H., and Futaki, S. (2012) Efficient intracellular delivery of nucleic acid pharmaceuticals using cell-penetrating peptides. *Acc. Chem. Res. 45*, 1132−1139.
(7) Futaki, S., Ohashi, W., Suzuki, T., Niwa, M., Tanaka, S., Ueda, K., Harashima, H., and Sugiura, Y. (2001) Stearylated arginine-rich peptides: a new class of transfection systems. *Bioconjugate Chem. 12*, 1005−1011.
(8) Khalil, I. A., Futaki, S., Niwa, M., Baba, Y., Kaji, N., Kamiya, H., and Harashima, H. (2004) Mechanism of improved gene transfer by the N-terminal stearylation of octaarginine: enhanced cellular association by hydrophobic core formation. *Gene Ther. 11*, 636−644.
(9) Tönges, L., Lingor, P., Egle, R., Dietz, G. P., Fahr, A., and Bähr, M. (2006) Stearylated octaarginine and artificial virus-like particles for transfection of siRNA into primary rat neurons. *RNA 12*, 1431−1438.
(10) Mäe, M., El, Andaloussi, S., Lundin, P., Oskolkov, N., Johansson, H. J., Guterstam, P., and Langel, U. (2009) A stearylated CPP for delivery of splice correcting oligonucleotides using a non-covalent co-incubation strategy. *J. Controlled Release 134*, 221−227.
(11) El-Sayed, A., Masuda, T., Khalil, I., Akita, H., and Harashima, H. (2009) Enhanced gene expression by a novel stearylated INF7 peptide derivative through fusion independent endosomal escape. *J. Controlled Release 138*, 160−167.
(12) Lehto, T., Abes, R., Oskolkov, N., Suhorutsenko, J., Copolovici, D. M., Mäger, I., Viola, J. R., Simonson, O. E., Ezzat, K., Guterstam, P., Eriste, E., Smith, C. I., Lebleu, B., El Andaloussi, S., and Langel, U. (2010) Delivery of nucleic acids with a stearylated (RxR)4 peptide using a non-covalent co-incubation strategy. *J. Controlled Release 141*, 42−51.
(13) Lehto, T., Simonson, O. E., Mäger, I., Ezzat, K., Sork, H., Copolovici, D. M., Viola, J. R., Zaghloul, E. M., Lundin, P., Moreno, P. M., Mäe, M., Oskolkov, N., Suhorutšenko, J., Smith, C. I., and Andaloussi, S. E. (2011) A peptide-based vector for efficient gene transfer in vitro and in vivo. *Mol. Ther. 19*, 1457−1467.
(14) Wang, H. Y., Chen, J. X., Sun, Y. X., Deng, J. Z., Li, C., Zhang, X. Z., and Zhuo, R. X. (2011) Construction of cell penetrating peptide vectors with N-terminal stearylated nuclear localization signal for targeted delivery of DNA into the cell nuclei. *J. Controlled Release 155*, 26−33.
(15) Veiman, K. L., Mäger, I., Ezzat, K., Margus, H., Lehto, T., Langel, K., Kurrikoff, K., Arukuusk, P., Suhorutšenko, J., Padari, K., Pooga, M., Lehto, T., and Langel, U. (2013) PepFect14 peptide vector for efficient gene delivery in cell cultures. *Mol. Pharmacol. 10*, 199−210.
(16) Mann, A., Thakur, G., Shukla, V., and Ganguli, M. (2008) Peptides in DNA delivery: current insights and future directions. *Drug Discovery Today 13*, 152−160.
(17) Ferrer-Miralles, N., Vázquez, E., and Villaverde, A. (2008) Membrane-active peptides for non-viral gene therapy: making the safest easier. *Trends Biotechnol. 26*, 267−275.
(18) Varkouhi, A. K., Scholte, M., Storm, G., and Haisma, H. J. (2011) Endosomal escape pathways for delivery of biologicals. *J. Controlled Release 151*, 220−228.
(19) Ogris, M., Carlisle, R. C., Bettinger, T., and Seymour, L. W. (2001) Melittin enables efficient vesicular escape and enhanced nuclear access of non-viral gene delivery vectors. *J. Biol. Chem. 276*, 47550−47555.
(20) Rozema, D. B., Ekena, K., Lewis, D. L., Loomis, A. G., and Wolff, J. A. (2003) Endosomolysis by masking of a membrane-active agent (EMMA) for cytoplasmic release of macromolecules. *Bioconjugate Chem. 14*, 51−57.
(21) Meyer, M., Zintchenko, A., Ogris, M., and Wagner, E. (2007) A dimethylmaleic acid-melittin-polylysine conjugate with reduced toxicity, pH-triggered endosomolytic activity and enhanced gene transfer potential. *J. Gene Med. 9*, 797−805.
(22) Legendre, J. Y., Trzeciak, A., Bohrmann, B., Deuschle, U., Kitas, E., and Supersaxo, A. (1997) Dioleoylmelittin as a novel serum-insensitive reagent for efficient transfection of mammalian cells. *Bioconjugate Chem. 8*, 57−63.
(23) Boeckle, S., Wagner, E., and Ogris, M. (2005) C- versus N-terminally linked melittin-polyethylenimine conjugates: the site of linkage strongly influences activity of DNA polyplexes. *J. Gene Med. 7*, 1335−1347.
(24) Lavignac, N., Lazenby, M., Franchini, J., Ferruti, P., and Duncan, R. (2005) Synthesis and preliminary evaluation of poly(amidoamine)-melittin conjugates as endosomolytic polymers and/or potential anticancer therapeutics. *Int. J. Pharm. 300*, 102−112.
(25) Chen, C. P., Kim, J. S., Steenblock, E., Liu, D., and Rice, K. G. (2006) Gene transfer with poly-melittin peptides. *Bioconjugate Chem. 17*, 1057−1062.
(26) Baumhover, N. J., Anderson, K., Fernandez, C. A., and Rice, K. G. (2010) Synthesis and in vitro testing of new potent polyacridine-melittin gene delivery peptides. *Bioconjugate Chem. 21*, 74−83.
(27) Juvvadi, P., Vunnam, S., Rotondi, K. S., and Merrifield, R. B. (1996) Synthetic melittin, its nantio, retro, and retroenantio isomers, and selected chimeric analogs: their antibacterial, hemolytic, and lipid bilayer action. *J. Am. Chem. Soc. 118*, 8989−8997.
(28) Salomone, F., Cardarelli, F., Di Luca, M., Boccardi, C., Nifosì, R., Bardi, G., Di Bari, L., Serresi, M., and Beltram, F. (2012) A novel chimeric cell-penetrating peptide with membrane-disruptive properties for efficient endosomal escape. *J. Controlled Release 163*, 293−303.
(29) Ma, Y., Yang, D., Ma, Y., and Zhang, Y. H. (2012) Novel cell-penetrating peptides based on α-aminoxy acids. *ChemBioChem 13*, 73−79.
(30) Doan, N. D., Létourneau, M., Vaudry, D., Doucet, N., Folch, B., Vaudry, H., Fournier, A., and Chatenet, D. (2012) Design and characterization of novel cell-penetrating peptides from pituitary adenylate cyclase-activating polypeptide. *J. Controlled Release 163*, 256−265.
(31) Wadia, J. S., Stan, R. V., and Dowdy, S. F. (2004) Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat. Med. 10*, 310−315.
(32) Lundberg, P., El-Andaloussi, S., Sütlü, T., Johansson, H., and Langel, U. (2007) Delivery of short interfering RNA using endosomolytic cell-penetrating peptides. *FASEB J. 21*, 2664−2671.
(33) Lo, S. L., and Wang, S. (2008) An endosomolytic Tat peptide produced by incorporation of histidine and cysteine residues as a non-viral vector for DNA transfection. *Biomaterials 29*, 2408−2414.
(34) Shaheen, S. M., Akita, H., Nakamura, T., Takayama, S., Futaki, S., Yamashita, A., Katoono, R., Yui, N., and Harashima, H. (2011) KALA-modified multi-layered nanoparticles as gene carriers for MHC class-I mediated antigen presentation for a DNA vaccine. *Biomaterials 32*, 6342−6350.
(35) Andaloussi, S. E., Lehto, T., Mäger, I., Rosenthal-Aizman, K., Oprea, I. I., Simonson, O. E., Sork, H., Ezzat, K., Copolovici, D. M., Kurrikoff, K., Viola, J. R., Zaghloul, E. M., Sillard, R., Johansson, H. J., Said Hassane, F., Guterstam, P., Suhorutšenko, J., Moreno, P. M., Oskolkov, N., Hälldin, J., Tedebark, U., Metspalu, A., Lebleu, B., Lehtiö, J., Smith, C. I., and Langel, U. (2011) Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically in vivo. *Nucleic Acids Res. 39*, 3972−3987.
(36) Vogelstein, B., and Kinzler, K. W. (1992) p53 function and dysfunction. *Cell 70*, 523−526.
(37) Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. (1991) p53 mutations in human cancers. *Science 253*, 49−53.
(38) Wen, P. Y., and Kesari, S. (2008) Malignant gliomas in adults. *N. Engl. J. Med. 359*, 492−507.
(39) Pulkkanen, K. J., and Yla-Herttuala, S. (2005) Gene therapy for malignant glioma: current clinical status. *Mol. Ther. 12*, 585−598.