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A biomimetic lipid library for gene delivery through thiol-yne click chemistry

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ABSTRACT

The delivery of nucleic acids such as plasmid DNA and siRNA into cells is a cornerstone of biological research and is of fundamental importance for medical therapeutics. Although most gene delivery therapeutics in clinical trials are based on viral vectors, safety issues remain a major concern. Non-viral vectors, such as cationic lipids and polymers, offer safer alternatives but their gene delivery efficiencies are usually not high enough for clinical applications. Thus, there is a high demand for more efficient and safe non-viral vectors. Here, we present a facile two-step method based on thiol-yne click chemistry for parallel synthesis of libraries of new biomimetic cationic thioether lipids. A library of novel lipids was synthesized using the developed method and more than 10% of the lipids showed highly efficient transfection in different cell types, surpassing the efficiency of several popular commercial transfection reagents. One of the new lipids showed highly efficient siRNA delivery to multiple cell types and could successfully deliver DNA plasmid to difficult-to-transfect mouse embryonic stem cells (mESC). Analysis of structure–activity relationship revealed that the length of the hydrophobic alkyl groups was a key parameter for efficient cell transfection and was more important for transfection efficiency than the nature of cationic head groups. The correlation of the size and surface charge of liposomes with transfection efficiency is described.

1. Introduction

The delivery of nucleic acids such as plasmid DNA and siRNA into cells is a cornerstone of biological research and is of fundamental importance for medical therapeutics. Indeed, since the first FDA-approved gene therapy experiment in 1990 [1], over 1700 clinical trials have been conducted for gene delivery [2]. Although most gene delivery therapeutics in clinical trials are based on viral vectors, safety issues remain a major concern [3]. Non-viral vectors, including cationic lipids [4,5], polymers [6,7], dendrimers [8], cationic proteins [9] and inorganic nanoparticles [10,11], offer safer alternatives but their gene delivery efficiencies are usually not high enough for clinical applications. Although lipid-based vectors have only 4.8% of all gene therapy clinical trials [12], they are already the most commonly used systems for in vitro delivery of nucleic acids into cells [4,13–15]. However, most of the lipid-based delivery systems are synthesized using a multi-step synthesis route, requiring protecting groups and excessive purifications [15], thus limiting the possibility for successful and fast structural optimizations. Recently Anderson et al. reported two important combinatorial approaches to synthesize libraries of alkyl amines for siRNA delivery using aza-michael addition [16] and epoxide-amine [17] reactions. However, there are still no convenient combinatorial methods that could lead to lipid-like molecules structurally similar to natural phospholipids, the main lipid components of the cell membrane.

Here we report a facile modular and scalable approach employing thiol-yne "click" chemistry [18–21] for the parallel synthesis of a library of cationic thioether lipids with two hydrophobic tails of variable lengths and possessing a linker group structurally mimicking the glycerol core of the phospholipids. We used the method to synthesize more than 100 novel lipids and found that more than 10% of all lipids showed highly efficient transfection in different cell types. Both siRNA delivery and transfection of difficult-to-transfect cell lines, such as mESC, was analyzed. Analysis of structure–activity relationship and the correlation of the size and surface charge of liposomes with transfection efficiency are described.

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2. Materials and methods

2.1. Chemicals, plasmids and siRNAs

1-Hexanethiol (95%), 1-heptanethiol (98%), 1-octanethiol (≥98.5%), 1-nonanethiol (95%), 1-decanethiol (96%), 1-undecanethiol (98%), 1-dodecanethiol (≥98%), 1-hexadecanethiol (≥95%), 2,2-dimethoxy-2-phenylacetophenone (99%), 4-pentynoic acid (95%), 5-hexynoic acid (97%), 4-(2-aminoethyl)morpholine (99%), 1-(2-aminoethyl)pyrrolidine (98%), N′,N′-diethylethane-1,2-diamine (99%), N′,N′-diethylpropane-1,3-diamine (≥99%), N′,N′-dimethylpropane-1,3-diamine (≥98%) and 1-[2-(dimethylamino)ethyl]piperazine (≥98%) were bought from Sigma–Aldrich (Steinheim, Germany). Dichloromethane (99.8%), tetrahydrofuran (99.8%) and *n*-hexane (98.5%) were bought from Merck (Darmstadt, Germany). Dimethylformamide (99.8%) was bought from Merck (Hohenbrunn, Germany). N′,N′-diisopropylcarbodiimide (99%) and N′,N′-dimethylethane-1,2-diamine (97%) were bought from Alfa Aesar (Karlsruhe, Germany); anhydrous hydroxybenzotriazole (≥95%) was bought from Molekula (Shaftesbury, Dorset, United Kingdom). 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine (DOPE) was bought from Corden Pharma Switzerland LLC (Liestal, Switzerland). Plasmid DNA consisting of 67.5 ng pCS2 + β-galagtosidase and 7.5 ng of pEGFP-1 (Clontech) was used per well of a 96-well plate. For siRNA transfection experiments siGENOME SMARTpool targeting human LRP6 (Dharmacon) was used at a final concentration of 40 nM to assess functional delivery of siRNA molecules in 24-well plates.

2.2. Library synthesis

First step: solutions of either 4-pentynoic or 5-hexynoic acid (0.5 mmol) in 0.5 ml tetrahydrofuran (THF), an alkyl thiol (2 eq.) in 0.5 ml of THF and 2,2-dimethoxy-2-phenylacetophenone (5 mg, 0.02 mmol) in 0.2 ml of THF were combined in a 20 ml glass vial protected from light and degassed by ultrasonication and purging with argon for 3 min. The samples were then irradiated with UV (365 nm, 1.87 mW/cm^2) for 1 h.

Second step: After evaporation of THF, the residue was redissolved in 8 ml of dichloromethane (DCM). The solution was separated into seven 1 ml batches for subsequent reactions with different amines. N,N′-Diisopropylcarbodiimide (DIC) (12 μl 0.075 mmol), an amine (0.063 mmol) and a 20 μl solution of 1-hydroxybenzotriazole (HOBt) in dimethylformamide (DMF) (c 0.5 g/ml) were added to each sample and vortexed. The solutions were protected from light with aluminum foil and shaked for 16 h under argon atmosphere. After evaporation of the solvent, 2 ml of *n*-hexane was added to each sample for extraction followed by centrifugation at 10,000 g for 5 min and separation of supernatant. Evaporation of hexane from supernatants gave final lipids as clear yellowish oils, which were then used for the screening. The average yield in library synthesis is 80%, and the estimated purity is about 70–80%.

2.3. ESI-MS and HRMS characterization

Electrospray ionization mass spectrometry (ESI-MS) was performed using an API 4000 Quadrupol mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo V™ electrospray ion source (TurboIonSpray® probe, Applied Biosystems) operating in positive ion mode at a source temperature of 400 °C. Nitrogen was used as nebulizer, curtain, collision and auxiliary gas. Instrument controlling and data acquisition were carried out using the Analyst Software V 1.4 (Applied Biosystems, Foster City, CA, USA). All parameter settings were optimized by flow injection experiments with standard solutions infused into the mass spectrometer using a syringe pump (Harvard Apparatus Inc, South Natick, USA) at an infusion flow rate of 10 μl/min. Lipids C6–C12 were dissolved in methanol, and tested in ESI-MS at concentration 5 μg/ml.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on a 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA). Peak lists were generated using Data Explorer Software 4.0 (Applied Biosystems). 2,5-Dihydroxybenzoic acid (DHB, 10 mg in 1 ml tetrahydrofuran containing 0.1% trifluoroacetic acid) was used as a matrix for C16 lipids. 10 μl lipids (1 mg in 1 ml THF) were mixed with 10 μl matrix, and 1 μl mixed solution was spotted on a stainless steel MALDI carrier.

2.4. NMR characterization

NMR spectra were obtained on a Bruker AMX 500 spectrometer, chemical shifts are reported in parts per million (ppm) on the δ scale, and were referenced to residual protonated solvent peaks.

2.5. Cationic liposome preparation in the initial screening

A 20 μl solution of DOPE (0.0067 mol/l) and 10 μl solution of a cationic lipid (0.0175 mol/l) in ethanol (for C16-lipids, 1:1 ratio of ethanol and THF was used to dissolve lipids) were mixed and vortexed. Then 70 μl of a 200 mM sodium acetate buffer (pH 5.0) was added and the solution was vortexed for 30 s and sonicated for 5 min to form liposomes.

2.6. Cationic liposome preparation during optimization of hits

Stock solutions of DOPE (7.44 mg/ml, 0.01 mol/l) and cationic lipids (0.01 mol/l) were made in absolute ethanol. Stock solutions of DOPE and lipids were mixed to produce 300 μl solutions with three different lipid/DOPE mole ratios (2:1, 1:1 and 1:2) in Eppendorf tubes, followed by vortexing for 10 s and mixing with 700 μl of 200 mM sodium acetate buffer (pH 5.0). The solution was then vortexed again for 10 s.

2.7. A1C11 cationic liposome preparation for mESC D3 transfection

Stock solutions of DOPE and the A1C11 lipid were mixed in a round-bottom glass flask to produce 300 μl solution with 1:1 lipid/DOPE mole ratio, followed by slow evaporation of ethanol using a rotary evaporator. Sodium acetate buffer (1000 μl, 200 mM, pH 5.0) was added to a clear lipid film formed on glass walls, followed by gentle rotation of the flask for 2 h to produce liposomes.

2.8. In vitro transfection assay in HEK293T and Hela cell with eGFP-pDNA

For the HEK293T cell transfection screen, 0.4 μl liposomal reagent, 75 ng plasmid DNA (67.5 ng pCS-LacZ and 7.5 ng pCMV-EGFP) and 4×10^4 cells were used per well in 96-well plates using a one-step (also termed reverse transfection) protocol. Both the liposomal reagent and DNA were diluted in a final volume of 10 μl with 50 mM sodium acetate buffer (pH 5.0), combined and mixed with pipette action to give 20 μl lipoplex solution and left at room temperature for 30 min. 80 μl of freshly trypsinized cell suspension in DMEM supplemented with 10% FCS was then added, mixed gently with pipette and immediately transferred to the 96 wells. After 16 h incubation at 37 °C in 5% CO_2, Hoechst 33342 was added to a final concentration of 1 μg/ml by pipetting 10 μl of a 10 μg/ml solution. After a further 30 min incubation to allow staining of DNA within nuclei, cells were transferred to an Olympus IX81 automated fluorescent imaging microscope and images for bright field, Hoechst and GFP acquired. Cell transfection efficiencies were estimated using the free software programme CellProfiler after counting the number of GFP positive cells and dividing by the total number of cells (nuclei). A detailed manual analysis of several images after overlaying the Hoechst and GFP channels in Photoshop showed that both CellProfiler as well as Image J software programmes underestimated the true transfection efficiency due to the inability of the software to always discern individual GFP transfected cells that were closely adjacent. This did not effect the overall results however as the error was constant for various transfection efficiencies. Relative transfection efficiency was determined by dividing the transfection efficiencies of lipids with transfection efficiency of Lipo2000.

2.9. In vitro transfection assay with siRNA

For siRNA mediated gene silencing experiments 1 μl liposomal reagents A1C11 and A7C11 and 20 pmol (1 μl of 20 μM) standard control or LRP6 siRNA (Genome SMARTpool, Dharmacon) were used per well in 24-well plates. Liposomes and siRNA were diluted in a final volume of 50 μl with 50 mM sodium acetate buffer (pH 5.0), combined and mixed with pipette action to give 100 μl lipoplex solution and left at room temperature for 20 min. 400 μl of DMEM supplemented with 10% FCS was then added, mixed gently with pipette and immediately transferred to the 24 wells containing mouse embryonic fibroblast (MEF) cells at 50% confluency, after first removing the existing media from the wells.

2.10. Western blot

48 h after siRNA transfection, MEF cells were lysed in 100 μl of 1% Triton X-100 buffer (50 mM Tris, pH 7.0, 0.15 M NaCl plus protease/phosphatase inhibitors). After centrifugation, clarified supernatants from these total cellular lysates were denatured in SDS-loading buffer and 5 μl loaded on a 10% SDS-PAGE gel. Proteins separated by SDS-PAGE were transferred to PVDF membrane and Western blot performed using a polyclonal rabbit antibody for total LRP6 protein (anti-T1479; at 1:2000 dilution in 5% BSA). Beta-Actin was used as a loading normalization control.

2.11. mESC D3 transfection assay

Mouse D3 Embryonic Stem (mES) Cell transfection was conducted in a 6-well plate coated with 0.1% gelatine. Effectene was chosen as a positive control. Cells were cultured in ES cell medium (DMEM-GlutaMAX™-I medium supplemented with 15% FCS, 0.1 mM β-mercaptoethanol, 1% penicillin/streptomycin and 1000 U/ml LIF) in culture dishes. 4 μl liposomal reagent, 1 μg plasmid DNA (0.9 μg pCS-LacZ and 0.1 μg pCMV-EGFP) and 2.5×10^5 cells were used per well using a one-step (also termed reverse transfection) protocol. Both the liposomal reagent and DNA were diluted in a final volume of 100 μl with 50 mM sodium acetate buffer (pH 5.0), combined and mixed with pipette action to give 200 μl lipoplex solution and left at room temperature for 30 min. 500 μl of freshly trypsinized cell suspension in ES cell medium was then added, mixed gently with pipette and immediately transferred to one well of a 6 well plate. 1300 μl of ES cell medium was further added to each well.