Tumor Regression in Mice by Delivery of Bcl-2 Small Interfering RNA with Pegylated Cationic Liposomes

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Abstract

The pharmacokinetics and antitumor activity of pegylated small interfering RNA (siRNA)/cationic liposome complexes were studied after systemic administration to mice. We designed pegylated-lipid carriers for achieving increased plasma concentrations of RNA and hence improved accumulation of RNA in tumors by the enhanced permeability and retention effect. We compared the pharmacokinetics of siRNA complexed with liposomes incorporating pegylated lipids with longer (C-17 or C-18), shorter (C-12 to C-16), or unsaturated (C-18:1) acyl chains. When longer acyl chains were used, the plasma concentrations of siRNA obtained were dramatically higher than when shorter or unsaturated chains were used. This may be explained by the higher gel-to-liquid-crystalline phase-transition temperature (Tc) of lipids with longer acyl chains, which may form more rigid liposomes with reduced uptake by the liver. We tested a siRNA that is sequence specific for the antiapoptotic bcl-2 mRNA complexed with a pegylated liposome incorporating a C-18 lipid (PEG-LIC) by i.v. administration in a mouse model of human prostate cancer. Three-fold higher accumulation of RNA in the tumors was achieved when PEG-LIC rather than nonpegylated liposomes was used, and sequence-specific antitumor activity was observed. Our siRNA/PEG-LIC complex showed no side effects on repeated administration and the strength of its antitumor activity may be attributed to its high uptake by the tumors. Pegylation of liposomes improved the plasma retention, uptake by s.c. tumors, and antitumor activity of the encapsulated siRNA. PEG-LIC is a promising candidate for siRNA cancer therapy. [Cancer Res 2008;68(21):8843–51]

Introduction

Sequence-specific gene silencing based on RNA interference (RNAi) holds great promise for molecular-targeted treatment of various diseases, including genetic disorders, cancer, and virus infection. RNAi is mediated by small interfering RNA (siRNA), which directs the sequence-specific degradation of mRNA by the RNA-induced silencing complex (1–3). RNA medicines have advantages in drug development over small molecules, antibodies, and other biological agents. They are easily synthesized and highly specific and their pharmacokinetics are expected to be largely independent of length and sequence. Nonviral carriers used in the delivery of plasmid DNA for in vitro transfection or gene therapy have been adapted for in vivo delivery of siRNA (4–9) because they are less likely than viral vectors to induce adverse events such as lethal immune reactions and lymphomas (10). Furthermore, because RNAi operates in the cytoplasm, it is desirable for a nonviral carrier to deliver siRNA to the cytoplasm rather than to the nucleus as viral carriers do.

There are several preclinical studies of siRNA complexed with cationic lipids, nanoparticles, cyclodextrin, polyethyleneimine, or atelocollagen (4, 11–13). In most clinical studies, chemically modified siRNA without a carrier has been administered locally, for example to the eye for age-related macular degeneration or intranasally for respiratory syncytial virus (14–16). For targeting tumors, systemic administration of siRNA is an attractive approach. However, many problems must be solved to ensure the success of this approach. First, the siRNA must be protected from nuclease degradation in the bloodstream. Second, the properties of the carrier must be carefully optimized because a carrier with a large particle size or excessive positive charge can aggregate or be opsonized in the blood, leading to its rapid uptake by the mononuclear phagocytic system and its failure to be taken up by the desired tissues or organs (17). Third, siRNA can induce an IFN response mediated by Toll-like receptors 3, 7, and 8 (TLR3, TLR7, and TLR8) when it is administered i.v. with a carrier (18–20).

In an effort to overcome these problems, we developed a cationic liposome incorporating a lipid derivatized with poly(ethylene glycol) (PEG) residues (pegylated lipid). The pegylated lipid provides the liposome with a PEG coating that both stabilizes the particle and protects it from opsonization (21), thereby preventing rapid systemic clearance by the mononuclear phagocytic system. The use of a pegylated lipid with a saturated 18-carbon acyl chain was associated with a reduced rate of clearance of the complex from the circulation and a greater uptake of RNA at tumor sites. High levels of expression of the antiapoptotic protein Bcl-2 are associated with resistance to radiation and chemotherapeutic agents in some tumor types (22–24); therefore, a drug that reduced Bcl-2 levels would be a promising chemotherapeutic candidate. In the present study, PEG-LIC complexed with siRNA targeting human bcl-2 mRNA showed sequence-specific antitumor activity with concomitant suppression of Bcl-2 protein in a mouse model of human prostate cancer.

Materials and Methods

Materials. Palmitoyl-oleophosphatidylcholine (POPC) was purchased from NOF; synthetic cholesterol (Chol) was from Sigma-Aldrich; and [2,5,6,3H]adenosine 5'-triphosphate, ammonium salt, was from Mandel NEN Products. All other chemicals were of reagent or analytical grade and were used without further purification.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/). Requests for reprints: Satoru Sonoke, Discovery Research Laboratories, Nippon Shinyaku Co. Ltd., 3-14-1 Sakura, Tsukuba, Ibaraki 305-0003, Japan. Phone: 81-29-850-6217; Fax: 81-29-850-6217; E-mail: s.sonoke@po.nippon-shinyaku.co.jp. ©2008 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-08-0127

www.aacrjournals.org 8843 Cancer Res 2008; 68: (21). November 1, 2008
Synthesis of CLZ-lipids. 1,3-O-Diacylglycerols were prepared according to modifications of literature procedures. CLZ-42 was prepared by coupling 1,3-O-dioleoylglycerol with N,N-diethylthelyenediamine and PEG-LIC(CARB) by coupling 1,3-O-dioleoylglycerol with α-methyl-ω-amino propyl polyoxethylene (MEPA-20H; average molecular weight, 2,000; NOF). In both syntheses, the coupling reagent was N,N-carbonyldimidazole. C-12 (1,3-O-dilauroylglycerol), C-14 (1,3-O-dimyristylglycerol), C-16 (1,3-O-dipalmitoylglycerol), C-17 (1,3-O-dihetadecanoylglycerol), and C-18 (1,3-O-diostearoylglycerol) conjugates were similarly synthesized. 1,3-O-Dioleoylglycerol was converted to its 2-O-phosphoramidite with 2-cyanoethyl-N,N,N,N′,N′-tetraisopropylphosphorodiamidite in the presence of N,N-diisopropylamine tetrazolide. The amidite was coupled with 2-N-(N-butoxycarbonyl)-2-aminoethanol in the presence of HCl-tetrazole, then oxidized with I₂ solution. After removal of the cyanoethyl and 2-N-(N-butoxy carbonyl) groups, the amine obtained was reacted with α-succinimidylsuccinyl-ω-methoxy-polyoxethylene (ME-02ICs; average molecular weight, 2,000; NOF) to afford PEG-LIC. All pegylated lipids were isolated as a single component on TLC and gave a mass spectrum consistent with the expected structure, as well as proton nuclear magnetic resonance (1H NMR) signals characteristic of the PEG methylene and lipid groups.

Synthesis of siRNA. Small interfering RNA strands were synthesized on an Expedite 900 DNA synthesizer (Applied Biosystems). The purity was >90% as determined by capillary gel electrophoresis. B043 is sequence-specific for human bcl-2 mRNA, and GL-3 and M-1 are sequence-specific for Photinus pyralis (firefly) luciferase mRNA. The siRNA sequences used were as follows: B043 sense strand, 5′-GUGAUGAGUAACUCAUUtdT-3′; B043 antisense strand, 5′-AUAUGAUGUAUCUAUCACdTdT-3′; GL3 sense strand, 5′-GUUACCCUGAUCUAGGdCdT-3′; GL3 antisense strand, 5′-UGCAAGAUCUGAUGGAdTdT-3′; M-1 sense strand, 5′-GCCUAAAGAGCCAUUGdCdT-3′; and M-1 antisense strand, 5′-GCCCUAUUGGUAUAGCAdTdT-3′.

Synthesis of tritium-labeled siRNA. Desalted DNA oligonucleotides were purchased from Hokkaido System Science. The T7 promoter primers were 5′-TAATACGACTCACTATAGGGGCTGAAAGCTATGGGCCCT-3′ (sense) and 5′-AAGCCCATATGCTTTTATACCCCTATAGTGCTGATTATTA-3′ (antisense). The T7 promoter primers used for the enzymatic synthesis of the tritium-labeled M-1 sense strand were 5′-TAATACGACTCACTATAGGGGCTGAAAGCTATGGGCCCT-3′ (sense) and 5′-AAGCCTATGCTTTTATACCCCTATAGTGCTGATTATTA-3′ (antisense). Tritium-labeled dsRNA 21-nt RNAs were generated in simultaneous reactions from DNA templates by in vitro transcription with a T7 transcription kit (Takara) with unlabeled nucleoside triphosphates and [2,5,8-3H]adenosine 5′-triphosphate, ammonium salt, as substrates. After transcription, dsRNA was purified by phenol extraction, desalted on a Microspin G-25 column (GE HealthCare), and annealed in 30 mM L-Hepes-KOH (pH 7.4) containing 100 mM/L potassium acetate and 2 mM/L magnesium acetate by heating at 90°C for 1 min followed by incubation at 37°C for 1 h. After concentration in a Microcon YM-10 centrifugal filter device (Millipore), RNA was determined by its absorbance at 260 nm. The purity of the RNA was checked by electrophoresis on a 15% nondenaturing polyacrylamide gel. The specific radioactivity of the [2,5,8-3H]adenosine-labeled M-1 was 7.2 × 10⁶ dpm/μg.

Preparation and characterization of LIC complexes. LIC complexes were prepared as previously described (4, 25, 26). Briefly, LIC with a lipid composition POPC/PEG-CLZ/CLZ-42 = 4:1:3 (w/w/w) was homogenized and emulsified in 10% (v/v) maltose with a probe-type sonicator (Branson Sonifier Model 220D; Branson Ultrasonics). In some experiments, siRNA was complexed with the cationic lipid–based transfection reagents Lipofectin or Oligofectamine (both from Invitrogen) according to the manufacturer’s instructions. The particle size distributions of the PEG-LIC complexes were determined by laser dynamic light scattering with a Nicomp 380 particle sizer, and their ζ potentials were determined with a Zetasizer 2000 (Malvern Instruments). For transmission electron microscopy, samples were placed on a specimen mesh coated with collodion film, negatively stained with sodium phosphotungstate (pH 7.0) and observed under a JEM1200EX electron microscope (JEOL) operated at 100 kV.

Human cell lines. A431 (epithelial carcinoma) and PC-3 (prostate carcinoma) cells were obtained from American Type Culture Collection. A431 cells were maintained in DMEM (Sigma-Aldrich), both supplemented with 10% (v/v) fetal bovine serum, at 37°C in 5% CO₂. Mouse tumor model. Male BALB/c nude mice (5 wk old) were purchased from Clea Japan. For a mouse model of s.c. tumor, mice were inoculated on day 0 under the dorsal skin with 3.0 × 10⁶ A431 or PC-3 cells in 100 μL of Nutrient Mixture F-12 Medium. After 10 d, when the tumor had reached a volume of 50 to 80 mm³, B043/PEG-LIC or GL-3/PEG-LIC at a dose of 10 mg/kg or 5% (w/v) maltose solution was administered i.v. once a day from day 10 to day 14 and once a day from day 17 to day 21. Tumor diameters were measured every 3 or 4 d with digital calipers and the tumor volume was calculated on days 13, 17, 20, and 24 as (width)² × length/2.

Female BALB/c nude mice (6 wk old) were purchased from Clea Japan. For a mouse model of peritoneal dissemination of ovarian cancer, mice were inoculated ip. on day 0 with 0.6 × 10⁶ mucinous cystadenocarcinoma (MCAS) cells, a human ovarian cancer cell line. After 4 d, each formulation was administered ip. at 10 mg/kg twice a week for 5 wk from day 4 to day 35 and the survival of the mice was monitored until day 91. Animal procedures were approved by the committee for the institutional care and use of animals of Nippon Shinyaku Co.

Pharmacokinetics of siRNA/PEG-LIC. Normal mice or mice bearing PC-3 cell xenografts were given tritium-labeled M-1/PEG-LIC at a dose of 2.5 or 10 mg/kg. After 5, 60, 120, or 240 min, the liver, lungs, kidneys, spleen, and tumors were removed. Tissue (0.1 g) was finely minced with scissors in Solvable (Perkin-Elmer), an alkaline aqueous-based tissue solubilizer. After overnight incubation at 40°C, samples were dissolved in Hionic-Fluor (Perkin-Elmer) and the radioactivity was measured in a Tri-Carb 2070TR liquid scintillation counter (Packard).

Histology and light microscopy. Tissue samples were mounted in optimum cutting temperature (O.C.T.) compound embedding medium (Sakura Fineteck) and frozen in dry ice–cooled ethanol. The mounted tissue was allowed to equilibrate in a cryostat (Coldtome CM-502; Sakura Fineteck) at −20°C for 20 min, cut into 7-μm sections, and placed on glass slides. The tissue sections were fixed in 10% formalin for 10 min and stained with Victoria blue (Muto Pure Chemicals) for 30 min. Sections were washed with 70% ethanol, stained with H&E dye (Myer-Hematoxylin solution; Wako Pure Chemical Industries), and mounted in Mount-Quick mounting medium (Dako). Micrographs were obtained using an Olympus BX-550 microscope.

Cell isolation and IFN-α assay. Human peripheral blood mononuclear cells (PBMC) were isolated from whole blood from healthy donors by density centrifugation with Ficoll-Paque Plus (GE Healthcare). For immunostimulation assays, 6 × 10⁶ freshly isolated PBMCs were seeded in 48-well plates and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. B043/PEG-LIC, B043/LIC, or B043 alone was added to cells at a final nucleic acid concentration of 30, 100, or 300 nmol/L and culture supernatants were collected after 24 h and assayed in duplicate for IFN-α with a Mouse Interferon Alpha ELISA kit (PBL Biomedical Laboratories).

Hemolysis assay. Blood was withdrawn from the inferior vena cava of rats and incubated with PEG-LIC, LIC, Lipofectin, or Oligofectamine at 37°C for 30 min. After incubation, plasma was prepared by centrifugation and hemoglobin was determined by its absorbance at 540 nm. One hundred percent lysis was defined as the lysis observed after 10-fold dilution of the blood with distilled water, and the results are presented as the concentration of liposome (μg/mL) required to produce 50% lysis.

Evaluation of cell viability. Cell viability was evaluated by tetrazolium dye metabolic assay by measuring the mitochondrial reduction of WST-8 [2-(2-methoxy-4-nitrophenyl)-5-(4-nitrophenyl)-2H-tetrazolium, monosodium salt; Cell Counting Kit-8; Dojindo] to the water-soluble formazan according to the manufacturer’s instructions. Human umbilical vein endothelial (HUVEC) cells (1 × 10⁶/cm²) were seeded on 24-well plates and incubated for 48 h. PEG-LIC, LIC, Lipofectin, or Oligofectamine were added and incubation continued for a further 48 h. The cells
were then incubated with WST-8 assay solution for 1 h and the amount of formazan dye generated was determined by the absorbance at 450 nm.

**Statistics.** The data were analyzed by Dunnett's multiple comparison test with SAS System version 8.2 (SAS Institute). A \( P \) value of <0.01 or <0.05 was considered to indicate a statistically significant difference.

**Results**

**Physicochemical properties of siRNA/PEG-LIC complexes.** Both B043/LIC and B043/PEG-LIC formed multilamellar vesicles observed by transmission electron microscopy (Fig. 1A). The dark-staining regions probably include the condensed hydrophobic siRNA. B043/PEG-LIC showed a narrow volume-weighted distribution of particle sizes with a mean around 100 nm (Fig. 1B). The B043/PEG-LIC particles had a cationic surface with a \( \zeta \) potential of 48 mV (Fig. 1C), slightly lower than the 55 mV observed for B043/LIC (data not shown). The particle diameter of both complexes measured by laser particle size analysis was consistent with that observed by transmission electron microscopy. The siRNA/PEG-LIC complexes were stable at room temperature during the time required to complete each measurement and showed no change in particle diameter during storage at 4°C for over a year.
Pharmacokinetics of siRNA/PEG-LIC complexes. To determine the effect of the length of the acyl chain anchor on the pharmacokinetics of the siRNA/PEG-LIC complex, a series of lipids containing acyl chains of 12 to 18 carbons was synthesized (Fig. 2A). PEG-LIC complexes incorporating [3H]M-1 siRNA were prepared and the tissue and plasma distribution of M-1 was investigated after administration of the complexes to mice. Each PEG-LIC complex was administered by a single injection into the tail vein at a dose of 2.5 mg/kg, and 4 h later the concentrations of M-1 in the plasma and liver were measured (Fig. 2B). The plasma concentrations of M-1 incorporated into PEG-LIC complexes with C-17 or C-18 acyl chains were 10 to 30 times higher than the plasma concentrations of M-1 in PEG-LIC complexes with shorter acyl chains. Conversely, the liver concentrations of M-1 in PEG-LIC complexes with C-17 or C-18 chains were lower than those observed with shorter chains. When a lipid containing an unsaturated C-18 chain was used, the plasma concentrations of M-1 were lower than when a saturated C-18 chain was used.

The time course of the plasma concentration of [3H]M-1 siRNA was investigated in mice with PEG-LIC complexes in which PEG was attached to the lipid by either a carbamate linker [PEG-LIC(CARB)] or a phosphodiester linker (PEG-LIC). Both complexes were administered by injection into the tail vein at 2.5 mg/kg. The plasma concentration profiles of the complexes were similar (Fig. 3A). The complexes also showed almost the same estimated area under the curve (AUC0-8): 132 mg/mL·h for PEG-LIC and 135 mg/mL·h for PEG-LIC(CARB)—values that are much higher than that of 28 mg/mL·h observed for nonpegylated LIC. No effect of linker chemistry was therefore observed. In a study of the biodistribution of PEG-LIC and LIC, the mononuclear phagocytic system organs (the liver and the spleen) showed significantly lower concentrations of M-1 when M-1/PEG-LIC was administered than when M-1/LIC was administered (Fig. 3B and C). The intactness of B043 siRNA in the liver was confirmed 4 h after administration by Northern blotting (Supplementary Fig. S1).

Targeting tumors by the enhanced permeability and retention effect. The enhanced permeability and retention (EPR) effect was confirmed in solid s.c. tumors in a mouse model by i.v. administration of Evans blue dye. The experiment was conducted to visualize the accumulation of macromolecules to the tumor at the tissue level. Nude mice bearing tumors derived from A431 cells were injected with 10 mg/kg Evans blue, and 24 h later a representative s.c. tumor was removed and photographed (Fig. 4A). Albumin-Evans blue complex was observed in the region surrounding the tumor, confirming the accumulation and retention of macromolecules in this region of the tumor. Histologic analysis of the tumor was done after staining with Victoria blue, which selectively stains the elastic fibers of blood vessels. Staining was observed in the region surrounding the tumor, indicating the presence of new blood vessels (Fig. 4B) and confirming that this region is the angiogenesis region of the tumor. These results provide assurance that this nude mouse model is suitable for investigating the distribution of RNA/PEG-LIC complexes to s.c. tumors.

Tumor-bearing mice were injected with 10 mg/kg M-1/PEG-LIC complexes incorporating [3H]M-1 siRNA. After 8, 24, or 72 h, mice...
Activity, effect on cell viability, and induction of IFN-α lipidd-based transfection reagents by measuring their hemolytic compared with that of LIC and commercially available cationic lipids (after i.v. administration of M-1/liposome complexes incorporating non-pegylated PEG-LIC was administered than when M-1/LIC was administered in the tumors at 8 and 24 h were about 3-fold higher when M-1/PEG-LIC than M-1/LIC was administered (Fig. 4D). The immunostimulatory activity of siRNA/LIC complexes was tested by measuring their induction of IFN-α in human PBMCs. B043/PEG-LIC, B043/LIC, or naked B043, at a final nucleic acid concentration of 30, 100, or 300 nmol/L, was incubated with PBMCs for 24 h and the culture supernatants were assayed for IFN-α (Table 1). Neither B043/PEG-LIC nor naked B043 induced IFN-α at any concentration tested, whereas B043/LIC induced IFN-α in a dose-dependent manner between 30 and 300 nmol/L. Serum chemistry and hematology were investigated in mice administered i.v. with B043/LIC, B043/PEG-LIC, or the standard chemotherapeutic agent Taxotere at doses of 3 or 10 mg/kg (Supplementary Table S1). Besides somewhat lower WBC counts in the Taxotere-treated groups, no significant toxic effects were observed for any of these test substances.

Antitumor effect of B043/PEG-LIC. To investigate the antitumor effect of B043/PEG-LIC, we used a mouse xenograft model bearing PC-3 human prostate cancer cells instead of A431 cells as a suitable animal model application for the development of B043 as an RNA medicine. siRNA/PEG-LIC was administered i.v. at a dose of 1, 3, or 10 mg/kg in two 5-day courses of daily injections and the tumor volume was monitored until day 24 (Fig. 5A). The average tumor volume on day 24 was 450 mm³ in the maltose-treated negative control group, 310 mm³ in the 1 mg/kg B043/LIC group, 220 mm³ in the 3 mg/kg B043/PEG-LIC group, 220 mm³ in the 3 mg/kg B043/LIC group, and 160 mm³ in the 10 mg/kg B043/PEG-LIC group, so that B043/PEG-LIC suppressed tumor growth in this mouse model in a dose-dependent manner.

The effect of B043/PEG-LIC on survival was investigated in a mouse model of peritoneal dissemination with the human ovarian cancer cell line MCAS. B043/PEG-LIC or B043/LIC was administered i.p. at 10 mg/kg twice weekly for 5 weeks and the survival of the mice was monitored for 91 days. Administration of B043/PEG-LIC resulted in a mean survival period of 70.8 days, slightly shorter than that of 77.8 days observed with B043/LIC. Both RNA/LIC complexes clearly prolonged survival relative to control maltose-treated mice, whose mean survival period was 42.4 days.

Discussion

Targeting tumors is an attractive application of RNAi, but there are few reports of therapeutic success (4,27). A possible reason for the lack of success is that the carrier reagents used, which were originally designed for in vitro transfection, tend to aggregate. In vitro, aggregation contributes to a higher rate of transfection; however, it is incompatible with in vivo applications. For example, Lipofectamine/plasmid DNA complexes aggregate to form relatively large particles >500 nm in diameter (28) and these particles accumulate in the lungs, the first-pass organ after i.v. injection.
To target other organs or tumor tissue, aggregation must be avoided. To address this problem, we designed a cationic-liposome complex, LIC, with a particle diameter <200 nm (25). LIC avoids becoming trapped in the lungs and accumulates in the liver so it is a good candidate for development for the delivery of RNA for the treatment of diseases such as metastatic liver cancer and hepatitis C (32). We have reported the anticancer activity of a siRNA/LIC complex systemically administered in a mouse model of liver metastasis (4). In the present study, we investigated the potential of pegylated lipid carriers based on LIC to achieve increased plasma concentrations of RNA and hence improved accumulation of RNA in s.c. tumors by the EPR effect.

The ζ potential is the electric potential at the interface between the hydrated particle and the bulk solution, and it is a measure of the magnitude of the electrostatic forces between the particles. The ζ potential is used to guide the development of liposome/nucleic acid formulations because it influences the interactions both between the particles and the cell surface and among the particles themselves. Zeta potentials compatible with the introduction of a nucleic acid into the cytoplasm are in the range 0 to 60 mV (7, 33, 34). The ζ potential of B043/LIC was 55 mV and that of B043/PEG-LIC was 48 mV, values that are compatible with both the intracellular delivery of nucleic acid and the avoidance of aggregation. Transmission electron micrographs of pegylated and nonpegylated LIC suggest that the particles form multilamellar vesicles whose stained aqueous regions include the condensed siRNA.

Incorporation of the RNA within the lamellar structure of the liposomes should provide a high encapsulation ratio as well as

<table>
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<tr>
<th>Liposome</th>
<th>Hemolysis (50%; μg/mL)</th>
<th>IC₅₀* (μg/mL)</th>
<th>siRNA/LIC</th>
<th>IFN-α induction in PBMCs (pg/mL)</th>
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<td>30 nmol/L †</td>
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<td>PEG-LIC</td>
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<td>12,000</td>
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<td>51</td>
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<tr>
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<td>Oligofectamine</td>
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Abbreviation: ND, not detected.

*IC₅₀ values are means of triplicate determinations.

†Final nucleic acid concentration.
with oleyl chains (C-18:1) in the 1- and 3-positions. However, the M-1/PEG-LIC complex incorporating C-18:1 did not yield higher plasma concentrations of M-1 than nonpegylated M-1/LIC, nor was there any reduction in clearance by the liver (data not shown). We next investigated the effect of changing the length and saturation status of the acyl chains of the pegylated lipid. When C-17 or C-18 acyl chains were used, the plasma concentrations of M-1 were dramatically lower than when C-12, C-14, or C-16 were used, and the saturated C-18 acyl chain yielded higher plasma concentrations of M-1 than the unsaturated C-18:1. The concentrations of M-1 in the liver showed the opposite trend, the longer acyl chains yielding significantly lower liver concentrations of M-1 than the shorter acyl chains. These results show that both the length and the saturation status of the acyl chain of the pegylated lipid component strongly influenced the pharmacokinetics of siRNA after i.v. administration of siRNA/PEG-LIC.

The physicochemical characteristics of the pegylated lipids, particularly their gel-to-liquid-crystalline phase-transition temperature (Tc), affect the physiologic stability of the liposomes (39). In the present study, liposomes whose pegylated lipid components had acyl chains of 16 or fewer carbons or a longer unsaturated acyl chain (C-18:1) yielded low plasma levels of RNA. Liposomes generally form soft capsules at temperatures above the Tc of their component lipids. When the Tc of the component lipids is near or below body temperature, the liposomes are expected to form soft capsules after administration. In contrast, when the Tc of the component lipids is above body temperature, the liposomes are expected to form hard capsules. For example, the Tc value of phosphatidylcholine containing C-18:1 is below 0°C and that of phosphatidylcholine containing C-16 is 41°C, whereas that of phosphatidylcholine containing C-18 is 58°C. To investigate this relationship directly in liposomes, we measured the Tc values of our liposomes by differential scanning calorimetry. Liposomes containing C-18:1 or C-14 did not show a clear Tc peak, but liposomes containing C-16 showed a Tc of −7°C, whereas liposomes containing C-18 showed a Tc of 67°C. This suggests that our pegylated liposomes form soft capsules when C-16 or C-18:1 is used, but form hard capsules when C-18 is used. Because soft liposomes are rapidly taken up by the liver, possibly due to a rapid loss of PEG, they would be expected to yield lower plasma concentrations of RNA. In contrast, retention of PEG by hard liposomes may help to avoid uptake by the liver and hence yield higher plasma concentrations of RNA. In this way, differences in the plasma concentrations of RNA delivered by the liposomes may be explained by differences in the Tc values of the liposomes.

We also tested the effect of the chemistry of the PEG-lipid linker on the pharmacokinetics of pegylated liposomes whose component pegylated lipids had C-18 acyl chains. Complexes of RNA with PEG-LIC(CARB), which has a carbamate linker, gave plasma concentrations of RNA very similar to those obtained with PEG-LIC, which has a phosphodiester linker. We selected the phosphodiester linker for further study because it is more easily cleaved in vivo than the carbamate linker (40), and so is more likely to allow release of PEG at the tumor site. In addition, the ethanalamine generated by cleavage can promote the release of liposomes from the endosomes to the cytosol. (We think that PEG is removed enzymatically by cleavage between the ethanalamine residue and the PEG chain, leaving the ethanalamine residue still attached to the lipid.) In the low-pH environment of the endosome, the ethanalamine can promote inversion of the liposome and thus endosomal escape (31, 41).
We tested the tumor-targeting potential and antitumor activity of our pegylated liposome carrier, PEG-LIC. When M-1/PEG-LIC M-1 RNA was administered i.v. in a mouse model of s.c. tumor, higher accumulation of RNA was observed in the tumors than when a nonpegylated carrier was used. And when PEG-LIC incorporating B043, a siRNA targeting the antiapoptotic bcl-2 mRNA, was administered i.v. in a mouse model of prostate cancer, sequence-specific antitumor activity was observed with concomitant suppression of Bcl-2 protein. No side effects were observed during repeated administration of B043/PEG-LIC, and the body weight (data not shown) and serum chemistry ( Supplementary Table S1) of the treated mice were the same as in control mice. When we compared Bcl-2 siRNA/PEG-LIC with Taxotere in the same mouse cancer model, 3 mg/kg Bcl-2 siRNA/PEG-LIC gave about the same efficacy as 10 mg/kg Taxotere (Supplementary Fig. S2). Whereas Bcl-2 siRNA/PEG-LIC showed no toxicity in the serum chemistry analyses, Taxotere caused a reduction in the WBC count (Supplementary Table S1). No increases in liver-enzyme levels in the plasma were observed with either drug.

siRNA can induce a TLR-mediated IFN response in mice when delivered with a carrier (18). This observation has raised concerns about possible side effects resulting from the therapeutic administration of siRNA. Our pegylated liposome carrier PEG-LIC showed no induction of IFN-α in PBMCs, a feature that should contribute to its safety during repeated administration. We also observed antitumor activity with B043/PEG-LIC in a mouse model of peritoneal dissemination of ovarian cancer by i.p. administration.

In conclusion, siRNA/PEG-LIC could be safely administered to mice and showed superior uptake by s.c. tumors and strong antitumor activity in mouse models of human cancer by systemic or local administration. Our pegylated cationic liposome, PEG-LIC, is therefore a promising tool for the safe and effective delivery of siRNA to tumors with potential for application to human therapy. Our results take cancer therapy that includes siRNA another step closer to the clinic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Accepted 8/20/2008. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. G.E. Smyth, Discovery Research Laboratories, Nippon Shinyaku Co., Ltd., for helpful discussions, suggestions, and support during the preparation of the manuscript.

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