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Research paper

Synthesis and evaluation of novel lipopeptide as a vehicle for efficient gene delivery and gene silencing

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ABSTRACT

Nucleic acid-based therapeutics have recently emerged as a new class of next generation agents for treatment and prevention of viral infection, cancer, and genetic disorders, but their wide use is limited by their relatively weak delivery into target cells. Usage of synthetic cationic amphiphiles with peptide hydrophilic domain as agents for non-viral gene delivery is an attractive approach. We developed the schemes for the synthesis of aliphatic peptides with different length of the hydrocarbon chains in hydrophobic domains and different amino acids in polar head. For the obtained derivatives we determined transfection efficiency, critical vesicle concentration, particle size, ζ-potential and aggregates stability. We have found that the transfection efficiency is increased if the ornithine is a part of polar head in an amphiphile. The most promising amphiphile for liposomal formation OrnOrnGlu($C_{16}H_{33}$)$_2$ was examined more carefully. It has been shown that the lipopeptide possesses low toxicity (*in vitro* and *in vivo*) and high transfection efficiency with pDNA and siRNA in different cell lines. In addition, the production of liposomes based on this lipopeptide is simple, quick and cheap. Thus OrnOrnGlu($C_{16}H_{33}$)$_2$ is a promising vehicle for gene delivery and gene silencing.

1. Introduction

Gene delivery is one of the most basic techniques of molecular biology, a technological basis for *in vitro* and *in vivo* gene therapy. The success of gene therapy is largely dependent on the development of a vector or vehicle that can selectively and efficiently deliver a gene to target cells with minimal toxicity [1]. The use of cationic liposome/DNA complexes (lipoplexes) and cationic polymers/DNA (polyplexes) for the transfer of genes into somatic cells has become very popular due to its limited toxicity and relative effectiveness *in vitro* [2]. Synthetic cationic lipids are widely used for non-viral gene delivery [3–7]. However, low efficiency of transfection in different cell lines determines the need in development of new agents. The transfection activity depends on the structural components of the amphiphile, such as polar and hydrophobic domains. Amphiphiles containing more than one charged group seem to be more effective in transfection, because of the reduced aggregate size, easy interaction with the cell membrane and ability to compact the genetic material [8–10]. Synthetic peptides are perspective substances for the construction of the multivalent polar domains due to their biocompatibility, several reactive groups for modification and increased binding affinity for nucleic acids [11–14]. Peptide-based vectors are advantageous over other nonviral strategies in that they are able to tightly compact and protect DNA, target specific cell-surface receptors, disrupt the endosomal membrane, and deliver the DNA cargo to the nucleus. Basic amino acids have specific side groups that are positively charged at neutral pH that helps particles to interact not only with the plasmid DNA, but also with the cellular membrane. Using lysine and ornithine is expected to enhance the gene delivery ability because the cationic charge of the head groups at physiological pH can contribute to the binding and condensation of DNA and they are widely used as part of cell penetrating peptides to deliver genes or drugs [15,16].

The process of lipofection is associated with overcoming of several biological barriers: infiltration of lipoplex through the cell membrane, penetration to the endosome, DNA escape from the endosome (to avoid the formation of lysosomes and destruction

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of plasmid by nucleases) and transfer to the nucleus. In this regard, hydrophobic domain of amphiphile plays an important role in the delivery of nucleic acid. It determines the phase transition temperature and fluidity of the liposomal bilayer, affects the stability of the liposomal dispersion, protects DNA from interaction with nucleases and affects the escape of the lipoplex from the endosome [17]. According to the recent data, the hydrophobic domain of the amphiphile also determines a toxicity of a compound [18].

In this study, we aimed to investigate the physiochemical properties, toxicity (*in vitro* and *in vivo*) and *in vitro* gene transfection activity of a series of aliphatic derivatives of di-, tri- and tetrapeptides with different amino acid sequences.

2. Methods

The synthesis of the lipopeptides is described in the Supplementary.

2.1. The critical packing parameter (CPP)

The critical packing parameter (CPP) was calculated to predict the most likely shape in aqueous solution for the particles based on lipopeptides by the formula:

$$p = V/(L \times S)$$

V – volume of the hydrocarbon chains of the amphiphile, L – length of the hydrocarbon chains, S – the area of the head group.

They were determined in the program HyperChem Pro 6.0.

2.2. Liposome preparation

A chloroform solution of the lipid (~2 mg) was added to a pear shaped flask. The solvent was removed under a vacuum by rotary evaporator, leaving a thin film deposited onto the flask wall. A total of 1 mL distilled water was then added and the film was hydrated and peeled off by vortexing and sonicating at 65 °C 3 times for 15 min. The solution was extruded through a polycarbonate membrane (100 nm) using an Avanti polar lipids mini-extruder until a homogeneous liposome solution was obtained (typically 5 extrusions).

2.3. Particle size and ζ-potential

The particle size distribution was determined by particle size analyzer series LSTM 13320 (Beckman Coulter, USA) according to the method of photonic correlation spectroscopy based on the principle of dynamic light scattering

2.4. Gel retardation assay

Complexes, diluted in 1× TBE (Tris–BorateEthylenediaminete traacetic acid (EDTA); Sigma–Aldrich) and gel loading solution (Sigma–Aldrich), were loaded in a 1% agarose gel containing 0.5 g/mL of ethidium bromide (Sigma–Aldrich). Electrophoresis was carried out at 100 V for 45 min in 1× TBE as running buffer. The gel was visualized under UV lamps using a Gel Imager-2.

2.5. Nucleic acids

Oligonucleotide sequence of siRNA against firefly luciferase was described previously (GenBank accession no. U47298, bp 206 (rational)) [19]. Control siRNA against phosphoprotein gene of respiratory syncytial virus was described previously [20]. siRNA samples were prepared by mixing of equimolar amounts of the self-complementary oligoribonucleotides, heating for 2 min at 60 °C, and cooling to room temperature. The dilution of siRNA to final concentration 0.1 μg/μL of the duplex was conducted by RNAase-free water. All oligonucleotides were purchased from Syntol (Russia). The plasmid "pGL3-Control Vector" (Promega) containing SV-40 promoter-driven firefly luciferase gene was used for gene transfer experiments.

2.6. Cell culture

A549 (Human lung adenocarcinoma epithelial cell line), HeLa (human adenocarcinoma), 293T (cells are isolated from human embryonic kidneys and are transformed with large T antigen), and Jurkat (human T lymphocyte, suspension) cells were maintained in DMEM, supplemented with 10% fetal bovine serum (FBS) and gentamicin (40 μg/mL) at 37 °C and 5% CO_2. The Chinese hamster ovary (CHO) cells were maintained in RPMI-1640 medium with 10% FBS and gentamicin (40 μg/mL). The cells were subcultured regularly using trypsin/EDTA. For siRNA delivery experiments, Huh-7 (human hepatocarcinoma) cells robustly replicating luciferase-encoding genotype 1b isolate Con1 hepatitis C virus (HCV) replicons were used. This cell line was maintained in 1/1 mixture of DMEM/F12 medium supplemented with 10% FBS and penicillin (10 units/mL), streptomycin (0.1 mg/mL) and G418 (500 μg/mL)в at 37 °C and 5% CO_2.

2.7. Cytotoxicity assay

CHO, Hela and 293T cell line were used. Cells were seeded into 96-well cell culture plates at 2×10^5 cells/mL 24 h before experiments. Cells were treated with 2-fold dilutions of lipopeptides in FBS-free culture media for 24 h. The cytotoxicity of lipopeptide was assessed by "CellTiter 96® Non-Radioactive Cell Proliferation Assay" kit (Promega, USA) as recommended by the manufacturer. The data were presented as IC_{50} value or, correspond to concentration of the lipopeptides that inhibits cell proliferation by 50% and as percentage of viable cells.

2.8. Transfection with plasmid DNA

In vitro gene transfer efficiency of the lipopeptides was estimated in A549, 293T, CHO and Jurkat cells by using firefly luciferase reporter gene as a part of pGL3-Control plasmid (pDNA) or pGFP (Clontech). For transfection with plasmid, cells were seeded in 24-well culture plates at 1×10^5 cells/well in appropriate medium (DMEM, supplemented with 10% fetal bovine serum (FBS) and gentamicin (40 μg/mL)) and cultured for 24 h prior to transfection. The lipoplexes were prepared directly before transfection by mixing of 1 μg per well of the plasmid with the lipopeptides at different DNA phosphate/OrnOrnGlu$(C16)_2$ nitrogen ratio (P:N) followed by 15 min incubation at room temperature. The complexes were added to the cells (in the same media with 10% FBS) and incubated for 24 h at 37 °C. 24 h after transfection cells were harvested. The level of luciferase protein expression was quantified by "Luciferase Assay System" (Promega) kit as recommended by the manufacturer. The data were presented as relative light units (RLU) per 10^5 cells. Cells transfected with pGFP were analyzed by flow cytometry to calculate GFP^+ cells. In the transfection experiments, Lipofectamine 2000 (Life Technologies) was used as a positive control.

2.9. Gene silencing with siRNA

For *in vitro* gene silencing experiments, Huh-7 cells robustly replicating luciferase-encoding genotype 1b HCV replicons were used. The protocol was the same as described for the plasmid transfection with two exception – the amount of used siRNA 0.5 μg per well and cells were cultured and transfected in the