Abstract. Background: Disulfide-linked oligodeoxyribonucleotide (ODN) liposomes were formulated and evaluated for the delivery of antisense ODN G3139 in KB human oral carcinoma cells. Materials and Methods: Liposomes composed of 1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane (DOTAP)/egg phosphatidylcholine/α-tocopheryl polyethylene glycol 1000 succinate were incorporated with hydrophobized disulfide-linked ODN. Disulfide-linked ODN liposomes were characterized for their size, ODN intracellular delivery, Bcl-2 mRNA and protein expression, growth inhibition, and chemosensitization. Results: Intracellular delivery of ODN with disulfide-linked ODN liposomes was more efficient than that with non-liposomal hydrophobized disulfide-linked ODN. Disulfide-linked ODN liposomes were characterized for their size, ODN intracellular delivery, Bcl-2 mRNA and protein expression, growth inhibition, and chemosensitization. Results: Intracellular delivery of ODN with disulfide-linked ODN liposomes was more efficient than that with non-liposomal hydrophobized disulfide-linked ODN. Treatment of the cells with disulfide-linked ODN liposomes resulted in efficient Bcl-2 down-regulation greater than that with hydrophobized disulfide-linked ODN and consistent with that of cellular growth inhibition and the sensitization to daunorubicin in KB cells. Disulfide-linked ODN liposomes exhibited superior colloidal stability during 5-week storage. Conclusion: Disulfide-linked liposomes are effective delivery vehicles for antisense ODN.

Nonviral gene therapy approach in the treatment of diseases has been increasingly attracting attention due to the limitations of the viral vectors, including virally-induced inflammatory responses, immunological reactions and oncogenicity (1-3). Cationic lipid delivery is a promising approach involving the use of oligodeoxyribonucleotide (ODN) complexes with cationic liposomes. Cationic liposomes have been reported to be used for ODN delivery in various cell lines both in vitro and in vivo (4-5).

Antisense ODNs, short single-strand DNA, are of potential interest for treatment of human diseases including cancer and viral infections (6-7). However, antisense ODNs are degradable in biological medium. Several approaches have been investigated to circumvent this disadvantage, including phosphorothioate modification of the ODN backbone (8) and 2'-sugar modifications (9). Furthermore, lower cellular uptake is another major problem of ODN administration, which limits its therapeutically activity (10-11). Several approaches have been investigated to improve uptake into the cells, including the association of ODN with carrier systems such as lipoprotein (12), cationic liposomes (13), pH-sensitive liposomes (14), ligand-targeted liposomes (15), and polymer nanoparticles (16).

In this study, we formulated disulfide-linked ODN liposomes and examined their ability to deliver ODN and their transfection efficiency in Bcl-2 down-regulation in KB cells. We also evaluated growth inhibition and chemosensitization with an anticancer drug, daunorubicin, in KB cells and monitored the colloidal stability of disulfide-linked ODN liposomes.

Materials and Methods

Materials. 1,2-Di-(9Z-octadecenoyl)-3-trimethylammonium-propylene (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphothanolamine-N-[3-(2-pyridyl)ethyldithio]propionate] (18:1 PDP PE or DOPE-PDP) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Egg phosphatidylcholine (PC) was purchased from Lipoid GMBH (Ludwigshafen, Germany).
α-Tocopheryl polyethylene glycol 1000 succinate (TPGS) was obtained as a gift from Eastman Chemical Ltd. (Llangefni, Anglesey, UK). Dithiothreitol (DTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640, fetal bovine serum (FBS), trypsin-EDTA and penicillin-streptomycin were purchased from Invitrogen (Grand Island, NY, USA). Six-well plates were purchased from TPP (Trasadingen, Switzerland).

**Antisense oligonucleotides.** Thiol-modified ODN, G3139 (G3139-SH), fully phosphorothioated 18mer oligonucleotide (sequence: 5'-TCT CCC AGC GTG CGC CAT-3'-thiol) was purchased from Operon Biotechnologies Inc. (Huntsville, AL, USA). G3139, fully phosphorothioated single-stranded 18mer oligonucleotide (sequence: 5'-TCT CCC AGC GTG CGC CAT-3'), fluorescein isothiocyanate (FITC)-labeled thiol G3139 (sequence: FITC-5'-TCT CCC AGC GTG CGC CAT-3'-thiol) and FITC-labeled G3139 (sequence: FITC-5'-TCT CCC AGC GTG CGC CAT-3') were purchased from Alpha DNA (Quebec, Canada). Thiol-modified G3139 was used immediately after being dissolved. In case of longer storage, thiol-modified G3139 was reactivated before use with DTT at a molar ratio of 1:100. The excess DTT was removed by ethanol precipitation (17).

**Preparation of hydrophobized disulfide-linked ODN.** DOPE-PDP was dissolved in ethanol and mixed with thiol-modified ODN solution (10 mM Tris, pH 8.0) at molar ratio of 5:5. The resulting DOPE-PDP-ODN was incubated for 1 h at room temperature prior to incorporation into liposome formulation or use in other experiments.

**Preparation of disulfide-linked ODN liposomes.** Cationic liposomes were prepared from DOTAP, PC and TPGS by ethanol dilution method described previously (18) with minor modification. DOTAP, PC and TPGS were dissolved in ethanol at a molar ratio of 58:40:2, and injected into HEPES buffered solution (20 mM HEPES, pH 7.4) upon stirring at room temperature. Ethanol was removed by dialysis using a molecular weight cut-off (MWCO) 10,000 Dalton Float-A-Lyser (Spectrum Laboratories Inc., Ranco Dominguez, CA, USA) and against HEPES buffer (20 mM HEPES, pH 7.4) for 2 h at room temperature. The resulting liposomes were then filter-sterilized using 0.22 μm pore-diameter filters (Fisher Scientific, Pittsburgh, PA, USA). Disulfide-linked ODN liposomes were prepared by mixing cationic liposomes with an equal volume of hydrophobized disulfide-linked ODN in HEPES buffer (20 mM HEPES, pH 7.4) (Figure 1) and incubated at room temperature for 15 min before use in transfection studies. The concentration of ODN was fixed at 0.9 μM.

**Particle characterization.** The particle size of disulfide-linked ODN liposomes was determined by dynamic light scattering using a particle sizer (NICOMP 370; Particle Sizing Systems Inc., Santa Barbara, CA, USA) in the volume weighing mode. The liposomes were prepared in 20 mM HEPES, pH 7.4 (300 μl). Prior to measurement, the samples were diluted with the same buffer to 500 μl. Cationic liposomes (DOTAP:PC:TPGS; 58:40:2) prepared by ethanol dilution without hydrophobized disulfide-linked ODN was used as the plain liposomes.

Encapsulation efficiency of ODN in disulfide-linked ODN liposomes was determined by centrifugation (14,000 rpm, 20 min) through a Microcon centrifugal filter device, MWCO 10,000 (Millipore Corporation, Bedford, MA, USA). FITC-labeled ODN disulfide-linked liposomes were prepared in 300 μl of 20 mM HEPES, pH 7.4. The fluorescence intensity (FI) of unencapsulated FITC-labeled ODN in the supernatant was determined by measuring at an excitation of 490 nm and emission of 520 nm on a luminescence spectrometer (LS 54B; Perkin Elmer, UK). The encapsulation efficiency (%) was calculated as follows. Empty liposomes were used as a background control. An ODN standard curve was generated between FI equivalent to 0.5 and 15 μg of ODN per ml in 20 mM HEPES, pH 7.4.

**RNA extraction and quantitative RT-PCR analysis of Bcl-2 mRNA expression.** Total RNA from cultured cells was extracted after 48 h incubation with the liposome formulation. Cells were harvested by trypsinization with 0.25% trypsin-EDTA and washed with PBS, pH 7.4. The cells were then lysed with 250 μl of Trizol reagent (Invitrogen, Grand Island, NY, USA) for 5 min and total RNA was extracted by adding 100 μl of chloroform. The total RNA was further purified by ethanol precipitation (19). The total RNA was transcribed into cDNA using the first-strand cDNA synthesis kit (Invitrogen Carlsbad, CA, USA). The resulting cDNA was amplified by quantitative RT-PCR StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using Taqman Universal Master
Mix (Applied Biosystems). Primer and probe sequences were designed by the Primer Express program (Applied Biosystems): \textit{Bcl-2} forward primer 5’ CCGCGGATCACCCTCCTGTC 3’; \textit{Bcl-2} reverse 5’ ACCGCGGATCACCCTCCTGTC 3’. The antisense effect of ODN in disulfide linked-ODN complexes was evaluated on \textit{Bcl-2} down-regulation in KB cells. Figure 2 shows the effect of ODN delivered by disulfide-linked ODN liposomes was dependent on their lipid-to-ODN ratio. The antisense effect of ODN in disulfide linked-ODN complexes was evaluated on \textit{Bcl-2} down-regulation in KB cells. Figure 2 shows the effect of ODN delivered by disulfide-linked ODN liposomes was dependent on their lipid-to-ODN ratio.

\textbf{Down regulation of \textit{Bcl-2} by disulfide-linked ODN liposomes.} The antisense effect of ODN in disulfide linked-ODN liposomes was observed when compared with untreated cells. In contrast, at lipid-to-ODN ratio of 4.6, disulfide-linked ODN liposomes achieved efficient \textit{Bcl-2} protein down-regulation \((p<0.05)\). The \textit{Bcl-2} protein down-regulation had a tendency to decrease at lipid-to-ODN ratios greater than 4.6. This finding shows that \textit{Bcl-2} down-regulation in KB cells by disulfide-linked ODN liposomes is lipid-to-ODN ratio dependent.

We compared disulfide-linked ODN liposomes with non-liposomal hydrophobized disulfide-linked ODN and Oligofectamine-ODN complexes with respect to their disrupting \textit{Bcl-2} mRNA and protein expression, as shown in Figure 3. Hydrophobized disulfide-ODN and free ODN did not reduce the \textit{Bcl-2} mRNA level but disulfide-linked ODN

\section*{Results}

\subsection*{Growth inhibition.} Evaluation of growth inhibition was performed by the trypan blue dye exclusion assay. KB cells were seeded in a 6-well plate at a density of 2×10^4 cells/cm^2 in 2 ml of growth medium and incubated for 24 h at 37°C under 5% CO\textsubscript{2} atmosphere. Untreated cells and cells transfected with free ODN, hydrophobized disulfide-linked ODN and Oligofectamine\textsuperscript{TM} Reagent (7.5 \mu l per well)-ODN complexes were used as controls. After transfection, the cells were washed with PBS and continued to culture in 2 ml of fresh growth medium at 37°C under 5% CO\textsubscript{2} atmosphere for 72 h. After incubation the cells were stained with 0.4% trypan blue stain solution (Lonza, Wakerville, MD, USA), and the cell number was counted on a hemacytometer (Hausser Scientific, Horsham, PA, USA). Relative growth inhibition \((\%)\) was calculated based on the density of viable cells. The viability of untreated control cells was arbitrarily defined as 100%.

\subsection*{Statistical analysis.} The results are represented as the mean\pm standard deviation (SD) of three repeat studies. Statistical significance of differences in \textit{Bcl-2} mRNA and protein expression, growth inhibition and chemosensitization were examined using one-way analysis of variance (ANOVA) followed by an LSD post hoc test. The significance level was set at \(p<0.05\).
liposomes down-regulated Bcl-2 by 58.25±4.66% (p<0.05). In comparison, oligofectamine-ODN complexes, used as a positive control, brought about 64.35±2.8% Bcl-2 mRNA down-regulation (Figure 3A).

The ability of disulfide-linked ODN liposomes to down-regulate Bcl-2 protein in KB cells was examined by Western blotting, and band intensities in treated cells relative to control untreated cells were quantified by using the ImageJ software. As shown in Figure 3B and 3C, in cells treated with disulfide-linked ODN liposomes, Bcl-2 protein level was reduced by 65.87±3.83% (p<0.05). Hydrophobized disulfide-linked ODN and free ODN slightly reduced the Bcl-2 protein level. The effect on Bcl-2 protein level correlated with the antisense ODN activity measured using the Bcl-2 mRNA level shown in Figure 3A. Oligofectamine-ODN complexes used as a positive control induced Bcl-2 protein down-regulation of 76.15±10.77%.

**Intracellular delivery of ODN by disulfide-linked ODN liposomes.** Cellular delivery of ODN by disulfide-linked ODN liposomes was examined by confocal fluorescence microscopy (Figure 4). FITC was incorporated into disulfide-linked ODN liposomes which were then incubated with KB cells for 4 h at 37°C under 5% CO2 atmosphere. Cells incubated with ODN alone and those with hydrophobized disulfide-linked ODN exhibited fluorescence around the cells, whereas cells incubated with disulfide-linked ODN liposomes exhibited ODN uptake as indicated by the fluorescence inside the cytoplasm. This finding demonstrates that ODN is more efficiently delivered into the cells by disulfide-linked ODN liposomes. Oligofectamine-ODN complexes as a positive control showed internalization of ODN.

**Effect of disulfide-linked ODN liposomes on KB cell growth.** The growth inhibition on KB cells following 72 h incubation with hydrophobized disulfide-linked ODN, free ODN and disulfide-linked ODN liposomes is shown in Figure 5A. Disulfide-linked ODN liposomes extensively inhibited cell growth to 47±4.2% (p<0.05) that of the control. There was no significant difference in cell growth inhibition between hydrophobized disulfide-linked ODN and free ODN. This finding shows that Bcl-2 down-regulation of disulfide-linked ODN liposomes was effective in inhibiting cancer cell growth. Oligofectamine-ODN complexes, as a positive control, caused cell growth inhibition of 49.5±3.5%.

**Sensitization of KB cells to daunorubicin treatment by ODN disulfide-linked liposomes.** Chemoresistance reversion and cell sensitization to chemotherapeutic drugs by Bcl-2 down-regulation was studied in KB cells with a commonly used
anticancer drug, daunorubicin. KB cells were pretreated with hydrophobized disulfide-linked ODN or disulfide-linked ODN liposomes for 24 h, prior to treatment with 0.5 μM of daunorubicin for another 24 h (Figure 5B). Treatment with daunorubicin alone had a moderate cytotoxicity of 51.8±4.4%, while pretreatment with hydrophobized disulfide-linked ODN and free ODN had no significant effect on cytotoxicity to daunorubicin in KB cells. The combination of pretreatment with disulfide-linked ODN liposomes followed by daunorubicin resulted in a significant improvement of cytotoxicity on KB cells to 65.3±4.7%. Oligofectamine-ODN complexes had a similar effect on cytotoxicity to disulfide-linked ODN liposomes.

Colloidal stability of disulfide-linked ODN liposomes. Cationic liposomes (DOTAP:PC:TPGS; 58:40:2) prepared by ethanol dilution had an average diameter of 27.1±3.6 nm as determined by dynamic light scattering. The particle size of disulfide-linked ODN liposomes was 176.2±11.2 nm. The efficiency of the oligonucleotide nanoencapsulation was 89.5±5.3%. The colloidal stability of the liposomes was evaluated by monitoring change in the average diameter of the formulation during storage at 4˚C. The particle size of the liposomes did not significantly change during 5 weeks at 4˚C (Figure 6).

Discussion

Cellular delivery is the major problem for ODN administration, which consequently affects its biological activities (10-11). In this study, we formulated and evaluated disulfide-linked ODN liposomes, and examined the cellular delivery of ODN and the transfection efficiency in Bcl-2 down-regulation in KB cells. We found that disulfide-linked ODN liposomes were efficient in delivery of ODN to KB cells and thus enhanced the transfection efficiency. The enhanced transfection efficiency was dependent on the lipid/ODN ratio. Hydrophobized disulfide-linked ODN itself did not penetrate the cells and thus suppressed the transfection efficiency.

Disulfide bond conjugation is a covalent linkage that occurs as a result of the reaction of two sulphhydryl (SH) groups of SH-containing substances. The linkage is stable in the extracellular environment but cleavable in the redox environment of the cytoplasm (20-21). The incorporation of disulfide bonds in delivery systems via disulfide bond conjugation has been reported in the application of drugs, genes and ODNs (22-25).

In this present study, we synthesized hydrophobized disulfide-linked ODN through thiol-disulfide exchange of thiol-modified ODN and DOPE-PDP, and incorporated hydrophobized disulfide-linked ODN into the liposomes.
Our study showed that non-liposomal hydrophobized disulfide-linked ODN did not trigger cellular uptake and delivery of ODN thereby biological activities, such as Bcl-2 down-regulation, growth inhibition and sensitization to daunorubicin were not evident. This could be due to the negative charge of hydrophobized disulfide-linked ODN that may inhibit its binding to the negatively charged cell surfaces. On the other hand, the disulfide-linked ODN liposome formulation enhanced cellular uptake and released the ODN, whereby inducing Bcl-2 down-regulation, growth inhibition and sensitization to daunorubicin in KB cells. The enhanced cellular uptake and release of ODN could be due to the combination of the components in the formulation. DOTAP, as a cationic lipid, may aid the cellular association and internalization of disulfide-linked ODN liposome in KB cells. Several reports have demonstrated on increase in cellular association and uptake of DNA and ODNs by cationic liposomes (10, 26). DOPE in our formulation may promote destabilizing of the endosomal membrane and release of the disulfide-linked ODN liposomes into the cytoplasm. DOPE, an adjuvant lipid, has been used in numerous liposome formulations to advance membrane disruption and nucleotide release, and thus enhance transfection efficiency (27, 28). The inclusion of DOPE in cationic liposomes did not affect DNA binding (29).

Subsequently, the ODN was cleavable from disulfide-linked ODN liposomes in the redox environment of the cytoplasm and was able to express its activities in KB cells. The introduction of disulfide bonds into the delivery systems may enhance the efficiency of gene transfection and have relatively low toxicity (30, 31).

Our successful delivery of ODN to KB cells could be due to the beneficial combination of cationic lipid DOTAP, adjuvant lipid DOPE and thiolytic cleavable disulfide-linked ODN. The biological activities of disulfide-linked ODN liposomes on Bcl-2 down-regulation, growth inhibition and sensitization to daunorubicin were evaluated relative to Oligofectamine-ODN complexes. In addition, the disulfide-linked ODN liposomes exhibited superior colloidal stability during 5 weeks. Our study demonstrated that disulfide-linked liposomes are a promising delivery vehicle for ODN.

**Conclusion**

Disulfide-linked ODN liposomes formulated in this study yielded nanosized particles of superior colloidal stability. A promising liposome formulation, DOTAP:Egg PC:TPGS:DOPE:ODN at a molar ratio of 58:40:2:5:5, exhibited high efficiency in cellular delivery of ODN, Bcl-2 mRNA and protein down-regulation, growth inhibition and sensitization to daunorubicin in KB oral carcinoma cells. This study suggests that disulfide-linked liposome is a highly effective delivery vehicle for antisense ODN.

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References


