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Targeted Delivery of Antisense Oligodeoxynucleotide and Small Interference RNA into Lung Cancer Cells

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Abstract: Selective gene inhibition by antisense oligodeoxynucleotide (AS-ODN) or by small interference RNA (siRNA) therapeutics promises the treatment of diseases that cannot be cured by conventional drugs. However, antisense therapy is hindered due to poor stability in physiological fluids and limited intracellular uptake. To address these problems, a ligand targeted and sterically stabilized nanoparticle formulation has been developed in our lab. Human lung cancer cells often overexpress the sigma receptor and, thus, can be targeted with a specific ligand such as anisamide. AS-ODN or siRNA against human survivin was mixed with a carrier DNA, calf thymus DNA, before complexing with protamine, a highly positively charged peptide. The resulting particles were coated with cationic liposomes consisting of DOTAP and cholesterol (1:1, molar ratio) to obtain LPD (liposome−polycation−DNA) nanoparticles. Ligand targeting and steric stabilization were then introduced by incubating preformed LPD nanoparticles with DSPE-PEG-anisamide, a PEGylated ligand lipid developed earlier in our lab, by the postinsertion method. Nontargeted nanoparticles coated with DSPE-PEG were also prepared as a control. Antisense activities of nanoparticles were determined by survivin mRNA down-regulation, survivin protein down-regulation, ability to trigger apoptosis in tumor cells, tumor cell growth inhibition, and chemosensitization of the treated tumor cells to anticancer drugs. We found that tumor cell delivery and antisense activity of PEGylated nanoparticles were sequence dependent and rely on the presence of anisamide ligand. The uptake of oligonucleotide in targeted, PEGylated nanoparticles could be competed by excess free ligand. Our results suggest that the ligand targeted and sterically stabilized nanoparticles can provide a selective delivery of AS-ODN and siRNA into lung cancer cells for therapy.

Keywords: Targeted delivery; antisense oligodeoxynucleotide; siRNA; survivin; lung cancer

Introduction

There are more than 170 000 new cases of lung cancer every year in the United States,[1] which is the leading cause of cancer death both in the United States and throughout the world. Non-small-cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for 80−85% of total populations. The main reason for the unfavorable prognosis of these tumors is their propensity to metastasize early and develop resistance to a wide range of anticancer drugs.[2] The 5-year survival rate for all stages of lung cancer combined is only 15%.[3] Therefore, new treatment modalities for lung cancer are urgently needed.

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In human cancer cells, the expression of four members of the antiapoptotic, inhibitor of apoptosis (IAP) protein family (XIAP, cIAP1, cIAP2, and survivin) has been investigated as a potential factor for chemoresistance.[4] Among the IAP family members, survivin, a 16.5 kDa protein with a single BIR domain, has drawn much attention. Survivin, an inhibitor of apoptosis and regulator of the cell cycle, is a nuclear-cytoplasm shuttling protein that is actively exported out of the nucleus by the export receptor CRM1.[5] Survivin is not usually detected in normal adult tissues, but is commonly expressed at high levels during fetal development, and in precancerous and cancerous lesions,[6] including NSCLC.[7] Overexpression of survivin indicates either an unfavorable course of the disease,[8] resistance to chemotherapy and radiotherapy,[9,10] or poor survival in NSCLC patients.[9] Moreover, suppression of survivin in lung cancer cells by antisense or siRNA results in spontaneous apoptosis.[11−13] These observations suggest that survivin is a useful prognostic marker of lung cancer and a potential molecular target for lung cancer treatment.

Selective oncogene inhibition mediated by AS-ODN or siRNA shows the potential for cancer treatment. However, oligonucleotide delivery, including AS-ODN and siRNA, is the rate-limiting step in antisense therapy.[14] With a highly charged and hydrophilic nucleotide backbone, AS-ODN and siRNA are extremely vulnerable to enzyme degradation and can hardly penetrate through cell membrane when administered into the human body. There are methods or reagents available for oligonucleotide delivery in vitro and in vivo, such as viral vectors,[15] hydrodynamic injection,[16] Oligofectamine,[17] and Lipofectamine 2000.[18] However, few have demonstrated clinical applicability because of the potential toxicity and poor stability in body fluid. Therefore, a suitable delivery system is still needed. LPD nanoparticles was developed earlier in our lab for plasmid DNA delivery.[19] It was engineered by combining cationic liposomes and polycation condensed plasmid DNA. When they were mixed, the components spontaneously rearranged to form a viruslike structure with the condensed DNA located inside the lipid membranes.[20] In this study, we attempted to formulate AS-ODN/siRNA in LPD and further introduced steric stabilization and tumor specificity by using a PEGylated lipid tethered to a targeting ligand. Tissue specificity, cellular uptake, and down-regulation of the survivin target have revealed the antitumor activity of the targeted LPD.

Experimental Section

Materials. DOTAP, cholesterol, and DSPE-PEG$_{2000}$ were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Protamine sulfate (fraction X from salmon) and calf thymus DNA (for hybridization, phenol−chloroform extracted and ethanol precipitated) were from Sigma-Aldrich (St. Louis, MO). DSPE-PEG$_{2000}$-anisamide (DSPE-PEG-AA) was synthesized in Dr. C. K. Lai's lab by the methods described previously,[21] and the chemical structure is shown in Figure 1.

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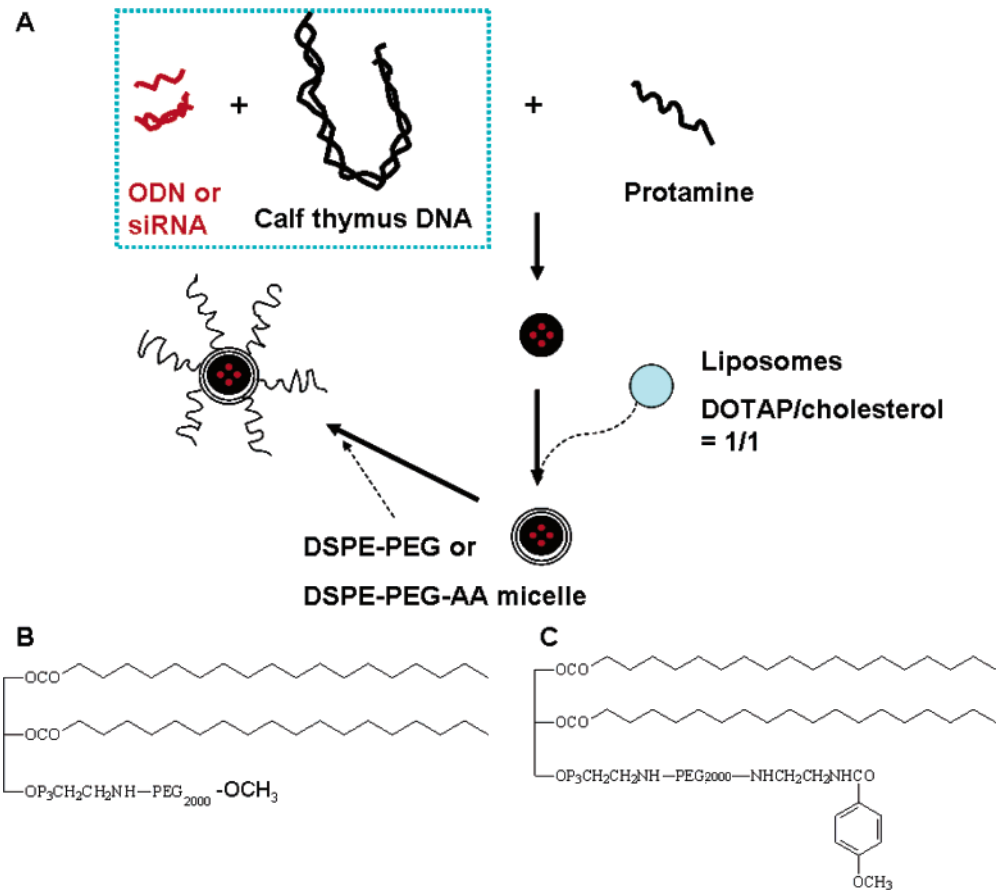

Figure 1. Illustration of preparation of PEGylated LPD (A) and chemical structures of DSPE-PEG_{2000} (B) and DSPE-PEG_{2000}-anisamide (C).

The AS-ODN and siRNA sequences are adopted from the previous studies.[2,22] The phosphorothioate oligodeoxynucleotides (ODN) with antisense sequence 5′-CCCAGCCTTC-CAGTCCCTTG-3′ and sense sequence 5′-CAAGGGACTG-GAAGGCTGGG-3′ were synthesized by Integrated DNA Technologies (Coralville, IA). 5′ FAM labeled antisense sequence was also obtained from Integrated DNA Technologies. Synthetic 19-nt RNAs with 3′ dTdT overhangs on both sequences were purchased from Dharmacon (Lafayette, CO) in deprotected, desalted, and annealed form. The sequence of survivin siRNA was 5′-GGCUGGCUUCAUCCAC-UGCdTdT-3′; 3′-dTdTCCGACCGAAGUAGGUGACG-5′. Scrambled control siRNA with sequence 5′-CAGUCGCGU-UUGCGACUGGUUdTdT-3′; 3′-dTdTGUCAGCGCAAACG-CUGACCAA-5′ was also synthesized in Dharmacon. For quantitative studies, FAM was conjugated to 5′ sense sequence.

H1299 cells, lung carcinoma, were obtained from ATCC and were maintained in RPMI1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA). H1299 cells overexpress sigma receptors and survivin[23−26] and were used as model cells in our study.

Preparation of Liposome, LPD, and PEGylated LPD Formulations (Figure 1). Liposomes and LPD were prepared as previously described with slight modifications.[27] Briefly, small unilamellar liposomes consisting of DOTAP

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and cholesterol (1:1 molar ratio) were prepared by thin film hydration followed by membrane extrusion. The total lipid concentration of the liposome was fixed at 10 mM. LPD was composed of DOTAP/cholesterol liposome, protamine, and the mixture of oligonucleotide and calf thymus DNA (1:1 weight ratio). To prepare LPD, 5 μL of protamine (0.8−4 mg/mL), 47 μL of deionized water, and 8 μL of a mixture of oligonucleotide and calf thymus DNA (2 mg/mL) were mixed in a 1.5 mL tube. The complex was allowed to stand at room temperature for 10 min before the addition of 40 μL of DOTAP/cholesterol liposome (total lipid concentration = 10 mM). LPD nanoparticles were kept at room temperature for another 10 min before further application.

PEGylated LPD formulations were prepared by the postinsertion method.[28,29] Briefly, 100 μL of preformed LPD was mixed with 0.63−16 μL of DSPE-PEG or DSPE-PEG-AA (20 mg/mL) and then incubated at 50−60 °C for 10 min. The resulting formulations were allowed to cool to room temperature before use.

The particle size of LPD and PEGylated LPD was measured by using a Coulter N4 Plus particle sizer (Beckman Coulter, San Francisco, CA). Particle sizes were reported as the mean ± standard deviation [polydispersity index (PI)].

Cellular Uptake Study. H1299 cells (1×10^5 per well) were seeded in 12-well plates (Corning Inc., Corning, NY) 20 h before experiments. Cells were treated with different formulations at a concentration of 200 nM for AS-ODN or 100 nM for siRNA in serum containing medium at 37 °C for 4 h. Cells were washed twice with PBS, followed by incubation with lysis buffer (0.3% Triton X-100 in PBS) at room temperature for 1 h. Fluorescence intensity of cell lysate was determined by a Perkin-Elmer LS 50B luminescence spectrometer (Norwalk, CT) (λex, 494 nm; λem, 519 nm). For free ligand competition study, cells were coincubated with 50 μM haloperidol with formulations. Cells were fixed with 1% paraformaldehyde in PBS at room temperature for 15 min, mounted onto a glass slide, and imaged by a Nikon fluorescence phase contrast optical microscope.

Serum Stability Study. Different formulations (10 μL) were mixed with 190 μL of FBS in 96-well plates. Aggregation in terms of turbidity increase of each sample was quantified by absorbance at 630 nm by a Bio-Rad Ultramark Microplate Imaging System (Hercules, CA).

Survivin mRNA Quantification by Real Time PCR. H1299 cells (2×10^5 per well) were seeded in 6-well plates (Corning Inc., Corning, NY) 20 h before experiments. Cells were treated with different formulations at a concentration of 1000 nM for AS-ODN or 500 nM for siRNA in serum containing medium at 37 °C for 4 h. Cells were washed once with PBS and maintained in fresh medium for 24 h. Total RNA were extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA) by following the manufacturer's protocol. cDNA was then reverse transcribed in the presence of reverse transcriptase (Promega, Madison, WI). Survivin mRNA levels were determined by an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA) as described previously.[30] The primer pairs for detecting the expression of survivin gene were survivin forward 5′-TCCACTGCCCCACTGAGAAC-3′ and survivin reverse 5′-TGGCTCCCAGCCTTCCA-3′. PCR primers for β-actin gene were β-actin forward, 5′-AAAGACCTGTACGCCAACACAGTGCTGTCTGG-3′, and β-actin reverse, 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′.

Histochemical Immunostaining. Five sterile round cover slips (1 cm × 1 cm) were placed into each well in 6-well plates. H1299 cells (1×10^5 per well) were then seeded into each well. Cells were treated with different formulations at a concentration of 1000 nM for AS-ODN or 400 nM for siRNA in serum containing medium at 37 °C for 4 h. Cells were washed once with PBS and maintained in fresh medium for 48 h. Cells were washed twice with PBS and fixed with 1% paraformaldehyde in PBS at room temperature for 15 min. Immunostaining was then performed with rabbit anti-human survivin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) with kits (DakoCytomation Envision + Dual Link System-HRP (DAB+), DakoCytomation, Carpinteria, CA) by following the product protocol. Cells were imaged by a Nikon phase contrast microscope. Positive cells were counted in 10 randomly chosen images per group. At least 300 cells were counted for each group.

Survivin Protein Assay. H1299 cells (1×10^5 per well) were seeded into 6-well plates. Cells were treated with different formulations at a concentration of 1000 nM for AS-ODN or 500 nM for siRNA in serum containing medium at 37 °C for 4 h. Cells were washed once with PBS and maintained in fresh medium for 48 h. Cells were rinsed twice with PBS and treated with 200 μL of lysis buffer (1 mM EDTA, 0.5% TritonX-100, 6 M urea, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 100 μM PMSF, 3 μg/mL aprotinin in PBS, pH 7.2−7.4) for 30 min on ice. Cell lysates were collected, vortexed briefly, and incubated on ice for another 15 min. Cell debris was removed by centrifugation at 2000*g* for 5 min, and protein concentrations were determined by Coomassie blue assay (Bradford method) after 6-fold dilution with 1% BSA in PBS. Samples of total protein of 10 mg were added into captured antibody precoated 96-well plates, and human survivin was assayed by ELISA (R&D systems, Minneapolis, MN).

Annexin V Staining and Quantification. H1299 cells (5×10^4 per well) were seeded into 6-well plates. Cells were

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treated with different formulations at a concentration of 1000 nM for AS-ODN or 500 nM for siRNA in serum containing medium at 37 °C for 72 h. Cells were washed once with PBS, trypsinized, and resuspended in PBS at a concentration of 1×10^6 cells/mL. Cells were then stained with Annexin V-FITC using a kit (BD Biosciences Pharmingen, San Jose, CA). Cells stained with Annexin V-FITC were detected and quantified by flow cytometry (Becton-Dickinson, Heidelberg, Germany). Results were processed using the Cellquest software (Becton-Dickinson).

Cellular Growth Inhibition Study. H1299 cells (1×10^4 per well) were seeded into 12-well plates. Cells were treated with different formulations at various concentrations in serum containing medium at 37 °C for 72 h. Cell viability was then detected by MTT assay. Briefly, 100 μL of MTT solution (15 mg in 50 mL of PBS) was added to each well, and the cells were incubated for 4 h at 37 °C. The media were removed, and the formazan crystals formed in cells were dissolved in DMSO. Absorbance at 570 nm was measured in an Ultramark Microplate Imaging System. The data are expressed as the percent of viable cells compared to the untreated control cells.

Chemosensitization Study. H1299 cells (1×10^4 per well) were seeded into 12-well plates. Cells were treated with different formulations at 500 nM for AS-ODN or 250 nM for siRNA in serum containing medium at 37 °C for 4 h. Cells were washed once with PBS and maintained in fresh medium for another 24 h. Cells were then incubated with cisplatin (Sigma-Aldrich, St. Louis, MO) containing medium at different concentrations. Cell viability was detected by the MTT assay 48 h later.

Results and Discussion

Targeted delivery is one of the focused areas in cancer gene therapy. Investigators have discovered that in vivo tumor targeting can be achieved by encapsulating genes into nanoparticles. Once injected into the blood stream, nanoparticles accumulate in the tumors due to its enhanced permeability and retention effect (EPR effect).[31] Moreover, further modification of ligands on the surface of nanoparticles has been shown to increase the delivery efficiency and tissue specificity.[32] In this study, we employed LPD, a nonviral vector developed in our lab,[19,20] as a nanocarrier for AS-ODN and siRNA. However, " naked" LPD tends to aggregate in the presence of serum proteins due to its highly positive charge content. To increase the serum stability of LPD, we introduced a steric barrier onto the LPD formulation by postinserting PEGylated lipids. Anisamide, a small molecular weight compound that specifically binds to sigma receptor, was tethered to the distal end of PEG for tumor targeting. This unique lipid, DSPE-PEG-AA, has been used by our group for the preparation of tumor targeted stealth liposomes for delivering anticancer drugs and showed great antitumor activity.[21] Its potential for providing stability and tumor targeting for the LPD formulation was examined in this study.

Sigma receptor is a well-known membrane-bound protein, which shows high affinity for neuroleptics[33] and benzamide derivatives,[34] such as anisamide. These receptors are overexpressed in a variety of human tumors including NSCLC, prostate cancer, melanoma, and breast cancer,[34−40] but also expressed on some normal tissues, such as liver, endocrine glands, kidney, lungs, gonads, central nervous system, and ovaries.[41,42] Our group was the first to demonstrate the success of use of sigma ligand to target anticancer drugs to

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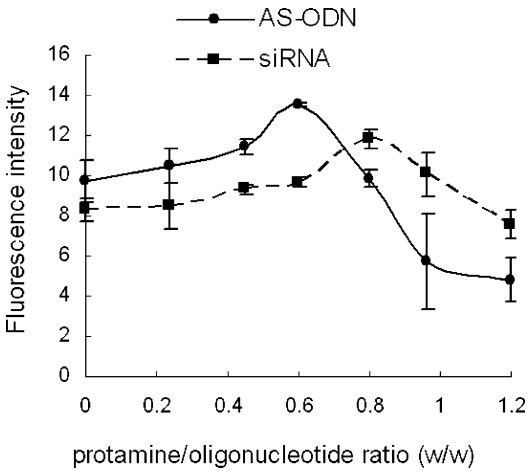

Figure 2. Effect of protamine/oligonucleotide ratio on the delivery efficiency of LPD. 5′ FAM labeled ODN or siRNA was formulated into LPD of different protamine/oligonucleotide ratios. H1299 cells were incubated with LPD formulations at 37 °C for 4 h. Delivery efficiencies of different LPD formulations were determined on the basis of the fluorescence intensities of cell lysates. Data = mean ± SD (n = 3).

tumors.[21] Although sigma receptors were found in some normal tissues, our previous results showed that anticancer drugs predominantly distributed to tumors due to the EPR effect of stealth liposomes (unpublished data). This suggests that anisamide can be applied as a safe ligand for tumor targeting once conjugated with nanoparticles. We hypothesize that the anisamide conjugated LPD can accumulate in tumors after iv injection, and then the anisamide ligand will facilitate the internalization of the nanoparticles and deliver oligonucleotide into tumor cells with high selectivity. In this study, we focused on the LPD formulation development and examined the potential of LPD for oligonucleotide delivery.

Calf thymus DNA serves as a carrier DNA in our LPD formulations and was shown to reduce the particle size by 10−30% and increase delivery efficiency by 20−80% (data not shown). This may be due to improved core compaction provided by high molecular weight DNA compared to oligonucleotide. Calf thymus DNA also contains limited amounts of immunostimulating CpG motif and provides greater advantage than the plasmid DNA. Figure 2 shows the protamine/nucleotide ratio optimization results. The optimized ratios for AS-ODN and siRNA are 0.6 and 0.8, respectively. Protamine is a highly positive charged peptide that acts as a DNA condensation reagent. By increasing the protamine concentration, the resulting formulation has increased delivery efficiency. However, too much protamine changed the net charge of protamine−DNA complex to slightly positive and interfered with the interaction of cationic lipids and, thus, reduced the delivery efficiency of the resulting formulation. Interestingly, different conformations of oligonucleotide require different amounts of protamine in the fixed +/− ratio for core condensation, since double stranded siRNA needed more protamine compared to single stranded AS-ODN. After postinsertion of DSPE-PEG onto the surface of the "naked" LPD, the particle size of the

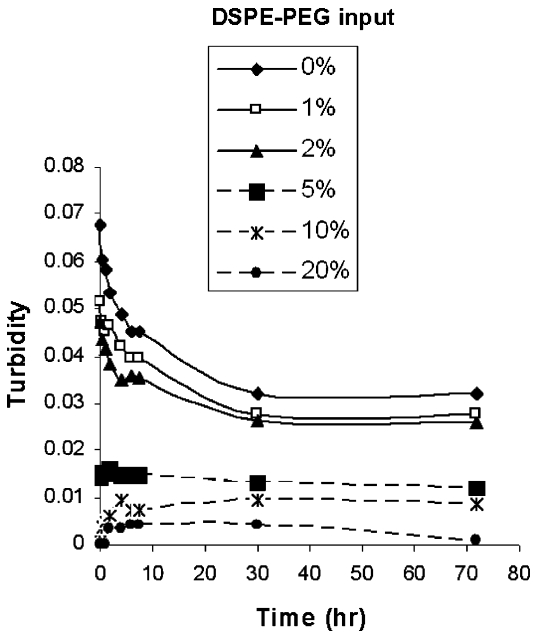

Figure 3. Effect of DSPE-PEG$_{2000}$ on preventing aggregation of LPD induced by FBS. LPD and PEGylated LPD were incubated with FBS (1:19 v/v) at 37 °C and turbidity (OD630) of the mixture was measured by a microplate reader. Data = mean (n = 3).

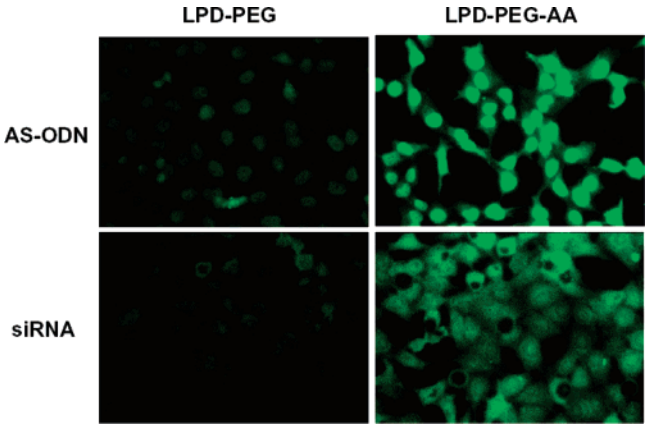

Figure 4. Fluorescence photographs of H1299 cells after treatment with 5′ FAM labeled AS-ODN or siRNA in LPD-PEG or LPD-PEG-AA. Cells were treated with different formulations at 37 °C for 4 h. Cells were rinsed, fixed, and imaged by a fluorescence microscope. Magnification = 400×.

formulation gradually reduced (data not shown). DSPE-PEG can impart a steric hindrance for lipid-based formulations and prevent particle aggregation.[43] It is reasonable to see a particle size reduction after PEGylation. We also demonstrated that PEGylation of the "naked" LPD can stabilize the formulation in the presence of FBS (see Figure 3). "Naked" LPD started to aggregate once mixed with serum. As time increased, large aggregates began to precipitate,

(43) Yoshioka, H. Surface modification of haemoglobin-containing liposomes with polyethylene glycol prevents liposome aggregation in blood plasma. *Biomaterials* **1991**, *12* (9), 861−4.

Table 1. Summary of Optimized Formulations for AS-ODN and siRNA[a]

formulation	protamine (2 mg/mL)	water	ODN or siRNA/calf thymus DNA (2 mg/mL)	10 mM liposome (DOTAP/cholesterol = 1:1)	DSPE-PEG_{2000} or DSPE-PEG_{2000}-AA (20 mg/mL)
AS-ODN	5	47	8	40	12.6
siRNA	6.4	40.6	8	40	12.6−16

[a] Units: μL.

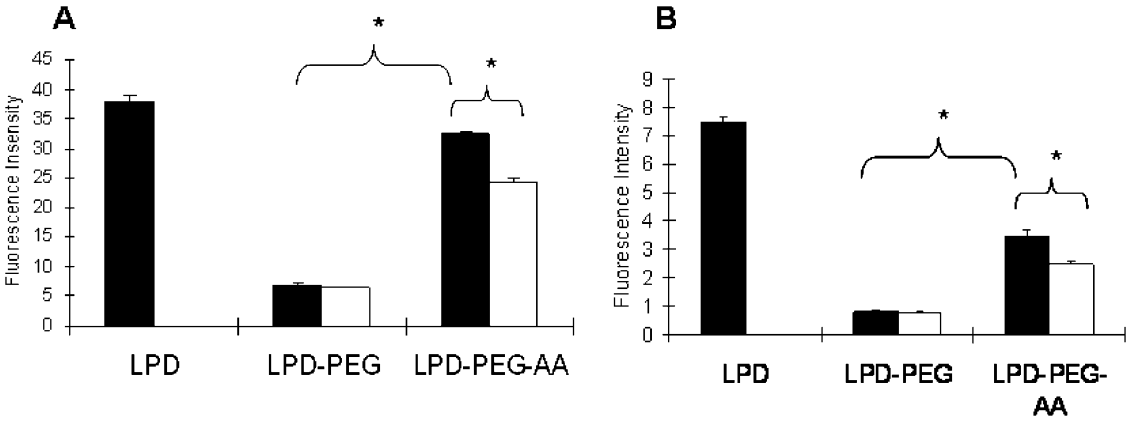

Figure 5. Fluorescence intensities of cells treated with 5′ FAM labeled AS-ODN (A) or siRNA (B) containing formulations. H1299 cells were incubated with different formulations at 37 °C for 4 h in the presence (white bars) or absence (black bars) of 50 μM haloperidol. Cells were washed and lysed. Cells lysates were analyzed for fluorescence intensities by a fluorescence spectrometer (λex, 494 nm; λem, 519 nm). * indicates a significant difference between two groups ($p < 0.05$).

resulting in turbidity decrease. By postinserting >20 mol % (of the total lipid) of DSPE-PEG, particle aggregation induced by FBS was completely prevented. Overall, the characteristics of the optimized formulation for AS-ODN and siRNA are summarized in Table 1. The particle sizes of AS-ODN formulation and siRNA formulation after PEGylation were 63.4 ± 14.0 nm (PI = 0.151) and 121 ± 22.9 nm (PI = 0.120), respectively.

The following experiments were performed with the optimized formulations as indicated in Table 1. As shown in Figure 4, the fluorescence signal in the cells treated with LPD-PEG-AA was much stronger than that of cells treated with LPD-PEG, a sterically stabilized formulation without any targeting ligand. It indicates that anisamide ligand increased the delivery efficiency of the nanoparticles for sigma receptor expressing cells, H1299. Interestingly, AS-ODN showed strong nucleus localization, while siRNA distributed homogeneously in the entire cells or predominantly in the cytoplasm. In quantitative results (see Figure 5), PEGylation introduced steric hindrance to LPD nanoparticles and reduced its delivery efficiency by 80%. However, the delivery efficiency of PEGylated LPD could be restored with the presence of the anisamide ligand. Ligand conjugation increased the delivery efficiency of PEGylated LPD by 4−7-fold. Besides, free haloperidol, a known high affinity ligand for the sigma receptor, competed with anisamide and partially inhibited the targeting effect of ligand modified LPD, suggesting that LPD-PEG-AA target to H1299 cells via a sigma receptor dependent pathway, i.e., receptor-mediated endocytosis.

To demonstrate the biological activity of the nanoparticle formulation, we first analyzed the survivin mRNA levels in cells after treatment with AS-ODN or siRNA by real time PCR (Figure 6A). Free AS-ODN and siRNA had little effect due to the poor cellular bioavailability of these negatively charged oligonucleotide. In the case of AS-ODN, AS-ODN containing LPD-PEG and LPD-PEG-AA down-regulated 10% and 60% survivin mRNA, respectively, while the sense ODN containing LPD-PEG-AA showed only a marginal effect (10%, not significant). The results suggest that the antisense effect of ODN formulation is sequence and formulation dependent. The antisense effect only shows when a sufficient amount of AS-ODN is delivered into the cells. Similar results were found with siRNA. siRNA formulated in LPD-PEG and LPD-PEG-AA down-regulated 30% and 70% survivin mRNA, respectively, while LPD-PEG-AA containing a scrambled siRNA showed relatively low effect (20%, not significant). Thus, the gene silencing effect mediated by siRNA was sequence specific and dependent on the efficient delivery by the targeted nanoparticle vector.

We employed two different methods to detect the survivin protein levels in the cells, i.e., immunohistochemistry (Figure 7) and ELISA (Figure 6B). As shown in Figure 7, only LPD-PEG-AA complexed with antisense sequence, for both AS-ODN and siRNA, significantly down-regulated survivin in H1299 cells. The survivin positive cells treated with medium only, free AS-ODN, AS-ODN in LPD-PEG, AS-ODN in LPD-PEG-AA, and sense ODN in LPD-PEG-AA were 87%, 90%, 80%, 36%, and 87%, respectively. Survivin positive

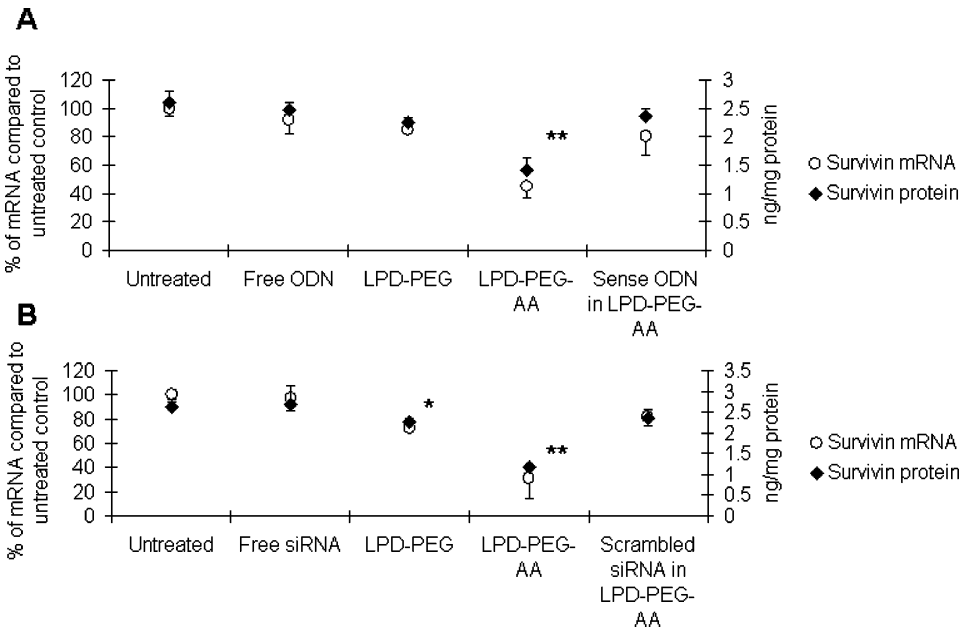

Figure 6. Survivin mRNA and protein levels in H1299 cells after AS-ODN (A) or siRNA (B) treatment. H1299 cells were treated with AS-ODN or siRNA in different formulations at 37 °C for 4 h. Survivin mRNA was quantified 24 h after treatment by real time PCR, and data are expressed as % of the untreated control. Survivin protein was quantified 48 h after treatment by the ELISA method. Data = mean ± SD ($n = 3$). * indicates $p < 0.05$, and ** indicates $p < 0.01$.

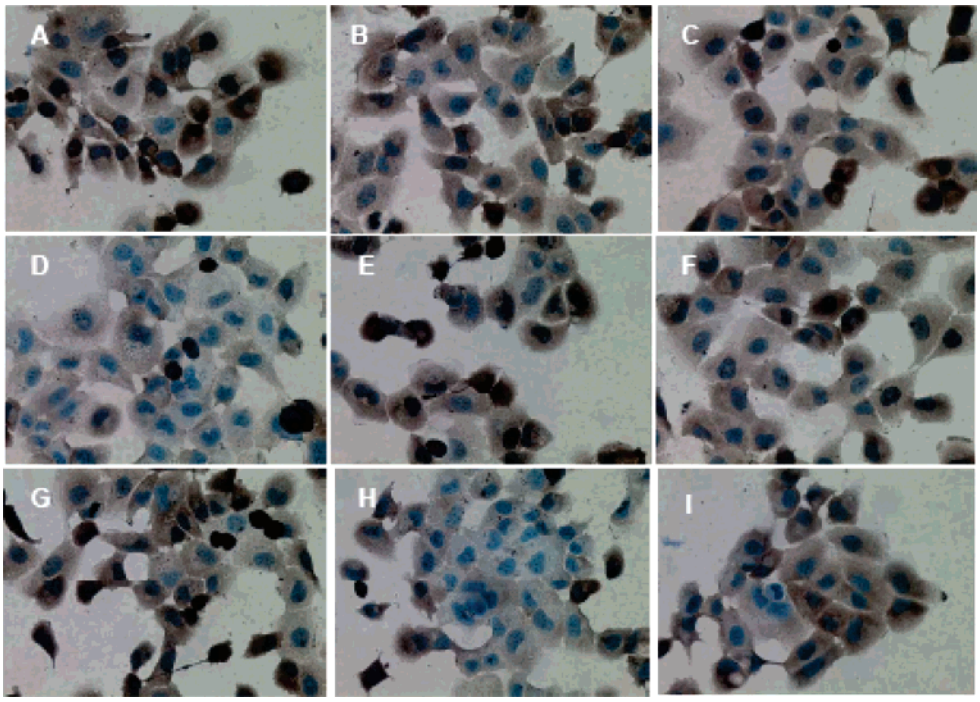

Figure 7. Microscopy photographs of fixed H1299 cells after immunostaining of survivin. Cells were treated with medium only (A), free AS-ODN (B), AS-ODN in LPD-PEG (C), AS-ODN in LPD-PEG-AA (D), Sense ODN in LPD-PEG-AA (E), free siRNA (F), siRNA in LPD-PEG (G), siRNA in LPD-PEG-AA (H), or scrambled siRNA in LPD-PEG-AA (I) at 37 °C for 4 h. Cells were fixed and immunostained for survivin expression. Magnification = 400×.

cells treated with medium only, free siRNA, siRNA in LPD-PEG, siRNA in LPD-PEG-AA, and scrambled siRNA in LPD-PEG-AA were 87%, 86%, 80%, 22%, and 87%, respectively. The results of the ELISA assays (Figure 6B) were consistent with those of the immunohistochemistry. Survivin down-regulation of free AS-ODN, AS-ODN in LPD-PEG, AS-ODN in LPD-PEG-AA, and sense ODN in LPD-PEG-AA was 5%, 10%, 40%, and 10%, respectively. In the case of siRNA, survivin down-regulation of free siRNA, siRNA in LPD-PEG, siRNA in LPD-PEG-AA, and scrambled siRNA in LPD-PEG-AA was 5%, 20%, 60%, and 20%, respectively. Despite a low level of nonspecific down-

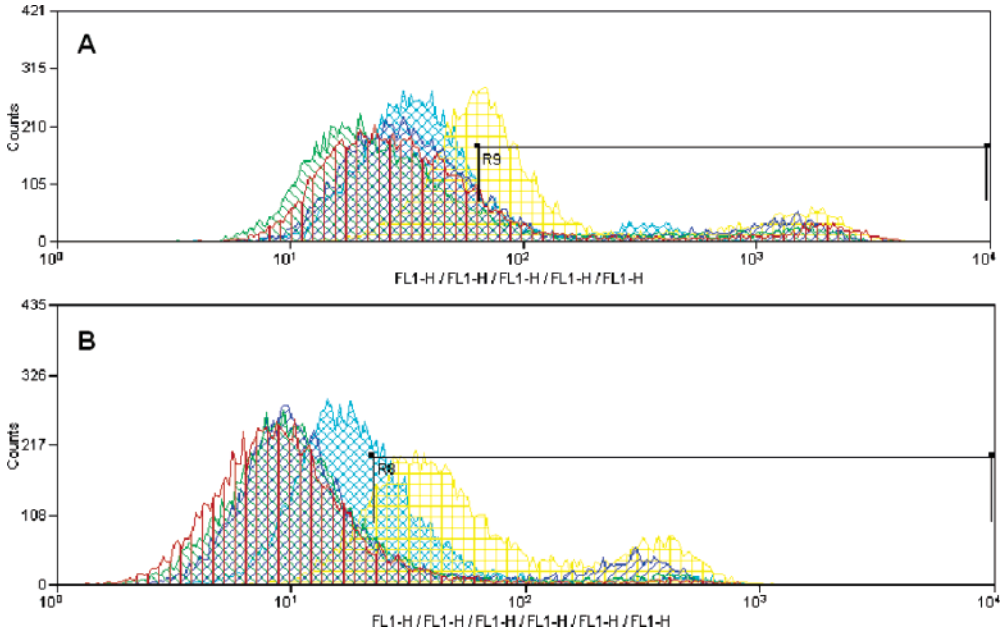

Figure 8. Flow cytometry analysis of Annexin V-FITC stained H1299 cells after treatment with AS-ODN (A) or siRNA (B). Cells were treated with medium only (red) or AS-ODN/siRNA in PBS (green), LPD-PEG (blue), LPD-PEG-AA (yellow), or sense ODN/scrambled siRNA in LPD-PEG-AA (light blue) at 37 °C for 72 h. Cells were trypsinized, resuspended, and stained with Annexin V-FITC. The FITC signal of cells was analyzed by a flow cytometer.

regulation in the control sense sequence and scrambled sequence (statistically insignificant), the antisense effect of the tested formulations were largely sequence and formulation dependent.

Phosphatidylserine (PS) is located in the inner leaflet of the cell membrane when cells are healthy. However, when cells are in the early stage of apoptosis, PS inverts to outer leaflet of the membrane and can be recognized by Annexin V.[44] We used flow cytometry to analyze the binding of FITC-labeled Annexin V with the apoptotic cells. As shown in Figure 8, the FITC-Annexin V signal shift was clearly seen when the cells were treated with AS-ODN or siRNA in LPD-PEG-AA, suggesting that cells were triggered to undergo apoptosis. However, we also detected some nonspecific toxicity of LPD-PEG-AA, since control sequence encapsulated in LPD-PEG-AA also induced a small degree of PS inversion. In the case of AS-ODN, as indicated in Table 2, Annexin V positive cells treated with medium only, AS-ODN in PBS, AS-ODN in LPD-PEG, AS-ODN in LPD-PEG-AA, and sense ODN in LPD-PEG-AA were 20%, 18%, 25%, 63%, and 28%, respectively. Annexin V positive cells treated with medium only, siRNA in PBS, siRNA in LPD-PEG, siRNA in LPD-PEG-AA, and scrambled siRNA in LPD-PEG-AA were 10%, 15%, 21%, 87%, and 38%, respectively.

The cellular growth inhibition effects of AS-ODN and siRNA delivered by different formulations followed a dose dependent manner as shown in Figure 9. At a concentration of 2000 nM, the growth inhibition effects of AS-ODN in PBS, AS-ODN in LPD-PEG, AS-ODN in LPD-PEG-AA, and sense ODN in LPD-PEG-AA were 5%, 15%, 60%, and 30%, respectively. For siRNA, at a concentration of 1000 nM, the growth inhibition effects of siRNA in PBS, siRNA in LPD-PEG, siRNA in LPD-PEG-AA, and scrambled siRNA in LPD-PEG-AA were 5%, 20%, 70%, and 30%, respectively. Although the cellular growth inhibition effect also showed sequence and formulation dependence, LPD-PEG-AA containing control sequences had nonspecific cytotoxicity when incubated with cells for 72 h. This finding is consistent with Annexin V staining assay, indicating that long time incubation with a LPD formulation is a stress for cells after large amounts have entered into the cells.

Table 2. Flow Cytometry Analysis of FITC-Annexin V Stained H1299 Cells after Treatment with AS-ODN or siRNA

treatment	diagram	signal	% annexin V positive[a]
medium	A	red	20.32
AS-ODN in PBS		green	18.23
AS-ODN in LPD-PEG		blue	25.03
AS-ODN in LPD-PEG-AA		yellow	63.03
sense ODN in LPD-PEG-AA		light blue	27.59
medium	B	red	10.64
siRNA in PBS		green	14.71
siRNA in LPD-PEG		blue	20.72
siRNA in LPD-PEG-AA		yellow	87.43
scrambled siRNA in LPD-PEG-AA		light blue	37.53

[a] Annexin V positive cells were analyzed on the basis of 10 000 cells.

(44) van Engeland, M.; Ramaekers, F. C.; Schutte, B.; Reutelingsperger, C. P. A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. *Cytometry* **1996**, *24* (2), 131−9.

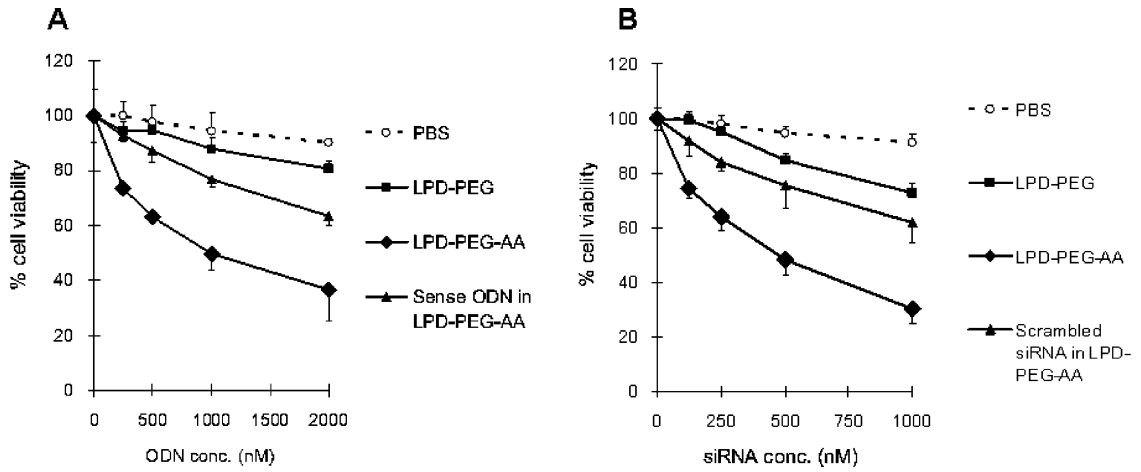

Figure 9. Cytotoxicity of AS-ODN (A) or siRNA (B) in different formulations. H1299 cells were incubated with various concentrations of AS-ODN/siRNA in PBS or AS-ODN/siRNA in LPD-PEG or LPD-PEG-AA. The viability of cells was measured 72 h later by MTT assay. Data = mean ± SD (n = 3).

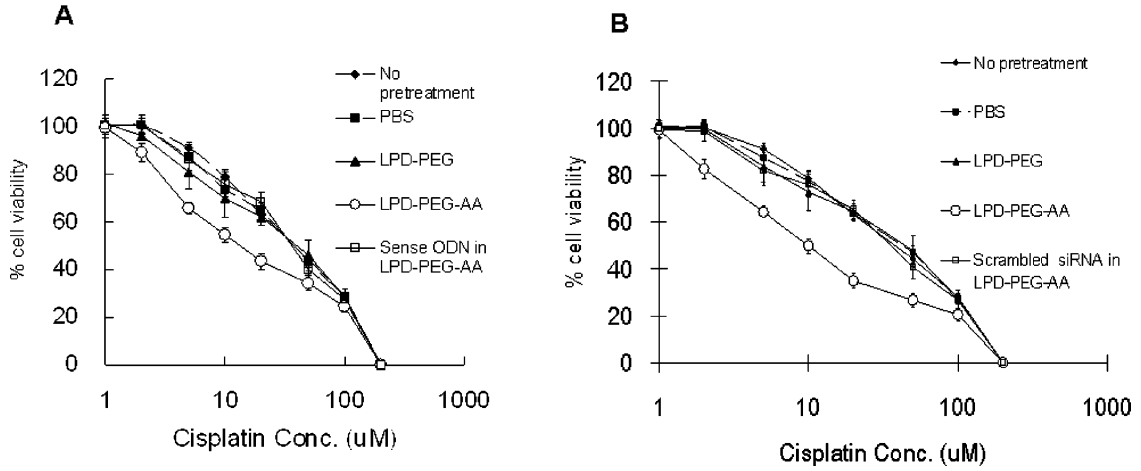

Figure 10. Chemosensitization of H1299 cells mediated by pretreatment of AS-ODN (A) or siRNA (B) in different formulations. Cells were incubated with AS-ODN/siRNA in different formulations at 37 °C for 4 h. Twenty four hours later, cells were challenged with various concentrations of cisplatin. The viability of cells was measured 48 h later by MTT assay. Data = mean ± SD (n = 3).

As shown in Figure 10, only AS-ODN or siRNA in LPD-PEG-AA could sensitize H1299 cells to cisplatin treatment. The IC50 was reduced from 40 μM to 20 μM and 10 μM after treatment with AS-ODN and siRNA in LPD-PEG-AA, respectively. This chemosensitization effect was highly sequence and formulation dependent, since LPD-PEG and control sequences had no effect. The results indicate that LPD-PEG-AA efficiently delivered AS-ODN or siRNA into H1299 cells, down-regulated survivin, an anti-apoptotic protein, and sensitized the cells to anticancer drug treatment.

In conclusion, we have demonstrated that the postinsertion method can be used to introduce PEGylated lipid to LPD and stabilize the nanoparticles in the presence of serum. Anisamide ligand increased delivery efficiency of LPD-PEG by 4−7-fold for sigma receptor overexpressing cells and provided strong antisense efficacies for down-regulating survivin mRNA and protein, inhibited tumor cell growth, and sensitized tumor cells to anticancer drugs. Thus, the PEGylated, anisamide targeted LPD shows a strong potential to deliver oligonucleotide for cancer therapy.

Abbreviations Used

LPD, liposome−polycation−DNA nanoparticles; DOTAP, 1,2-dioleoyl-3-(trimethyl)-ammonium propane; DSPE-PEG$_{2000}$, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000]; AA, anisamide; antisense oligodeoxynucleotide, AS-ODN; small interference RNA, siRNA; FAM, carboxyfluorescein; FITC, fluorescein 5(6)-isothiocyanate; LPD-PEG, PEGylated LPD; LPD-PEG-AA, anisamide conjugated PEGylated LPD.

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