Effect of the combination of cationic lipid and phospholipid on gene-knockdown using siRNA lipoplexes in breast tumor cells and mouse lungs

YOSHIYUKI HATTORI[1], MIN TANG[1], AYA AOKI[1], MOMOKA EZAKI[1], HANA SAKAI[1] and KEI-ICHI OZAKI[2]

[1]Department of Molecular Pharmaceutics, Hoshi University, Tokyo 142-8501; [2]Department of Molecular Pathology, Faculty of Pharmaceutical Sciences, Doshisha Women's College of Liberal Arts, Kyotanabe, Kyoto 610-0395, Japan

Received March 1, 2023; Accepted July 27, 2023

DOI: 10.3892/mmr.2023.13067

Abstract. Previously, using three types of cationic lipids, the effect of phospholipids in liposomal formulations on gene-knockdown efficacy was determined after *in vitro* and *in vivo* transfection with small interfering RNA (siRNA)/cationic liposome complexes (siRNA lipoplexes) containing various cationic lipids and phospholipids. In the present study, six other types of cationic lipids, namely *N*,*N*-dimethyl-*N*-tetradecyltetradecan-1-aminium bromide, *N*-hexadecyl-*N*,*N*-dimethylhexadecan-1-aminium bromide (DC-1-16), 2-[bis{2-(tetradecanoyloxy)ethyl}amino]-*N*,*N*,*N*-trimethyl-2-oxoethan-1-aminium chloride (DC-6-14), 1,2-di-*O*-octadecenyl-3-trimethylammonium propane chloride (DOTMA), 1,2-distearoyl-3-trimethylammonium-propane chloride (DSTAP) and 1,2-dioleoyl-3-dimethylammonium-propane were selected, and the effect of phospholipids in liposomal formulations containing each cationic lipid on gene-knockdown was evaluated. A total of 30 types of cationic liposomes composed of each cationic lipid with phosphatidylethanolamine containing unsaturated or saturated diacyl chains (C14, C16 or C18) were prepared. Regardless of the type of cationic lipid, the inclusion of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) in the liposomal formulations resulted in injectable size of siRNA lipoplexes after mixing of siRNA and cationic liposomes. Transfection of their lipoplexes with luciferase (Luc) siRNA into human breast cancer MCF-7-Luc cells stably expressing Luc led to a strong knockdown of Luc. Furthermore, the systemic injection of siRNA lipoplexes composed of DC-1-16, DC-6-14, DOTMA or DSTAP with DOPE resulted in siRNA accumulation in the lungs. Significant gene-knockdown was observed in the lungs of mice following the systemic injection of siRNA lipoplexes containing DC-1-16 and DOPE. Cationic liposomes composed of DC-1-16 and DOPE serve as potential carriers for *in vitro* and *in vivo* siRNA transfection.

Correspondence to: Professor Yoshiyuki Hattori, Department of Molecular Pharmaceutics, Hoshi University, 2-4-41 Ebara, Shinagawa, Tokyo 142-8501, Japan
E-mail: yhattori@hoshi.ac.jp

Key words: cationic liposome, small interfering RNA delivery, phospholipid, lung, gene-knockdown

Introduction

Small interfering RNA (siRNA) therapeutics are a novel class of drugs that inhibit gene expression by cleaving mRNA with a sequence complementary to the siRNA (1). However, a major limitation of siRNA therapeutics is the delivery of siRNAs to the target cells. siRNA is not readily taken up by cells because of membrane impermeability due to the hydrophilicity of negatively charged siRNA and the enzymatic degradation of siRNA by serum endonucleases (2). Therefore, an siRNA delivery system is essential to protect the siRNA molecules until they reach the target cell and efficiently introduce them into the cells through the cell membrane (2-5).

Among the available delivery systems, siRNA/cationic liposome complexes (siRNA lipoplexes) are attractive carriers (6,7). Cationic lipids and phospholipids are commonly used as components of cationic liposomal formulations to control transfection efficiency and stability (8,9). In particular, cationic lipids in liposomes are essential for interaction with negatively charged siRNAs (10). In addition, the structures of cationic lipids and phospholipids, such as the head group, alkyl or acyl chain length and saturation and the linker between the head group and the lipid anchor, affect the transfection efficiency of siRNA lipoplexes (8,9,11). Therefore, for achieving efficient siRNA transfection using siRNA lipoplexes, the optimal combination of cationic lipids and phospholipids in liposomal formulations must be determined.

Previously, three types of cationic lipid, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), dimethyldioctadecylammonium bromide (DDAB) and 11-[{1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl)propan-2-yl}amino]-*N*,*N*,*N*-trimethyl-11-oxoundecan-1-aminium bromide (TC-1-12) were selected, and the effect of phospholipids in cationic liposome formulations on the gene-knockdown efficacy of siRNA lipoplexes was examined (9). DOTAP and DDAB are cationic diacyl (C18) and dialkyl (C18) lipids, respectively, and TC-1-12 is a cationic triacyl (C12) lipid.

Among them, siRNA lipoplexes composed of DDAB/1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), TC-1-12/DOPE and TC-1-12/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) result in significant suppression of targeted mRNA both *in vitro* and *in vivo*, indicating that the optimal phospholipids in cationic liposomes for siRNA transfection are notably affected by the type of cationic lipid used. In the present study, to examine whether alkyl or acyl chain length and saturation and the linker between the head group and lipid anchor in cationic lipids affect optimal combination with phospholipid for gene-knockdown by siRNA lipoplexes, six other types of cationic lipid, namely *N*,*N*-dimethyl-*N*-tetradecyltetradecan-1-aminium bromide (DC-1-14), *N*-hexadecyl-*N*,*N*-dimethylhexadecan-1-aminium bromide (DC-1-16), 2-[bis{2-(tetradecanoyloxy)ethyl}amino]-*N*,*N*,*N*-trimethyl-2-oxoethan-1-aminium chloride (DC-6-14), 1,2-di-*O*-octadecenyl-3-trimethylammonium propane chloride (DOTMA), 1,2-distearoyl-3-trimethylammonium-propane chloride (DSTAP) and 1,2-dioleoyl-3-dimethylammonium-propane (DODAP) were selected, and the effect of cationic lipids and phospholipids in liposomal formulations on *in vitro* and *in vivo* gene-knockdown after transfection with siRNA lipoplexes were evaluated.

Materials and methods

Materials. *N*,*N*-Dimethyl-*N*-tetradecyltetradecan-1-aminium bromide (cat. no. DC-1-14), *N*-hexadecyl-*N*,*N*-dimethylhexadecan-1-aminium bromide (cat. no. DC-1-16) and 2-[bis{2-(tetradecanoyloxy)ethyl}amino]-*N*,*N*,*N*-trimethyl-2-oxoethan-1-aminium chloride (cat. no. DC-6-14) were obtained from Sogo Pharmaceutical Co., Ltd. 1,2-Di-*O*-octadecenyl-3-trimethylammonium propane chloride (DOTMA; cat. no. 14476) and 1,2-distearoyl-3-trimethylammonium-propane chloride (DSTAP; cat. no. 14486) were obtained from Polysciences, Inc. 1,2-Dioleoyl-3-dimethylammonium-propane (DODAP; cat. no. TRC-D483000) was obtained from Toronto Research Chemicals, Inc (LGC Group). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE; cat. no. ME-8080), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE; cat. no. ME-6060), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE; cat.no.ME-4040),1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE; cat. no. ME-8181) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE; cat. no. ME-6081) were obtained from NOF Co., Ltd. All other chemicals used were of the highest grade available.

siRNAs. Firefly luciferase siRNA (Luc siRNA), non-silencing siRNA (Control #1 siRNA) and cyanine 5-conjugated Control #1 siRNA (Cy5-siRNA) were designed as previously reported (hLuc sequence: GenBank accession no. AY535007.1) (9) and synthesized by Sigma-Aldrich Japan K.K. (Merck KGaA). siRNA sequences were as follows: Luc siRNA passenger strand, 5'-CCGUGGUGUUCGUGUCUAAGA-3', and guide strand, 5'-UUAGACACGAACACCACGGUA-3'; Control #1 siRNA passenger strand, 5'-GUACCGCACGUCAUU CGUAUC-3', and guide strand, 5'-UACGAAUGACGUGCG GUACGU-3'. For Cy5-siRNA, Cy5 dye was conjugated at the 5'-end of the passenger strand of Control #1 siRNA. siRNAs for mouse Tie2 and firefly Luc (Control #2 siRNA) were designed as previously reported (pGL2 Luc sequence: GenBank accession no. X65323.2; Tie2 sequence: GenBank accession no. NM_013690.3) (9) and synthesized by Japan Bio Services Co., Ltd. The siRNA sequences were as follows: Tie2 passenger strand, 5'-CcAuCaUuUgCcCaGaUaU-3', and guide strand, 5'-aUaUcUgGgCaAaUgAuGg-3'; and Control #2 siRNA passenger strand, 5'-AuCaCgUaCgCgGaAuAc UuCgA-3', and guide strand, 5'-uCgAaGuAuUcCgCgUaC gUgAu-3'. Lowercase letters represent 2'-*O*-methyl-modified nucleotides. Control #2 siRNA was used as a negative control for Tie2 siRNA.

Preparation of cationic liposomes and siRNA lipoplexes. Cationic liposomes were prepared using cationic lipids and phospholipids at a molar ratio of 1:1 as described previously (9). To prepare siRNA lipoplexes, a cationic liposome suspension was added to 50 pmol siRNA at a charge ratio (+:-) of 4:1, vortexed for 10 sec and left at room temperature for 15 min, as previously described (9).

Particle size and distribution [polydispersity index (PDI)] of cationic liposomes and siRNA lipoplexes were measured using a light-scattering photometer (cat. no. ELS-Z2; Otsuka Electronics Co., Ltd.), as previously described (9).

Cell culture. Human breast cancer MCF-7 cells stably expressing firefly Luc (MCF-7-Luc) were donated by Dr. Kenji Yamato (University of Tsukuba, Tsukuba, Japan) (12). Using STR DNA profile analysis, it was confirmed that the MCF-7-Luc cells used in the present study were identical to the MCF-7 cells registered in the database of Japanese Collection of Research Bioresources Cell Bank (JCRB, National Institute of Biomedical Innovation). MCF-7-Luc cells were cultured in RPMI-1640 medium (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific) and 1.2 mg/ml G418 sulfate (geneticin; FUJIFILM Wako Pure Chemical Corporation) at 37°C in a humidified atmosphere with 5% CO_2.

Gene-knockdown in MCF-7 cells using siRNA lipoplexes. MCF-7-Luc cells were seeded in six-well culture plates at a density of $3x10^5$ cells/well at 37°C. After 24 h, each siRNA lipoplex containing 50 pmol Control #1 siRNA or Luc siRNA was diluted in 1 ml of RPMI-1640 medium supplemented with 10% FBS (final concentration, 50 nM siRNA) and added to the cells. MCF-7-Luc cells without the addition of cationic liposomes or siRNA lipoplexes were used as untreated cells. Following 48 h of incubation at 37°C, the cells were lysed by the addition of 250 μl of cell lysis buffer (Pierce™ Luciferase Cell Lysis Buffer; Pierce; Thermo Fisher Scientific, Inc.) after washing with PBS, and subjected to one cycle of freezing (-80°C) and thawing at 37°C, followed by centrifugation at 13,000 x g for 10 sec at 4°C. Aliquots of 10 μl of the supernatants of cell lysates were mixed with 50 μl of PicaGene MelioraStar-LT Luminescence Reagent (Toyo Ink Mfg. Co., Ltd.) and the luminescence was measured as counts per sec (cps) with a chemoluminometer (ARVO X2; PerkinElmer, Inc.). Protein concentrations of the supernatants were determined with bicinchoninic acid (BCA) reagent (Pierce™ BCA Protein Assay Kit; Pierce; Thermo Fisher Scientific, Inc.),

using bovine serum albumin (Thermo Fisher Scientific, Inc.) as a standard, and the luciferase activity (cps/μg protein) was calculated. Luc activity (%) was calculated relative to that of untreated cells according to the formula below. Luc activity (%)=(Luc activity (cps/μg protein) in siRNA transfected cells/Luc activity (cps/μg protein) in untreated cells) x100.

Cytotoxicity of siRNA lipoplexes. Each siRNA lipoplex sample with 5 pmol Control #1 siRNA was diluted in 100 μl of RPMI-1640 medium supplemented with 10% FBS (final concentration, 50 nM siRNA) and added to MCF-7-Luc cells at 50% confluency in 96-well plates. MCF-7-Luc cells without the addition of cationic liposomes or siRNA lipoplexes were used as untreated cells. At 24 h post-transfection, cell viability was measured using a Cell Counting Kit-8 (Dojindo Laboratories, Inc.), as previously reported (9). WST-8 substrate was incubated with cells at 37˚C for 60 min. The cell viability (%) was calculated relative to that of untreated cells according to the formula below. Cell viability (%)=(absorbance at 450 nm in siRNA transfected cells/absorbance at 450 nm in untreated cells) x100.

Biodistribution of siRNA following intravenous injection of siRNA lipoplexes into mice. Ethical approval was obtained from the Institutional Animal Care and Use Committee of Hoshi University (approval no. P22-016). A total of six female *BALB/c* mice (weight, 18-20 g; age, 8 weeks; Sankyo Labo Service Corporation, Inc.) were housed at 24˚C and 55% humidity under a 12/12-h light/dark cycle (lights on at 8:00 a.m.) with *ad libitum* access to food and water. siRNA lipoplexes with 20 μg of Cy5-siRNA were systemically administered to mice via the lateral tail vein (n=1/siRNA lipoplex). At 1 h after injecting the siRNA lipoplexes, the mice were euthanized by cervical dislocation, and death was confirmed by cessation of the heartbeat. Tissues (lungs, heart, liver, spleen and kidneys) were analyzed through Cy5 fluorescence imaging using the NightOWL LB981 NC100 system (Berthold Technologies GmbH & Co. KG) as previously described (9,13). Thereafter, the tissue samples were frozen on dry ice and sliced into 16-μm sections. The localization of the Cy5-siRNA was examined using a fluorescence microscope (Eclipse TS100-F; Nikon Corporation).

Hemolysis and agglutination assay. Blood (0.2 ml) was collected from the jugular vein of one female BALB/c mouse (age, 8 weeks; Sankyo Labo Service Corp., Inc.) under anesthesia induced by isoflurane inhalation (1.5% gas-air mixture; FUJIFILM Wako Pure Chemical Corporation). After the blood collection, the mice were euthanized by cervical dislocation. Erythrocytes were collected from the blood at 4˚C by centrifugation at 300 x g for 3 min and resuspended in PBS as a 2% (v/v) suspension of erythrocytes. siRNA lipoplexes with 2 μg of siRNA were added to 100 μl of a 2% (v/v) erythrocyte suspension. Following incubation for 15 min at 37˚C, the sample was placed on a glass plate and agglutination was observed using a light microscope (Eclipse TS100-F; Nikon Corporation). For hemolysis of erythrocytes, 100 μl of the 2% (v/v) erythrocyte suspension was centrifugated at 200 x g for 3 min after incubation with 2 μg of siRNA lipoplexes for 15 min at 37˚C and hemolysis of erythrocytes was observed. Hemolysis (%) was calculated as described previously (14).

Expression of Tie2 mRNA in the lung following systemic injection of siRNA lipoplexes. siRNA lipoplexes with 20 μg of Control #2 or Tie2 siRNA were administered systemically to 8-week-old female BALB/c mice via the lateral tail veins (n=3-4/siRNA lipoplex). All mice were euthanized by cervical dislocation 48 h after injection of siRNA lipoplexes and then the lungs were excised. The lungs from mouse without the injection of siRNA lipoplexes were used as untreated lungs (n=4). Total RNA was isolated from the lungs (a total of 31 mice) using Isogen II (Nippon Gene Co., Ltd.). cDNA was synthesized from total RNA using PrimeScript™ RT Master Mix (Takara Bio, Inc.) according to the manufacturer's protocol and quantitative PCR was performed using a Roche Light Cycler 96 system with FastStart Essential DNA Probes Master (Roche Diagnostics GmbH) and TaqMan™ Gene expression assays (cat. no. 4331182; Applied Biosystems; Thermo Fisher Scientific, Inc.) [FAM™ dye-labeled TaqMan® MGB probe and PCR primers: Tie2, cat. no. Mm00443243_m1; phosphatase and tensin homolog (PTEN); cat. no. Mm00477208_m1; Applied Biosystems; Thermo Fisher Scientific, Inc.; primer sequences not available]. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 600 sec, followed by 45 cycles of denaturation at 95˚C for 10 sec and primer annealing and extension at 60˚C for 30 sec (two-step amplification). Tie2 and PTEN are expressed in normal endothelial cells (15,16), and PTEN has been used as a reference gene of endothelial cells (9,17,18). Therefore, the expression levels of Tie2 mRNA (threshold cycle (Cq) value, ~23-24) were normalized to those of PTEN mRNA (Cq value, ~22-23) in each sample and analyzed using the comparative Cq ($2^{-\Delta\Delta Cq}$) method (19).

Statistical analysis. Data are presented as the mean ± SD of triplicate measurements. Statistical significance was determined using an unpaired Student's t-test or one-way ANOVA followed by Tukey's post-hoc test using GraphPad Prism (version 4.0; GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Size of cationic liposomes and siRNA lipoplexes. To examine the effects of cationic lipids and phospholipids in liposomal formulations on gene-knockdown efficacy after treatment with siRNA lipoplexes, DC-1-14, DC-1-16, DC-6-14, DOTMA, DSTAP and DODAP were used as cationic lipids for the preparation of cationic liposomes (Fig. 1). In our previous study on cationic liposomes containing cationic lipids DDAB and DOTAP, compared with phosphatidylcholines, the inclusion of phosphatidylethanolamines in liposomal formulations results in high gene-knockdown activity in cells (9); therefore, in the present study, DSPE, DPPE, DMPE, POPE and DOPE were used as phospholipids (Fig. 1). Cationic liposomes were prepared from cationic lipid and phosphatidylethanolamine at a molar ratio of 1:1 (Table I).

The size of cationic liposomes was 80-293 nm (PDI, 0.19-0.31) (Table I) although both LP-DC-1-14/DSPE and LP-DODAP/DSPE aggregated during preparation (>1 μm in size) (Table I). Our previous study determined that the optimal charge ratio (+:-) for the preparation of siRNA lipoplexes was 4:1 for LP-DC-1-14/DOPE, LP-DC-1-16/DOPE

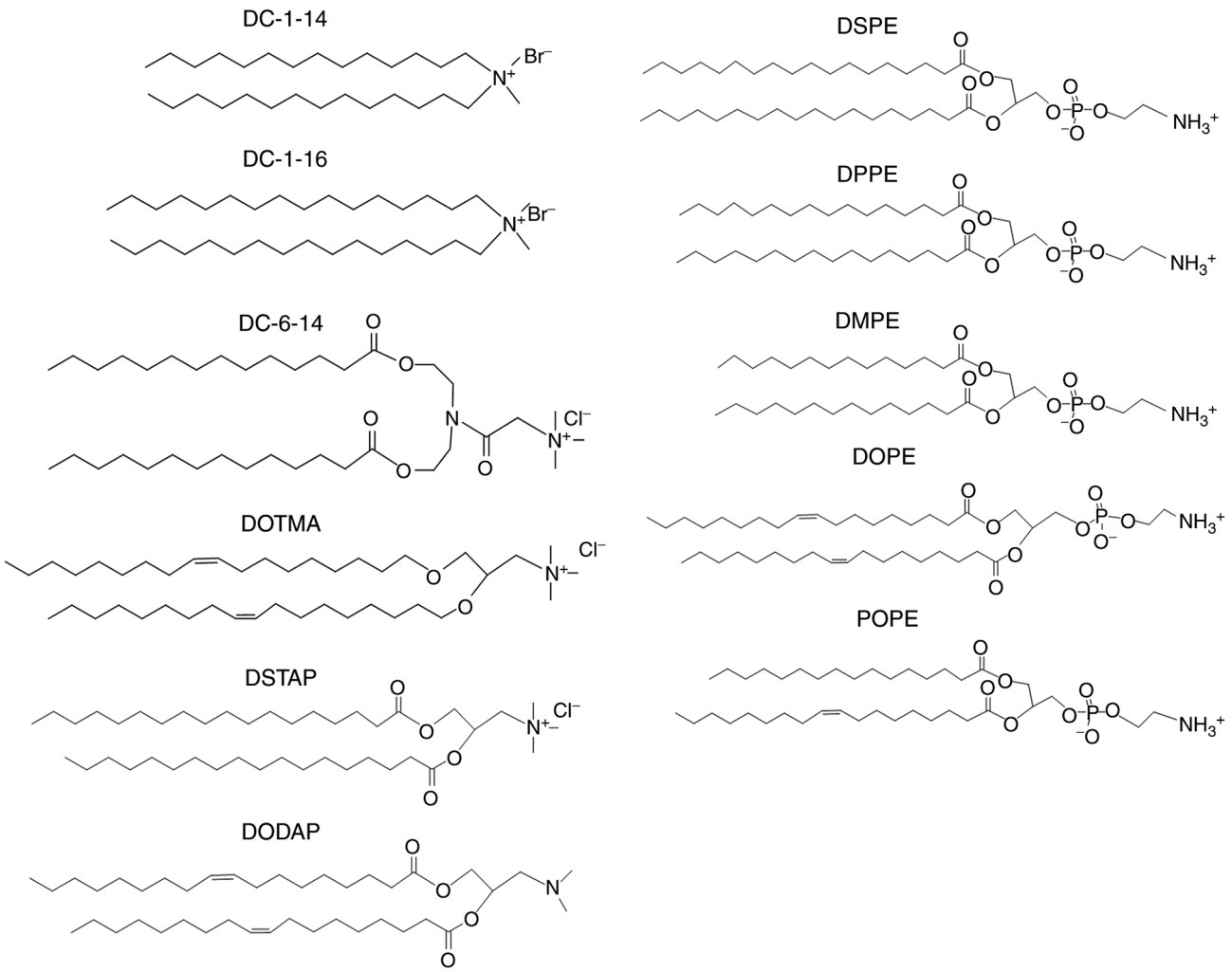

Figure 1. Structure of cationic lipids and phospholipids. DC-1-14, *N*,*N*-dimethyl-*N*-tetradecyltetradecan-1-aminium bromide; DC-1-16, *N*-hexadecyl-*N*,*N*-dimethylhexadecan-1-aminium bromide; DC-6-14, 2-[bis{2-(tetradecanoyloxy)ethyl}amino]-*N*,*N*,*N*-trimethyl-2-oxoethan-1-aminium chloride; DSTAP, 1,2-distearoyl-3-trimethylammonium-propane chloride; DOTMA, 1,2-di-*O*-octadecenyl-3-trimethylammonium propane chloride; DODAP, 1,2-dioleoyl-3-dimethylammonium-propane; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine.

and LP-DC-6-14/DOPE (8,11). Therefore, the present study employed a charge ratio (+:-) of 4:1 to prepare the siRNA lipoplexes. LP-DC-1-16/DSPE, LP-DC-6-14/DSPE, LP-DSTAP/DSPE, LP-DODAP/DPPE and LP-DODAP/DMPE lipoplexes aggregated (>1 μm in size) when the liposomes were mixed with the siRNA solution (Table I). The size of LP-DC-1-14/DPPE, LP-DC-1-14/POPE, LP-DC-1-16/DPPE, LP-DC-1-16/POPE, LP-DC-6-14/DPPE, LP-DOTMA/DSPE, LP-DOTMA/DPPE, LP-DOTMA/DMPE, LP-DOTMA/POPE, LP-DSTAP/DPPE and LP-DODAP/POPE lipoplexes increased to 428-937 nm (PDI, 0.20-0.39). However, the sizes of the other lipoplexes were 181-339 nm (PDI, 0.15-0.22). Overall, the inclusion of DSPE or DPPE in the liposomal formulation increased the size of the siRNA lipoplexes after preparation.

Effect of phosphatidylethanolamines in cationic liposomes on gene-knockdown efficacy in MCF-7 cells. To determine the effect of phosphatidylethanolamine in liposomal formulations on gene-knockdown efficacy using siRNA lipoplexes, each Luc siRNA lipoplex was transfected into MCF-7-Luc cells and gene-knockdown efficacy was evaluated by measuring Luc activity. LP-DC-1-16/DOPE, LP-DC-6-14/DOPE, LP-DOTMA/DPPE, LP-DOTMA/DMPE, LP-DOTMA/DOPE, LP-DOTMA/POPE, LP-DSTAP/DOPE and LP-DODAP/DOPE lipoplexes with Luc siRNA strongly suppressed Luc activity (>70% knockdown) compared with Control #1 siRNA (Fig. 2 and Table II). LP-DC-1-14/DOPE, LP-DC-1-16/DSPE, LP-DOTMA/DSPE, LP-DODAP/DPPE and LP-DODAP/POPE lipoplexes with Luc siRNA moderately suppressed Luc activity (50-70% knockdown) compared with Control #1 siRNA (Fig. 2 and Table II). Furthermore, LP-DC-1-14/DMPE, LP-DC-1-14/POPE, LP-DC-1-16/DPPE, LP-DC-1-16/POPE and LP-DODAP/DMPE lipoplexes with Luc siRNA slightly suppressed Luc activity (30-50% knockdown) compared with Control #1 siRNA (Fig. 2 and Table II).

Cytotoxicity after treatment with siRNA lipoplexes. The toxicity of phosphatidylethanolamines in the liposomal formulations on MCF-7-Luc cells was evaluated 24 h

Table I. Particle size of cationic liposomes and siRNA lipoplexes.

		Liposomes		Lipoplex[a]	
Liposome	Formulation (mol ratio)	Size[b] (nm)	PDI	Size[b] (nm)	PDI
LP-DC-1-14/DSPE	DC-1-14:DSPE (1:1)	Aggregation	-	N.D.	N.D.
LP-DC-1-14/DPPE	DC-1-14:DPPE (1:1)	123.4±1.5	0.20±0.02	428.1±86.6	0.20±0.03
LP-DC-1-14/DMPE	DC-1-14:DMPE (1:1)	104.5±6.6	0.25±0.00	240.1±14.2	0.16±0.06
LP-DC-1-14/DOPE	DC-1-14:DOPE (1:1)	102.4±2.6	0.23±0.01	290.2±18.2	0.25±0.04
LP-DC-1-14/POPE	DC-1-14:POPE (1:1)	80.4±2.3	0.24±0.01	540.9±87.8	0.24±0.03
LP-DC-1-16/DSPE	DC-1-16:DSPE (1:1)	134.8±4.7	0.21±0.01	Aggregation	-
LP-DC-1-16/DPPE	DC-1-16:DPPE (1:1)	195.7±6.7	0.29±0.00	579.2±85.0	0.25±0.04
LP-DC-1-16/DMPE	DC-1-16:DMPE (1:1)	99.3±1.0	0.22±0.01	207.6±38.7	0.15±0.07
LP-DC-1-16/DOPE	DC-1-16:DOPE (1:1)	107.1±1.9	0.22±0.01	180.8±7.3	0.19±0.04
LP-DC-1-16/POPE	DC-1-16:POPE (1:1)	116.9±1.4	0.26±0.00	517.6±91.8	0.23±0.03
LP-DC-6-14/DSPE	DC-6-14:DSPE (1:1)	107.6±1.7	0.25±0.00	Aggregation	-
LP-DC-6-14/DPPE	DC-6-14:DPPE (1:1)	157.7±2.3	0.26±0.02	593.1±18.4	0.26±0.01
LP-DC-6-14/DMPE	DC-6-14:DMPE (1:1)	92.3±0.5	0.22±0.01	195.1±14.1	0.20±0.03
LP-DC-6-14/DOPE	DC-6-14:DOPE (1:1)	113.4±2.6	0.26±0.01	260.3±27.6	0.19±0.06
LP-DC-6-14/POPE	DC-6-14:POPE (1:1)	109.3±0.5	0.23±0.01	223.0±22.1	0.16±0.08
LP-DOTMA/DSPE	DOTMA:DSPE (1:1)	124.3±1.1	0.25±0.00	937.3±89.8	0.39±0.04
LP-DOTMA/DPPE	DOTMA:DPPE (1:1)	232.3±10.8	0.29±0.01	650.8±51.2	0.28±0.02
LP-DOTMA/DMPE	DOTMA:DMPE (1:1)	202.6±9.3	0.29±0.01	922.4±96.1	0.37±0.03
LP-DOTMA/DOPE	DOTMA:DOPE (1:1)	199.0±1.6	0.29±0.01	338.7±16.8	0.15±0.01
LP-DOTMA/POPE	DOTMA:POPE (1:1)	292.5±4.9	0.31±0.00	631.2±28.4	0.27±0.01
LP-DSTAP/DSPE	DSTAP:DSPE (1:1)	160.6±38.1	0.19±0.01	Aggregation	-
LP-DSTAP/DPPE	DSTAP:DPPE (1:1)	133.7±1.5	0.24±0.01	794.0±110.0	0.33±0.04
LP-DSTAP/DMPE	DSTAP:DMPE (1:1)	116.1±1.0	0.23±0.01	261.0±17.9	0.16±0.03
LP-DSTAP/DOPE	DSTAP:DOPE (1:1)	150.8±1.8	0.25±0.01	335.1±11.6	0.15±0.00
LP-DSTAP/POPE	DSTAP:POPE (1:1)	148.1±2.8	0.20±0.01	206.8±0.2	0.22±0.00
LP-DODAP/DSPE	DODAP:DSPE (1:1)	Aggregation	-	N.D.	N.D.
LP-DODAP/DPPE	DODAP:DPPE (1:1)	182.1±2.2	0.26±0.00	Aggregation	-
LP-DODAP/DMPE	DODAP:DMPE (1:1)	177.8±2.0	0.22±0.01	Aggregation	-
LP-DODAP/DOPE	DODAP:DOPE (1:1)	121.1±0.8	0.22±0.01	255.7±31.5	0.23±0.03
LP-DODAP/POPE	DODAP:POPE (1:1)	149.6±0.6	0.21±0.00	528.2±121.7	0.23±0.05

[a]Charge ratio (+:-) of cationic lipid to siRNA phosphate=4:1. [b]In water. Aggregation indicates >1 μm in size. Each value represents the mean±S.D (n=3). PDI, polydispersity index; N.D., not determined; LP, liposome; DC-1-14, *N*,*N*-dimethyl-*N*-tetradecyltetradecan-1-aminium bromide; DC-1-16, *N*-hexadecyl-*N*,*N*-dimethylhexadecan-1-aminium bromide; DC-6-14, 2-[bis{2-(tetradecanoyloxy)ethyl}amino]-*N*,*N*,*N*-trimethyl-2-oxoethan-1-aminium chloride; DSTAP, 1,2-distearoyl-3-trimethylammonium-propane chloride; DOTMA, 1,2-di-*O*-octadecenyl-3-trimethylammonium propane chloride; DODAP, 1,2-dioleoyl-3-dimethylammonium-propane; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine.

post-transfection with siRNA lipoplexes. LP-DC-1-14/DPPE, LP-DC-1-14/POPE, LP-DC-1-16/POPE, LP-DOTMA/DSPE, LP-DSTAP/DMPE and LP-DODAP/DPPE lipoplexes showed slight cytotoxicity (70-80% cell viability), whereas the other lipoplexes did not exhibit cytotoxicity (>87% cell viability compared to untreated cells; Fig. 3). These results suggest that cationic liposomes composed of DOPE can be used for efficient gene-knockdown using siRNA lipoplexes with minimal toxicity.

Biodistribution of siRNA after intravenous injection of siRNA lipoplexes. The present study investigated the effect of cationic lipids in liposomal formulations on the biodistribution of siRNA 1 h after the systemic injection of lipoplexes with Cy5-siRNA. LP-DC-1-14/DOPE, LP-DC-1-16/DOPE, LP-DC-6-14/DOPE, LP-DOTMA/DOPE, LP-DSTAP/DOPE and LP-DODAP/DOPE were selected because their lipoplexes showed high gene-knockdown efficacy in cells (Table II) and were relatively small (<340 nm). The LP-DC-1-14/DOPE

Table II. Summary of *in vitro* gene knockdown efficacy after treatment with siRNA lipoplexes.

	Cationic lipid					
Phospholipid	DC-1-14	DC-1-16	DC-6-14	DOTMA	DSTAP	DODAP
DSPE	N.D.	++	-	++	-	N.D.
DPPE	-	+	-	+++	-	++
DMPE	+	-	-	+++	-	+
DOPE	++	+++	+++	+++	+++	+++
POPE	+	+	-	+++	-	++

+++, >70% knockdown; ++, 50-70% knockdown; +, 30-50% knockdown; -, <30% knockdown, compared with Control #1 siRNA. N.D., not determined; DC-1-14, *N*,*N*-Dimethyl-*N*-tetradecyltetradecan-1-aminium bromide; DC-1-16, *N*-hexadecyl-*N*,*N*-dimethylhexadecan-1-aminium bromide; DC-6-14, 2-[bis{2-(tetradecanoyloxy)ethyl}amino]-*N*,*N*,*N*-trimethyl-2-oxoethan-1-aminium chloride; DSTAP, 1,2-distearoyl-3-trimethylammonium-propane chloride; DOTMA, 1,2-di-*O*-octadecenyl-3-trimethylammonium propane chloride; DODAP, 1,2-dioleoyl-3-dimethylammonium-propane; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine.

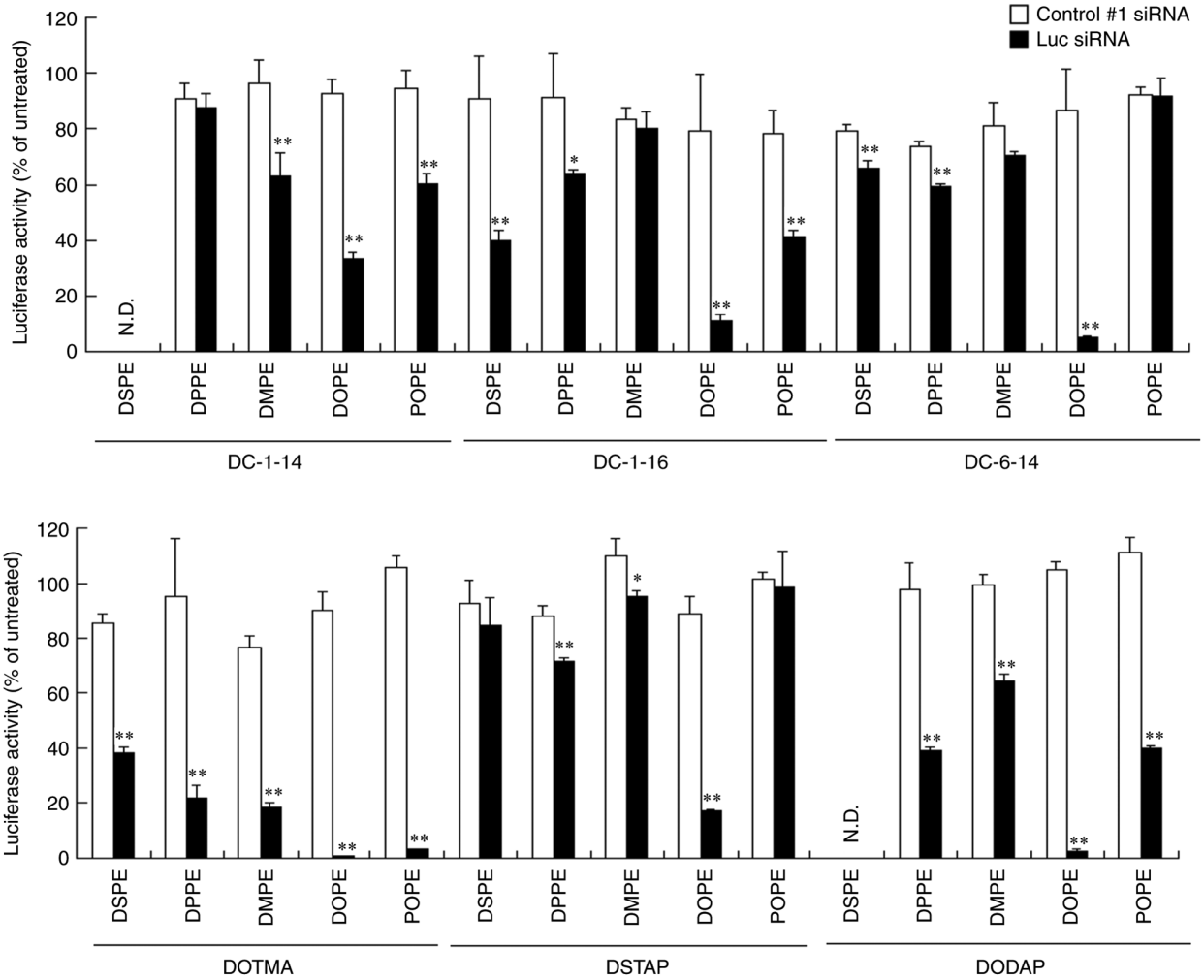

Figure 2. Effect of cationic lipids and phosphatidylethanolamines in liposomal formulations on the efficacy of gene-knockdown after transfection of siRNA lipoplexes into MCF-7-Luc cells. siRNA lipoplexes with Control #1 or Luc siRNA were added to MCF-7-Luc cells at a concentration of 50 nM and Luc assay was performed 48 h post-transfection. (n=3). *P<0.05, **P<0.01 vs. Control #1 siRNA. Luc, luciferase; si, small interfering; DC-1-14, *N*,*N*-dimethyl-*N*-tetradecyltetradecan-1-aminium bromide; DC-1-16, *N*-hexadecyl-*N*,*N*-dimethylhexadecan-1-aminium bromide; DC-6-14, 2-[bis{2-(tetradecanoyloxy)ethyl}amino]-N,N,N-trimethyl-2-oxoethan-1-aminium chloride; DSTAP, 1,2-distearoyl-3-trimethylammonium-propane chloride; DOTMA, 1,2-di-*O*-octadecenyl-3-trimethylammonium propane chloride; DODAP, 1,2-dioleoyl-3-dimethylammonium-propane; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine.

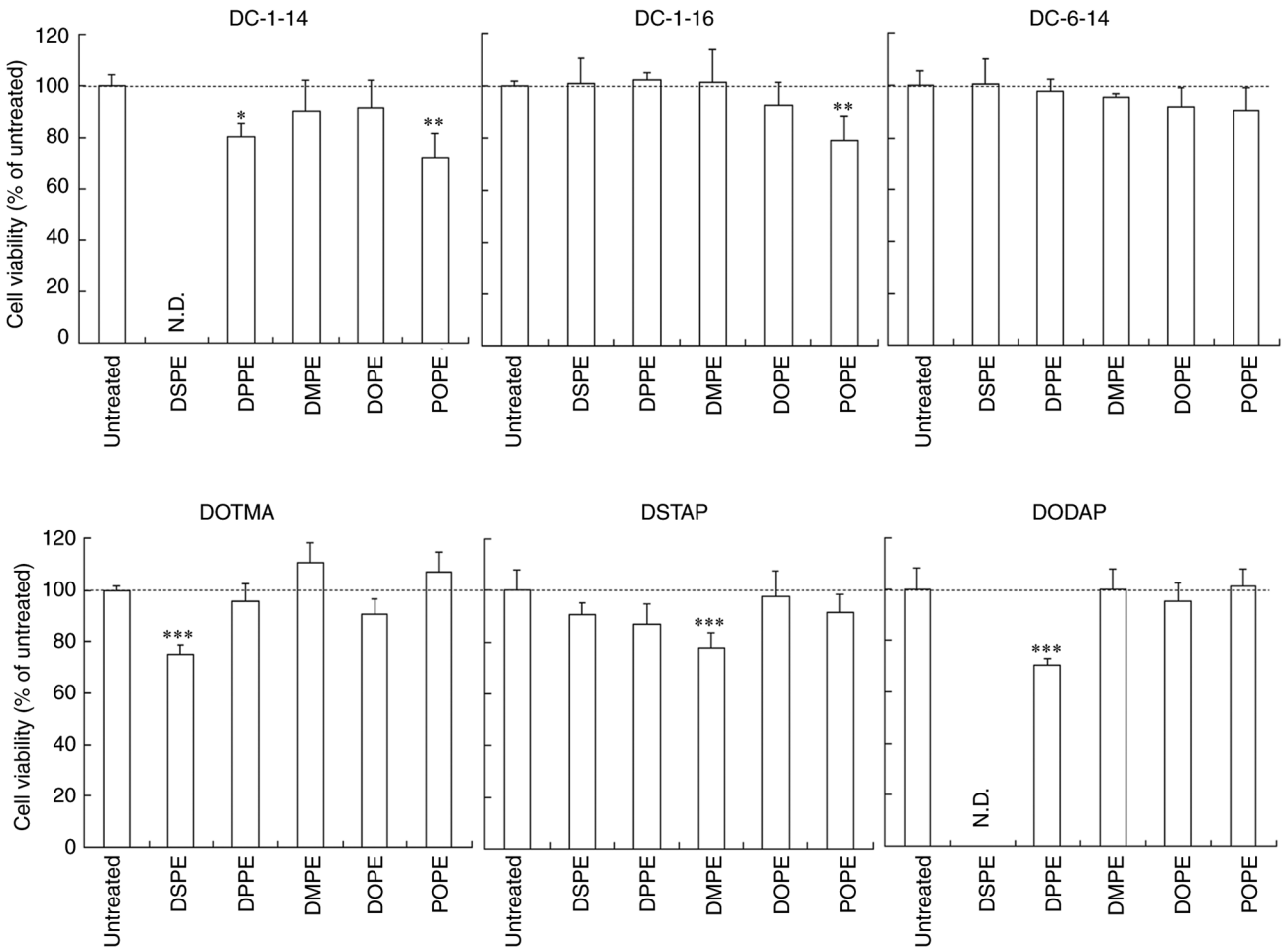

Figure 3. Effect of cationic lipids and phosphatidylethanolamines in liposomal formulations on cell viability at 24 h after transfection of siRNA lipoplexes into MCF-7-Luc cells. siRNA lipoplexes were transfected into MCF-7-Luc cells at a concentration of 50 nM. (n=4-6). *P<0.05, **P<0.01, ***P<0.001 vs. untreated. si, small interfering; DC-1-14, *N*,*N*-dimethyl-*N*-tetradecyltetradecan-1-aminium bromide; DC-1-16, *N*-hexadecyl-*N*,*N*-dimethylhexadecan-1-aminium bromide; DC-6-14, 2-[bis{2-(tetradecanoyloxy)ethyl}amino]-*N*,*N*,*N*-trimethyl-2-oxoethan-1-aminium chloride; DSTAP, 1,2-distearoyl-3-trimethylammonium-propane chloride; DOTMA, 1,2-di-*O*-octadecenyl-3-trimethylammonium propane chloride; DODAP, 1,2-dioleoyl-3-dimethylammonium-propane; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine.

and LP-DODAP/DOPE lipoplexes caused siRNA accumulation in the liver (Figs. 4 and 5). The LP-DC-1-16/DOPE and LP-DC-6-14/DOPE lipoplexes caused siRNA accumulation in the lungs and liver. In particular, the LP-DOTMA/DOPE and LP-DSTAP/DOPE lipoplexes led to high siRNA accumulation in the lungs. For all lipoplexes, siRNAs were weakly detected in the spleen but not in the heart. All siRNA lipoplexes led to a relatively high accumulation of siRNA in the kidneys.

Aggregation and hemolysis after mixing siRNA lipoplexes with erythrocytes. The effect of cationic lipids in cationic liposomes on aggregation and hemolysis was evaluated by mixing siRNA lipoplexes with an erythrocyte suspension. The LP-DC-1-14/DOPE, LP-DC-1-16/DOPE, LP-DC-6-14/DOPE, LP-DOTMA/DOPE and LP-DSTAP/DOPE lipoplexes exhibited agglutination after mixing with the erythrocyte suspension (Fig. 6A). In particular, the LP-DC-1-16/DOPE, LP-DC-6-14/DOPE and LP-DOTMA/DOPE lipoplexes formed large aggregates after mixing with the erythrocyte suspension. By contrast, the LP-DODAP/DOPE lipoplexes did not agglutinate erythrocytes. The LP-DC-1-14/DOPE and LP-DOTMA/DOPE lipoplexes exhibited moderate levels of hemolysis (28.6 and 24.1%, respectively) after mixing with the erythrocyte suspension. However, other lipoplexes exhibited low levels of hemolysis (<14%).

Gene-knockdown in the lungs after systemic injection of siRNA lipoplexes. Tie2 has previously been used to evaluate the gene-knockdown efficacy of siRNA lipoplexes in the vascular endothelium of the lungs (17). The present study evaluated the effect of liposomal formulations on Tie2 mRNA knockdown in the pulmonary vascular endothelium 48 h after a single systemic injection of Tie2 siRNA lipoplexes into mice (Fig. 7). LP-DC-1-16/DOPE, LP-DC-6-14/DOPE, LP-DOTMA/DOPE and LP-DSTAP/DOPE were selected to evaluate the knockdown efficacy, as their lipoplexes exhibited siRNA accumulation in the lungs (Figs. 4 and 5). LP-DC-1-16/DOPE lipoplexes with Tie2 siRNA highly suppressed Tie2 mRNA expression (37.4% knockdown) compared with Control #2 siRNA. However, LP-DOTMA/DOPE lipoplexes with Tie2 siRNA slightly

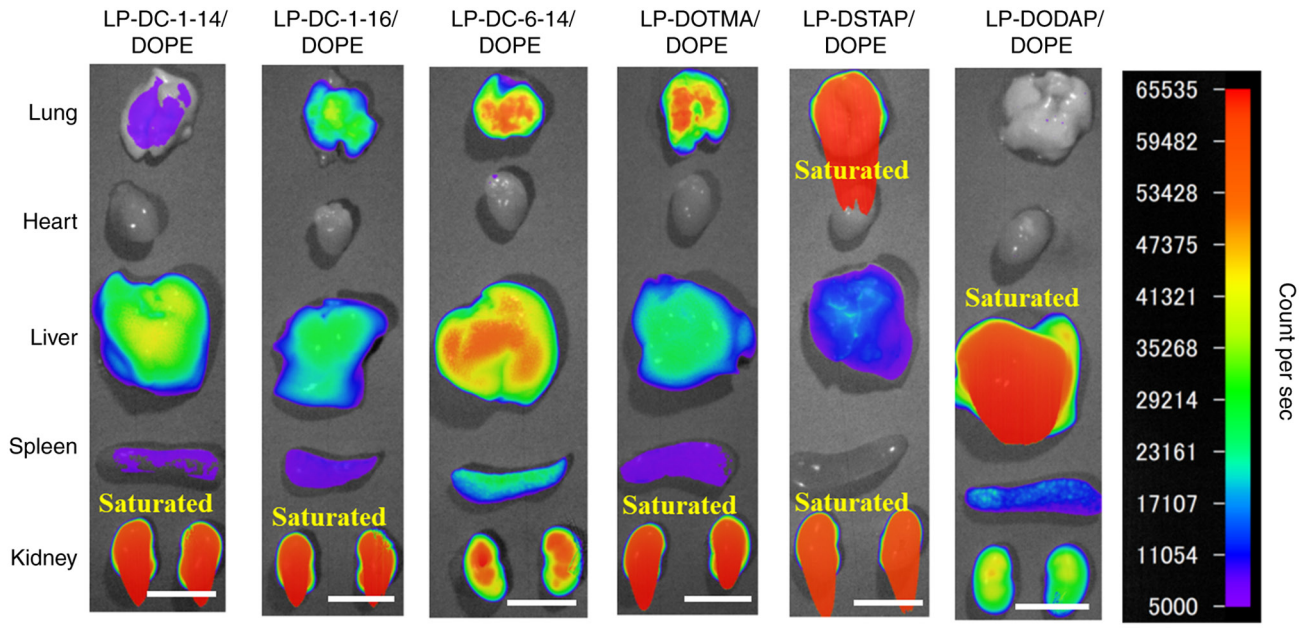

Figure 4. Effect of cationic lipids in liposomal formulations on the biodistribution of siRNA in mice at 1 h after systemic injection of siRNA lipoplexes. siRNA lipoplexes with 20 μg of Cy5-siRNA were administered systemically to mice. Cy5 fluorescence imaging of tissue was performed 1 h post-injection. Fluorescence intensity is illustrated using a color-coded scale (red, maximum; purple, minimum). Scale bar, 1 cm. si, small interfering; Cy5, cyanine 5; DC-1-14, *N*,*N*-dimethyl-*N*-tetradecyltetradecan-1-aminium bromide; DC-1-16, *N*-hexadecyl-*N*,*N*-dimethylhexadecan-1-aminium bromide; DC-6-14, 2-[bis{2-(tetradecanoyloxy)ethyl}amino]-*N*,*N*,*N*-trimethyl-2-oxoethan-1-aminium chloride; DSTAP, 1,2-distearoyl-3-trimethylammonium-propane chloride; DOTMA, 1,2-di-*O*-octadecenyl-3-trimethylammonium propane chloride; DODAP, 1,2-dioleoyl-3-dimethylammonium-propane; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine.

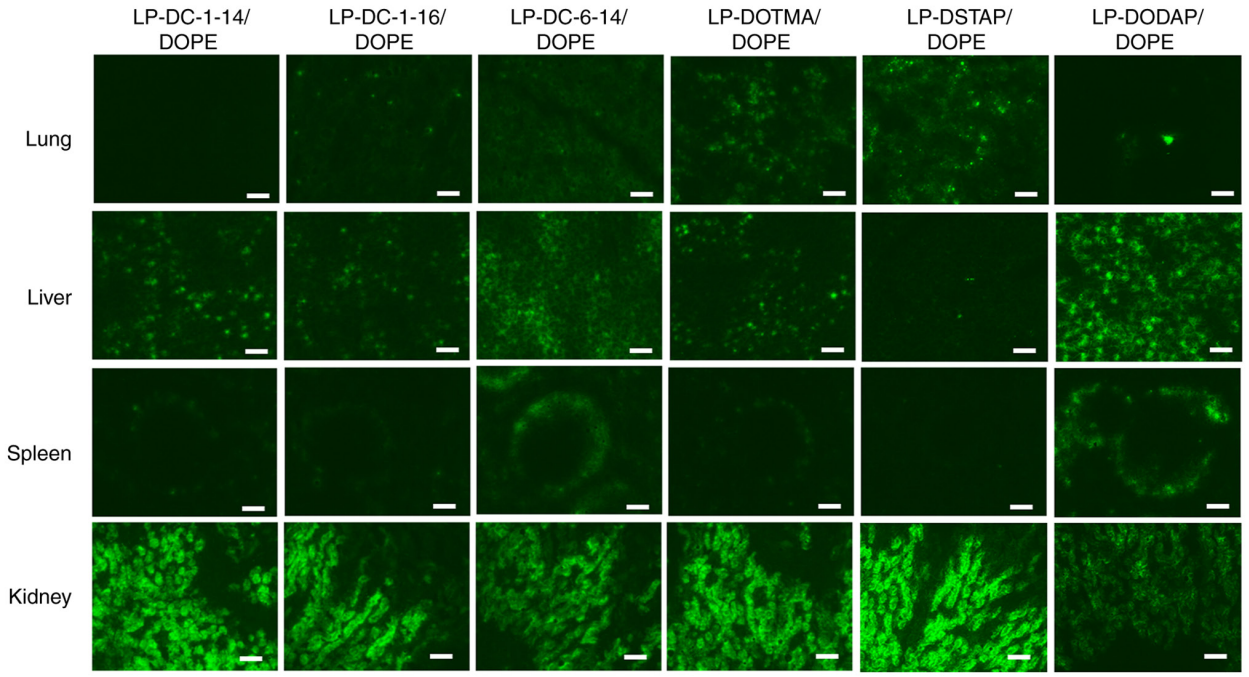

Figure 5. Effect of cationic lipids in liposomal formulation on the biodistribution of siRNA in mice 1 h following systemic injection of siRNA lipoplexes. siRNA lipoplexes with 20 μg of Cy5-siRNA were administered intravenously to mice. At 1 h post-injection, tissues were collected, frozen, and sliced to observe the localization of Cy5-siRNA (green) using a fluorescent microscope. Scale bar, 100 μm. si, small interfering; Cy5, cyanine 5; DC-1-14, *N*,*N*-dimethyl-*N*-tetradecyltetradecan-1-aminium bromide; DC-1-16, *N*-hexadecyl-*N*,*N*-dimethylhexadecan-1-aminium bromide; DC-6-14, 2-[bis{2-(tetradecanoyloxy)ethyl}amino]-*N*,*N*,*N*-trimethyl-2-oxoethan-1-aminium chloride; DSTAP, 1,2-distearoyl-3-trimethylammonium-propane chloride; DOTMA, 1,2-di-*O*-octadecenyl-3-trimethylammonium propane chloride; DODAP, 1,2-dioleoyl-3-dimethylammonium-propane; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine.

suppressed Tie2 mRNA expression in the lungs (14.8% knockdown) compared with Control #2 siRNA and LP-DC-6-14/DOPE, and LP-DSTAP/DOPE lipoplexes with Tie2 siRNA did not significantly suppress Tie2 mRNA expression. None of the siRNA lipoplexes caused mice mortality after systemic injection.

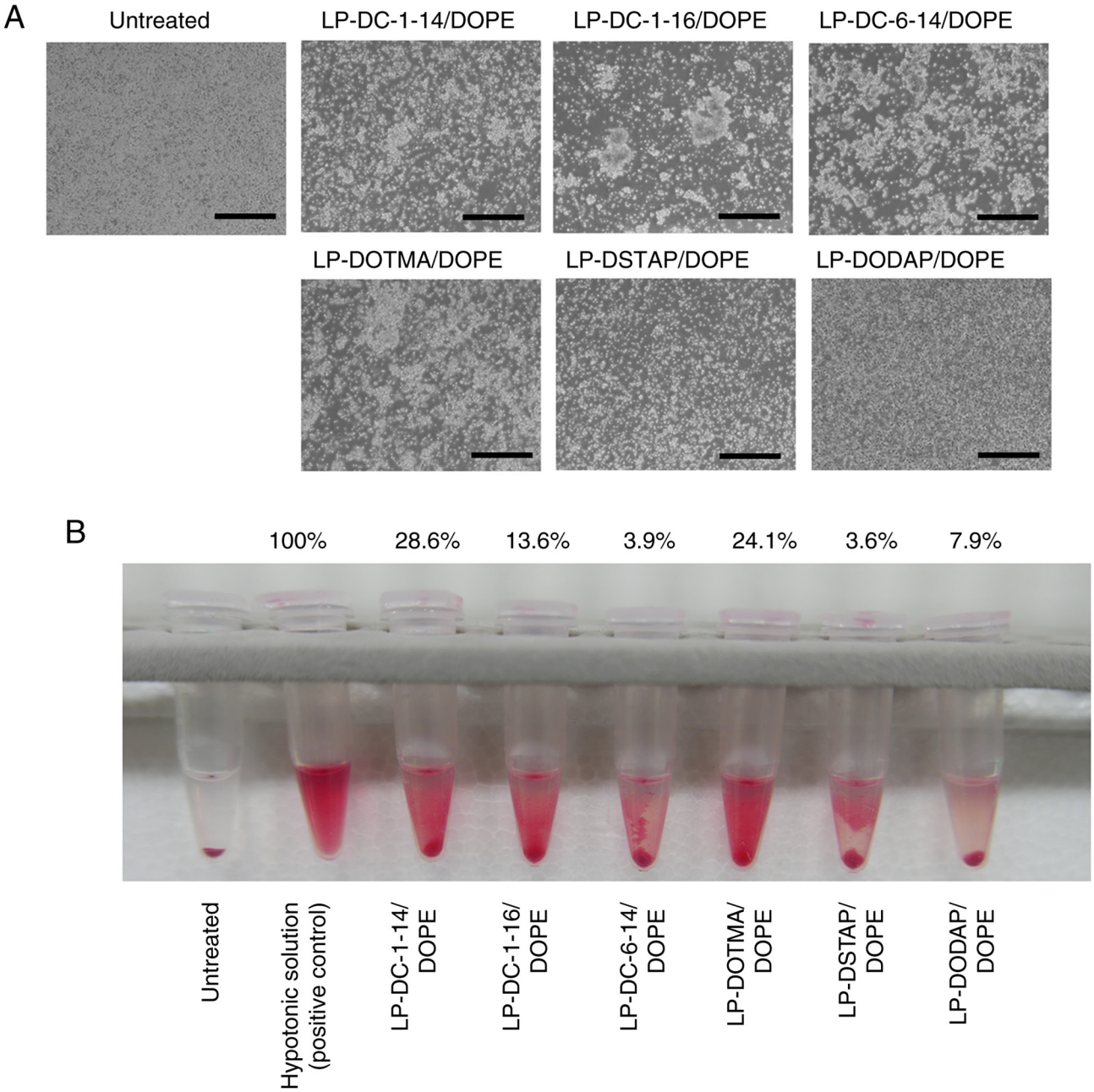

Figure 6. Effect of cationic lipids in liposomal formulations on the agglutination and hemolysis of erythrocytes after treatment with siRNA lipoplexes. Lipoplexes with 2 μg of siRNA were incubated with erythrocyte suspension. (A) Agglutination was observed under a light microscope (scale bar, 200 μm). (B) Hemolysis of erythrocytes after treatment with siRNA lipoplexes was observed. As a positive control for hemolysis (100% hemolysis), erythrocytes were suspended in hypotonic solution (water). Hemolysis (%) was calculated relative to the absorbance of treatment with hypotonic solution, and it is presented as mean (n=4). DC-1-14, *N*,*N*-dimethyl-*N*-tetradecyltetradecan-1-aminium bromide; DC-1-16, *N*-hexadecyl-*N*,*N*-dimethylhexadecan-1-aminium bromide; DC-6-14, 2-[bis{2-(tetradecanoyloxy)ethyl}amino]-*N*,*N*,*N*-trimethyl-2-oxoethan-1-aminium chloride; DSTAP, 1,2-distearoyl-3-trimethylammonium-propane chloride; DOTMA, 1,2-di-*O*-octadecenyl-3-trimethylammonium propane chloride; DODAP, 1,2-dioleoyl-3-dimethylammonium-propane; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine.

Discussion

Generally, cationic liposomes are prepared by combining cationic lipids with neutral helper lipids. However, to achieve efficient siRNA transfection, identifying the optimal combination of cationic and neutral helper lipids in the liposomal formulations is important. In the present study, the effect of phosphatidylethanolamines in cationic liposomes on the gene-knockdown efficacy of siRNA lipoplexes was determined using six types of cationic lipids. In *in vitro* transfection, LP-DC-1-16/DOPE, LP-DC-6-14/DOPE, LP-DOTMA/DPPE, LP-DOTMA/DMPE, LP-DOTMA/DOPE, LP-DOTMA/POPE, LP-DSTAP/DOPE and LP-DODAP/DOPE lipoplexes containing Luc siRNA strongly suppressed Luc activity (>70% knockdown) compared with Control #1 siRNA. Regardless of the type of cationic lipid, the inclusion of DOPE induced strong Luc knockdown in MCF-7-Luc cells. DOPE contains two unsaturated acyl chains and a relatively small head group, which forms a hexagonal phase at acidic pH (20) and destabilizes the liposomal

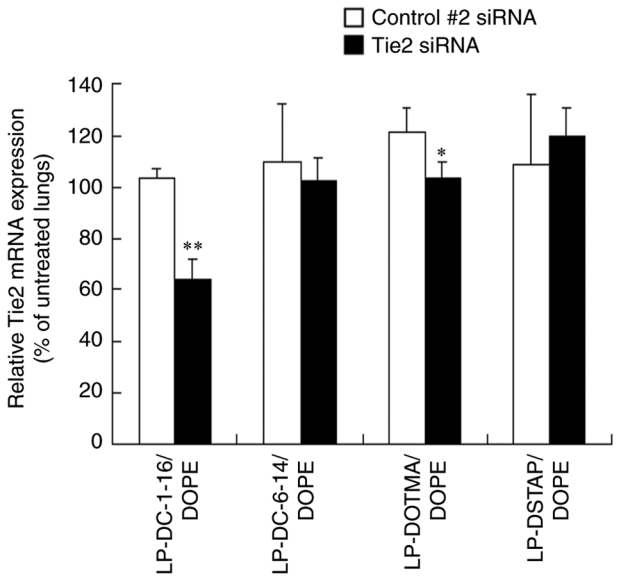

Figure 7. Effect of cationic lipids in liposomal formulations on the knockdown of Tie2 mRNA in the lung following systemic injection of Tie2 siRNA lipoplexes into mice. Tie2 mRNA level in the lung was normalized to PTEN mRNA level at 48 h after systemic administration of siRNA lipoplex with 20 μg of Control #2 or Tie2 siRNA (n=3-4). Tie2 expression (%) was calculated relative to that in untreated lungs. *P<0.05, **P<0.01 vs. Control #2 siRNA. si, small interfering; PTEN, phosphatase and tensin homolog; DC-1-16, *N*-hexadecyl-*N*,*N*-dimethylhexadecan-1-aminium bromide; DC-6-14, 2-[bis{2-(tetradecanoyloxy)ethyl}amino]-*N*,*N*,*N*-trimethyl-2-oxoethan-1-aminium chloride; DSTAP, 1,2-distearoyl-3-trimethylammonium-propane chloride; DOTMA, 1,2-di-O-octadecenyl-3-trimethylammonium propane chloride; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine.

structure (21). Therefore, DOPE may improve gene-knockdown efficiency by destabilizing the endosomal membrane after cellular uptake of siRNA lipoplexes via endocytosis, thereby resulting in the release of siRNA into the cytoplasm. However, in DOTMA-based cationic liposomes, the inclusion of DMPE, DPPE and POPE resulted in high gene-knockdown activity in the present study. DOTMA is a stable diether analog of DOTAP which has hydrolysable ester bonds as a linker between a quaternary ammonium head group and acyl chains (22). In our recent study, siRNA lipoplexes composed of DOTAP and DOPE or DOTAP and POPE demonstrated strong gene-knockdown efficacy, whereas siRNA lipoplexes composed of DOTAP and DPPE or DOTAP and DMPE did not (9). This finding suggests that differences in the linkers in cationic lipids may affect the transfection efficacy of siRNA lipoplexes in combination with DMPE and DPPE.

DOTMA and DSTAP contain a quaternary ammonium head group with alkyl and acyl chains (C18), respectively, and are positively charged irrespective of pH (23). Regarding siRNA biodistribution, systemic injection of LP-DOTMA/DOPE and LP-DSTAP/DOPE lipoplexes resulted in high siRNA accumulation in the lungs. Generally, in systemic circulation, positively charged siRNA lipoplexes form agglutinates with negatively charged erythrocytes (24), which are entrapped by pulmonary capillaries soon after systemic injection (25). These findings indicate that LP-DOTMA/DOPE and LP-DSTAP/DOPE lipoplexes may form stable agglutinations with blood components, resulting in efficient entrapment in lung capillaries. However, systemic injection of LP-DODAP/DOPE lipoplexes resulted in high siRNA accumulation in the liver in the present study. DODAP is an ionizable cationic lipid with a pKa of 6.6 (26) and contains a ternary ammonium head group with acyl chains (C18). The LP-DODAP/DOPE lipoplexes did not agglutinate erythrocytes. Dilliard *et al* (27) reported that the inclusion of DODAP in lipid nanoparticles (LNPs) increases apolipoprotein E (ApoE)-binding to LNPs in the plasma, and then enhances the uptake by hepatocytes via the low density lipoprotein (LDL) receptor. This finding suggests that LP-DODAP/DOPE lipoplexes might interact with ApoE, but not with erythrocytes in the systemic circulation, resulting in accumulation in the liver via the LDL receptor. By contrast, the systemic injection of siRNA lipoplexes containing cationic lipids with shorter dialkyl chains (C14 or C16) tend to increase siRNA accumulation in the liver. The systemic injection of LP-DC-1-16/DOPE and LP-DC-6-14/DOPE lipoplexes in the present study caused siRNA accumulation in both the lungs and liver, whereas LP-DC-1-14/DOPE lipoplexes caused siRNA accumulation in the liver. Generally, liposomes composed of lipids with short dialkyl chains are unstable because lipids with short dialkyl chains weaken the hydrophobic interactions between the alkyl chains by decreasing the hydrophobic area in the liposomal membrane (28). Therefore, the LP-DC-1-14/DOPE lipoplexes might be lysed soon after systemic injection, followed by their capture by Kupffer cells in the liver.

Regarding *in vivo* gene-knockdown, in the present study, LP-DC-1-16/DOPE lipoplexes with Tie2 siRNA induced a significant knockdown of Tie2 mRNA (37% knockdown) compared with Control #2 siRNA. By contrast, LP-DOTMA/DOPE lipoplexes exhibited a slight gene-knockdown, whereas LP-DC-6-14/DOPE and LP-DSTAP/DOPE lipoplexes did not show knockdown efficacy. Tagami *et al* (29) reported that some genes are upregulated or downregulated by treatment with non-specific siRNA lipoplexes, suggesting that the expression in some genes might be affected by inflammatory cytokines induced by a non-specific cellular immune response for siRNA lipoplexes via toll-like receptors (30). It has been reported that Tie2 expression in endothelial cells is stimulated by inflammatory cytokines (31). Therefore, the LP-DOTMA/DOPE lipoplexes with Control #2 siRNA lipoplexes might increase slightly Tie2 expression in the lungs (120%, compared with untreated lungs). However, further studies are required to investigate whether the injection of siRNA lipoplexes induce immune reactions such as inflammation and complement activation in mice. In our recent study, siRNA lipoplexes composed of DDAB and DOPE containing Tie2 siRNA induced significant knockdown of Tie2 mRNA (50% knockdown) compared with Control #2 siRNA, but those of DOTAP and DOPE did not (9). It is unclear why cationic liposomes composed of DC-1-16/DOPE exhibit high gene-knockdown efficiency *in vivo*, but the linker between the hydrophilic and hydrophobic lipid moieties in cationic lipid might be an important factor in transfecting siRNA lipoplexes into the lungs (32-34).

In our previous study, inclusion of dialkyl cationic lipid (DDAB) with a non-biodegradable linker into liposomal formulation exhibited high *in vivo* gene-knockdown by siRNA lipoplexes compared with that of diacyl cationic lipid (DOTAP) with a biodegradable linker (9). However, it was not clear the

effect of alkyl or acyl chain length and saturation in cationic lipid on a gene-knockdown by siRNA lipoplexes. The present study revealed that cationic lipids with a non-biodegradable linker and dialkyl saturated chains (C16-C18) are potential lipids for *in vivo* gene-knockdown by cationic liposomes.

In conclusion, the present study determined the effect of phosphatidylethanolamines in liposomal formulations on gene-knockdown efficacy in breast tumor cells and mouse lungs using siRNA lipoplexes. Differences in the structures of alkyl or acyl chains, head groups and linkers in cationic lipids may affect the optimal combination of phospholipids for gene-knockdown using siRNA lipoplexes. Overall, the present study provides insights onto the optimal liposomal formulation for siRNA delivery using cationic liposomes.

Acknowledgements

The authors would like to thank Ms Kumi Kawano (Department of Molecular Pharmaceutics, Hoshi University, Tokyo, Japan) for assistance with the animal experiments.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

Authors' contributions

YH conceived and designed the study, and wrote the manuscript. MT, AA, ME, HS and KO performed the experiments. YH and MT confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Institutional Animal Care and Use Committee of Hoshi University (approval no. P22-016).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Zimmermann TS, Lee AC, Akinc A, Bramlage B, Bumcrot D, Fedoruk MN, Harborth J, Heyes JA, Jeffs LB, John M, *et al*: RNAi-mediated gene silencing in non-human primates. Nature 441: 111-114, 2006.
2. Wang J, Lu Z, Wientjes MG and Au JLS: Delivery of siRNA therapeutics: Barriers and carriers. AAPS J 12: 492-503, 2010.
3. Barba AA, Cascone S, Caccavo D, Lamberti G, Chiarappa G, Abrami M, Grassi G, Grassi M, Tomaiuolo G, Guido S, *et al*: Engineering approaches in siRNA delivery. Int J Pharm 525: 343-358, 2017.
4. Khalil IA, Yamada Y and Harashima H: Optimization of siRNA delivery to target sites: Issues and future directions. Expert Opin Drug Deliv 15: 1053-1065, 2018.
5. Yan Y, Liu XY, Lu A, Wang XY, Jiang LX and Wang JC: Non-viral vectors for RNA delivery. J Control Release 342: 241-279, 2022.
6. Zhang Y, Satterlee A and Huang L: In vivo gene delivery by nonviral vectors: Overcoming hurdles? Mol Ther 20: 1298-1304, 2012.
7. Xue HY, Guo P, Wen WC and Wong HL: Lipid-based nanocarriers for RNA delivery. Curr Pharm Des 21: 3140-3147, 2015.
8. Hattori Y, Tamaki K, Ozaki KI, Kawano K and Onishi H: Optimized combination of cationic lipids and neutral helper lipids in cationic liposomes for siRNA delivery into the lung by intravenous injection of siRNA lipoplexes. J Drug Deliv Sci Technol 52: 1042-1050, 2019.
9. Hattori Y, Tang M, Torii S, Tomita K, Sagawa A, Inoue N, Yamagishi R and Ozaki KI: Optimal combination of cationic lipid and phospholipid in cationic liposomes for gene knockdown in breast cancer cells and mouse lung using siRNA lipoplexes. Mol Med Rep 26: 253, 2022.
10. Xia Y, Tian J and Chen X: Effect of surface properties on liposomal siRNA delivery. Biomaterials 79: 56-68, 2016.
11. Hattori Y, Nakamura M, Takeuchi N, Tamaki K, Shimizu S, Yoshiike Y, Taguchi M, Ohno H, Ozaki KI and Onishi H: Effect of cationic lipid in cationic liposomes on siRNA delivery into the lung by intravenous injection of cationic lipoplex. J Drug Target 27: 217-227, 2019.
12. Yamato K, Yamada T, Kizaki M, Ui-Tei K, Natori Y, Fujino M, Nishihara T, Ikeda Y, Nasu Y, Saigo K and Yoshinouchi M: New highly potent and specific E6 and E7 siRNAs for treatment of HPV16 positive cervical cancer. Cancer Gene Ther 15: 140-153, 2008.
13. Hattori Y, Tamaki K, Sakasai S, Ozaki KI and Onishi H: Effects of PEG anchors in PEGylated siRNA lipoplexes on *in vitro* gene-silencing effects and siRNA biodistribution in mice. Mol Med Rep 22: 4183-4196, 2020.
14. Tang M, Sakasai S, Onishi H, Kawano K and Hattori Y: Effect of PEG anchor in PEGylation of folate-modified cationic liposomes with PEG-derivatives on systemic siRNA delivery into the tumor. J Drug Target 31: 74-88, 2023.
15. Suzuki A, Hamada K, Sasaki T, Mak TW and Nakano T: Role of PTEN/PI3K pathway in endothelial cells. Biochem Soc Trans 35: 172-176, 2007.
16. Dumont DJ, Gradwohl GJ, Fong GH, Auerbach R and Breitman ML: The endothelial-specific receptor tyrosine kinase, tek, is a member of a new subfamily of receptors. Oncogene 8: 1293-1301, 1993.
17. Fehring V, Schaeper U, Ahrens K, Santel A, Keil O, Eisermann M, Giese K and Kaufmann J: Delivery of therapeutic siRNA to the lung endothelium via novel lipoplex formulation DACC. Mol Ther 22: 811-820, 2014.
18. Gutbier B, Kube SM, Reppe K, Santel A, Lange C, Kaufmann J, Suttorp N and Witzenrath M: RNAi-mediated suppression of constitutive pulmonary gene expression by small interfering RNA in mice. Pulm Pharmacol Ther 23: 334-344, 2010.
19. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
20. Cheng X and Lee RJ: The role of helper lipids in lipid nanoparticles (LNPs) designed for oligonucleotide delivery. Adv Drug Deliv Rev 99: 129-137, 2016.
21. Paliwal SR, Paliwal R and Vyas SP: A review of mechanistic insight and application of pH-sensitive liposomes in drug delivery. Drug Deliv 22: 231-242, 2015.
22. Sun D and Lu ZR: Structure and function of cationic and ionizable lipids for nucleic acid delivery. Pharm Res 40: 27-46, 2023.
23. Chen H, Ren X, Xu S, Zhang D and Han T: Optimization of lipid nanoformulations for effective mRNA delivery. Int J Nanomedicine 17: 2893-2905, 2022.
24. Eliyahu H, Servel N, Domb AJ and Barenholz Y: Lipoplex-induced hemagglutination: Potential involvement in intravenous gene delivery. Gene Ther 9: 850-858, 2002.
25. Hattori Y, Nakamura A, Hanaya S, Miyanabe Y, Yoshiike Y, Kikuchi T, Ozaki K and Onishi H: Effect of chondroitin sulfate on siRNA biodistribution and gene silencing effect in mice after injection of siRNA lipoplexes. J Drug Deliv Sci Technol 41: 401-409, 2017.

26. Thi TTH, Suys EJA, Lee JS, Nguyen DH, Park KD and Truong NP: Lipid-based nanoparticles in the clinic and clinical trials: From cancer nanomedicine to COVID-19 vaccines. Vaccines (Basel) 9: 359, 2021.
27. Dilliard SA, Cheng Q and Siegwart DJ: On the mechanism of tissue-specific mRNA delivery by selective organ targeting nanoparticles. Proc Natl Acad Sci USA 118: e2109256118, 2021.
28. Funakoshi Y, Iwao Y, Noguchi S and Itai S: Effect of alkyl chain length and unsaturation of the phospholipid on the physicochemical properties of lipid nanoparticles. Chem Pharm Bull (Tokyo) 63: 731-736, 2015.
29. Tagami T, Hirose K, Barichello JM, Ishida T and Kiwada H: Global gene expression profiling in cultured cells is strongly influenced by treatment with siRNA-cationic liposome complexes. Pharm Res 25: 2497-2504, 2008.
30. Ma Z, Li J, He F, Wilson A, Pitt B and Li S: Cationic lipids enhance siRNA-mediated interferon response in mice. Biochem Biophys Res Commun 330: 755-759, 2005.
31. Willam C, Koehne P, Jürgensen JS, Gräfe M, Wagner KD, Bachmann S, Frei U and Eckardt KU: Tie2 receptor expression is stimulated by hypoxia and proinflammatory cytokines in human endothelial cells. Circ Res 87: 370-377, 2000.
32. Rajesh M, Sen J, Srujan M, Mukherjee K, Sreedhar B and Chaudhuri A: Dramatic influence of the orientation of linker between hydrophilic and hydrophobic lipid moiety in liposomal gene delivery. J Am Chem Soc 129: 11408-11420, 2007.
33. Srujan M, Chandrashekhar V, Reddy RC, Prabhakar R, Sreedhar B and Chaudhuri A: The influence of the structural orientation of amide linkers on the serum compatibility and lung transfection properties of cationic amphiphiles. Biomaterials 32: 5231-5240, 2011.
34. Zhi D, Bai Y, Yang J, Cui S, Zhao Y, Chen H and Zhang S: A review on cationic lipids with different linkers for gene delivery. Adv Colloid Interface Sci 253: 117-140, 2018.