Introduction

Cancer is one of the most deadly diseases. Conventional treatments such as radiation therapy and chemotherapy have side-effects. Gene-based therapy is a new approach that selectively targets cancer cells, while reducing toxicity on other cells (Pagnan et al., 2000). A potential strategy is to use short stretches of single-stranded oligodeoxyribonucleotides (ODNs) to block unwanted gene expression. The specificity of this strategy has been explored as an alternative therapy with potential clinical application (Herrington et al., 2011; Schimmer et al., 2011).

One of main cell death machinery is regulated by the opposing action of bcl-2 family member (Igney and Krammer, 2002; Green, 2005). Bcl-2 protein is a target for cancer gene therapy because it exhibits antiapoptotic activity and is overexpressed in many types of cancers. Bcl-2 protein can block the release of cytochrome c into the cytosol after the initiation of apoptosis, which prevents the downstream propagation of the death signal, thereby promoting cell survival (Beck et al., 2002). Overexpression bcl-2 also causes the resistance of cancer cells to a variety of anticancer agents (Sartorius and Krammer, 2002; Zangemeister-Wittke, 2003). Preclinical and clinical studies have established that downregulation of bcl-2 protein leads to an increase in apoptosis and improved response to chemotherapy (Chi et al., 2000; Kirkwood et al., 2002).

Abstract

Previous study has shown human serum albumin (HSA) coated liposomes can deliver bcl-2 antisense oligodeoxyribonucleotide (ODN) into KB carcinoma cells, and decrease bcl-2 mRNA and protein expression level. In the current study, cell growth inhibition and chemosensitization of KB cells were evaluated. Liposomes composed of dimethyldioctadecyl ammonium bromide/egg phosphatidylcholine/α-tocopheryl polyethylene glycol 1000 succinate (58:40:2 molar ratio) complexed with bcl-2 antisense ODN and coated with HSA were examined for cell growth inhibition and sensitization to a commonly used chemotherapeutic drug, doxorubicin. HSA-coated liposome–ODN complexes effectively inhibited cell growth in the range of ODN concentration of 0.45–7.2 μM. Upon posttreatment with doxorubicin, the cytotoxicity was further significantly increased compared to the ODN complexes alone. The cytotoxicity was dependent on antisense ODN concentration, incubation time and doxorubicin concentration, and relatively independent on HSA concentration. This study suggests that HSA-coated liposomes are effective delivery vehicles for antisense ODN with potential therapeutic application and can be effectively combined with doxorubicin.

Keywords: Liposome, human serum albumin, antisense oligodeoxyribonucleotide, bcl-2, lipoplex, growth inhibition, chemosensitization, doxorubicin
Several antisense oncology trials could not fulfill the initial goals, which have raised doubts about the clinical potential of this technology (Gleave and Monia, 2005). A major problem of antisense ODN administration, which limits its therapeutic activity, is low cellular uptake (Arima et al., 1997; Le Doan, 2001). Patients with locally advanced breast cancer received bcl-2 antisense ODN by continuous intravenous infusion had limited bcl-2 down regulation in the tumors, related with insufficient delivery to the intact tumors (Moulder et al., 2008).

Improving the efficacy of therapy, drug delivery systems based on lipids, such as cationic liposomes, have potential usefulness to improve the intracellular delivery of antisense ODNs (Pastorino et al., 2008). For example, a cholesterol-derived cationic lipid for delivery of plasmid DNA (Kim et al., 2009), membrane sensitive peptide-derived cationic lipid for delivery of antisense ODN (Jääskeläinen et al., 2000), and amino acid-derived cationic lipid has been used for delivery of siRNA (Suh et al., 2009).

Our previous study has investigated human serum albumin (HSA)-coated liposomes could deliver bcl-2 antisense ODN into KB carcinoma cells and decrease bcl-2 mRNA and protein expression. (Weecharangsan et al., 2009). In current study, we examined cell growth inhibition and sensitization induced by a commonly used chemotherapeutic drug, doxorubicin, after transfecting bcl-2 antisense ODN by HSA-coated liposomes.

Materials and methods

Dimethyldioctadecyl ammonium bromide (DDAB), α-tocopheryl polyethylene glycol 1000 succinate (TPGS), and HSA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Egg phosphatidylcholine (PC) was obtained from Lipoid GmbH (Ludwigshafen, Germany). Doxorubicin was purchased from Pfizer (Bentley, Australia). Bcl-2 antisense ODN, fully phosphorylated 18-mer oligonucleotide (Sequence 5′-3′: TCT CCC AGC GTG CGC CAT) was purchased from Alpha DNA (Quebec, Canada). Lipofectamine™ 2000 reagent, MEM media and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY, USA). 96-well plates were purchased from Corning Inc. (Corning, NY, USA).

Preparation of cationic liposomes and HSA liposome–ODN complexes

Cationic liposomes were prepared from DDAB, PC, and TPGS by ethanol dilution as described previously (Maurer et al., 2001) with minor modification. Briefly, DDAB, PC, and TPGS were dissolved in ethanol at a molar ratio of 58:40:2 and injected into a stirring HEPES buffered solution (20 mM HEPES, pH 7.4) at room temperature. Cationic liposome–ODN complexes were prepared by mixing cationic liposomes with an equal volume of ODN at a lipid-to-ODN ratio of 15:1 in MEM and incubated at room temperature for 15 min. HSA was directly added to the liposome–ODN complexes at molar ratios to lipid of 0.15:100, 0.75:100, 1.5:100, 3:100, 4.5:100, and 9:100, and incubated for 15 min before use in transfection. The concentration of ODN was 0.45, 0.9, 1.8, 3.6, and 7.2 µM.

Cell culture

KB human oral carcinoma cells were obtained from Natural Products Research Section, Reasearch Division, National Cancer Institute, Bangkok, Thailand. KB cells have been reported to display HeLa markers and thus are possibly a subline of HeLa cervical cancer cells. The cells were grown in MEM containing 10% FBS, 100 µg/mL streptomycin, 100 U/mL penicillin, and 1% amphotericin B. The cells were maintained at 37°C in a humidified incubator with 5% CO₂.

Growth inhibition

Evaluation of growth inhibition was performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. KB cells were seeded in a 96-well plate at a density of 7 × 10⁴ cells/cm² in 100 µL of growth medium and incubated for 16h at 37°C under 5% CO₂ atmosphere. Prior to treatment, the medium was removed and the cells were rinsed with PBS. The cells were incubated with 62.5 µL of HSA liposome–ODN complexes and blank liposomes for 6h at 37°C under 5% CO₂ atmosphere. Untreated cells and cells transfected with free ODN and Lipofectamine™ 2000 (0.23 µL per well)–ODN complexes were used as controls. After transfection, the cells were washed with PBS and continued to culture in 100 µL of fresh growth medium at 37°C under 5% CO₂ atmosphere for 72 h. After incubation the viability of cell was determined by the MTT assay. Relative growth inhibition (%) was calculated based on absorbance at 570 nm using a microplate spectrophotometer (Zenyth 200 rt; Anthos Labtech Instruments GmbH, Salzburg, Austria). The viability of untreated control cells was arbitrarily defined as 100%.

Chemosensitization

KB cells were seeded into 96-well plates at a density of 7 × 10⁴ cells/cm² in 100 µL of growth medium and incubated for 16h. Prior to treatment, the medium was removed and the cells were rinsed with PBS. The cells were incubated with 62.5 µL of HSA liposome–ODN complexes for 6h at 37°C under 5% CO₂ atmosphere. Untreated cells and cells transfected with free ODN were used as controls. After transfection, the medium was replaced with 100 µL of fresh growth medium, and the cells were incubated for 24 h at 37°C under 5% CO₂ atmosphere. Cells were then incubated with medium containing doxorubicin at 0.1, 0.25, 0.5, or 0.75 µM. After another 24, 48, or 72 h, cytotoxicity was determined by the MTT assay. Relative cytotoxicity (%) was calculated based on absorbance at 570 nm using a microplate spectrophotometer (Zenyth 200 rt; Anthos Labtech Instruments GmbH, Salzburg, Austria). The viability of untreated control cells was arbitrarily defined as 100%.
GmbH, Salzburg, Austria). The cytotoxicity of untreated control cells was arbitrarily defined as 0%.

Statistical analysis
The results are represented as the mean ± SD of three repeat studies. Statistical significance of differences in growth inhibition and cytotoxicity were examined using one-way ANOVA followed by an LSD post hoc test.

Results
HSA-coated liposome–ODN complexes on KB cell growth inhibition
The growth inhibition on KB cells following 72h incubation with HSA-coated liposome–ODN complexes, Lipofectamine-ODN complexes, free ODN at ODN concentration of 1.8 and 3.6 μM, and liposome alone is shown in Figure 1. HSA-coated liposome–ODN complexes inhibited cell growth at ODN concentration of 1.8, and extensively at 3.6 μM. The cell growth inhibition level was relatively independent on the molar ratio of HSA-to-lipid of 0.15:100 to 9:100 in ODN complex formulation. HSA-coated liposome–ODN complexes inhibited cell growth to 29.0±15.7 to 53.5±5.6% and 61.8±14.4 to 72.8±8.8% that of the control at ODN concentrations of 1.8 (p<0.05) and 3.6 μM (p<0.001), respectively. This finding shows that bcl-2 downregulation of HSA-coated liposome–ODN complexes was effective in inhibiting cancer cell growth with increasing ODN concentration. Our previous study showed that HSA enhanced the uptake liposome–ODN complexes (Weecharangsan et al., 2009), however, the cytotoxicity relatively independent on the concentrations of HSA. HSA-coated liposomes at the molar ratios of HSA of 3:100 to 9:100 slightly inhibited cell growth to 23.9±9.5 to 35.5±13.3% (p>0.05). HSA-coated liposomes at the molar ratios of HSA of 0.15:100 to 1.5:100 had no effect on the level of cell growth. We, therefore, selected HSA-coated liposome at the HSA of molar ratio of 1.5:100 for further study. Lipofectamine-ODN complexes, as a positive control, at ODN concentrations of 1.8 and 3.6 μM caused cell growth inhibition of 84.4±7.0 (p<0.05) and 94.1±1.1% (p<0.001), respectively. Lipofectamine alone inhibited cell growth to 61.1±14.9% (p<0.05).

Sensitization of KB cells to doxorubicin treatment by HSA-coated liposome–ODN complexes
Chemosensitivity reversion and cell sensitization to anticancer chemotherapy drug by bcl-2 downregulation was studied in KB cells with a commonly used anticancer drug, doxorubicin. KB cells were pretreated with HSA-coated liposome–ODN complexes for six following in growth medium for 24h, prior to treatment with doxorubicin for another 48h (Figure 2). When the bcl-2 ODN was delivered as HSA-coated liposome complexes, KB cells became more susceptible to the cytotoxic effect of doxorubicin compared to the cells treated with doxorubicin and bcl-2 ASO. HSA-coated liposome–ODN complexes significantly improved cytotoxicity at ODN concentration of 1.8 μM (p<0.05), and extensively at 3.6 μM (p<0.001) in combination with 0.5 μM doxorubicin.

Effect of HSA concentration
Treatment with doxorubicin alone had a slight cytotoxicity of 22.3±2.9%, whilepretreatment with free ODN at a concentration of 1.8 μM followed by doxorubicin had a slight cytotoxicity of 34.7±14.0% (p>0.05) in KB cells. The combination of pretreatment with HSA-coated liposome–ODN complexes at the molar ratio of HSA of 0.15:100 to 9:100 followed by doxorubicin resulted in an improvement of cytotoxicity on KB cells from 42.2±14.4 to 69.6±8.3%. The cytotoxicity level was relatively independent on the ratio of HSA-to-lipid from 0.15:100 to 9:100 followed by doxorubicin resulted in an improvement of cytotoxicity at ODN concentration of 1.8 μM (p<0.05), and extensively at 3.6 μM (p<0.001) when compared with cells treated with ODN

Figure 1. Effect of HSA concentration of HSA-coated liposome/ODN complexes on the growth of KB cells. Cells were incubated with HSA-coated liposome/ODN complexes for 6h and in growth medium for 72h. White bar: HSA-coated liposome; dash bar: ODN concentration of 1.8 μM; dark bar: ODN concentration of 3.6 μM. *p<0.05; #p<0.001 compared with cells treated with ODN; #p<0.05 when compared with untreated cells. HSA, human serum albumin; ODN, oligodeoxyribonucleotide.

Figure 2. Effect of HSA concentration of HSA-coated liposome/ODN complexes on the sensitization to doxorubicin in KB cells. Cells were incubated with HSA-coated liposome/ODN complexes for 6h, in growth medium for 24h, and in growth medium containing 0.5 μM doxorubicin for 48h. Dash bar: ODN concentration of 1.8 μM; dark bar: ODN concentration of 3.6 μM. *p<0.05; #p<0.001 when compared with cells treated with 0.5 μM doxorubicin. HSA, human serum albumin; ODN, oligodeoxyribonucleotide.
cytotoxicity of 56.2 ± 1.0% (p > 0.001), while the combination of pretreatment with HSA-coated liposome–ODN complexes followed by doxorubicin treatment resulted in a significant improvement of cytotoxicity on KB cells from 88.2 ± 2.6 to 90.7 ± 2.5% (p < 0.001). The different ratio of HSA in HSA-coated liposome–ODN complexes had no effect on the level of cytotoxicity (Figure 2).

Effect of ODN concentration
The cytotoxicity of HSA-coated liposome–ODN complexes was studied following treatment with and without doxorubicin at a concentration of ODN of 0–7.2 μM (Figure 3). Treatment the cells with HSA-coated liposome–ODN complexes followed by doxorubicin treatment slightly improved the cytotoxicity at ODN concentration from 0.45 μM to 9.9 ± 12.4% (p > 0.05). Increasing the concentration of ODN of 0.9–7.2 μM in HSA-coated liposome formulation dramatically increased the cytotoxicity on KB cells from 25.0 ± 8.3 to 69.4 ± 6.6%. The cytotoxicity on KB cells treated with HSA-coated liposome–ODN complexes followed by doxorubicin significantly improved from 50.2 ± 4.7 to 90.7 ± 1.0% at ODN concentrations of 0.9–7.2 μM, respectively. This shows that the cytotoxicity level was dependent on the concentration of ODN in the liposome formulation and the KB cells treated with doxorubicin was significantly sensitized by HSA-coated liposome–ODN complexes at a concentration of ODN of 0.9–7.2 μM, respectively. The IC50 of KB cells treated with HSA-coated liposome–ODN complexes was 3.2 ± 1.3 μM, and reduced to 0.9 ± 0.1 μM in the cells treated with HSA-coated liposome–ODN complexes followed by doxorubicin treatment.

Effect of incubation time
Effect of incubation time of doxorubicin of 24, 48, and 72 h on KB cytotoxicity after pretreatment with HSA-coated liposome–ODN complexes at an ODN concentration of 0.9 μM is shown in Figure 4. Cytotoxicity after pretreatment with HSA-coated liposome–ODN complexes significantly increased with increased incubation time of doxorubicin from 24 to 72 h.

Effect of doxorubicin concentration
The cytotoxicity of KB cells pretreated with HSA-coated liposome–ODN complexes at an ODN concentration of 0.9 μM after 24 h ODN treatment following 48 h incubation with doxorubicin at concentrations of 0.1, 0.25, 0.5, and 0.75 μM is shown in Figure 5. The cytotoxicity of KB cells pretreated with HSA-coated liposome–ODN complexes significantly improved following 48 h incubation with doxorubicin at the concentrations of 0.5 and 0.75 μM (p < 0.001). The IC50 of KB cells treated with doxorubicin was >0.75 μM, and reduced to 0.25 ± 0.14 μM in the cells treated with HSA-coated liposome–ODN complexes following doxorubicin treatment.

Discussion
Our previous study showed that HSA-coated liposomes are effective delivery vehicle for bcl-2 antisense

Figure 3. Effect of ODN concentration of HSA-coated liposome/ODN complexes on the sensitization to doxorubicin in KB cells. Cells were incubated with HSA-coated liposome/ODN complexes at HSA to liposome molar ratio of 1.5:100 for 6 h, in growth medium for 24 h, and in growth medium containing 0.5 μM doxorubicin for 48 h. Dash bar: without 0.5 μM doxorubicin; dark bar: with 0.5 μM doxorubicin. NS, not significant; †p < 0.05; ‡p < 0.005; ††p < 0.001 when compared with untreated cells. HSA, human serum albumin; ODN, oligodeoxyribonucleotide.

Figure 4. Effect of incubation time of doxorubicin treatment on KB cell after treatment with HSA-coated liposome/ODN complexes. Cells were incubated with HSA-coated liposome/ODN complexes at HSA to liposome molar ratio of 1.5:100 and ODN concentration of 0.9 μM for 6 h, in growth medium for 24 h, and in growth medium containing 0.75 μM doxorubicin for 24, 48 and 72 h. *p < 0.005; †p < 0.001 compared with cells treated with ODN. HSA, human serum albumin; ODN, oligodeoxyribonucleotide.

Figure 5. Effect of doxorubicin concentration on KB cells after treatment with HSA-coated liposome/ODN complexes. Cells were incubated with HSA-coated liposome/ODN complexes at HSA to liposome molar ratio of 1.5:100 and ODN concentration of 0.9 μM for 6 h, in growth medium for 24 h, and in growth medium containing 0.75 μM doxorubicin for 48 h. Dash bar: untreated cells; dark bar: HSA liposome/ODN complexes. *p < 0.001 when compared with untreated cells. HSA, human serum albumin; ODN, oligodeoxyribonucleotide.
ODN and induced downregulation of bcl-2 in KB cells (Weecharangsan et al., 2009). In the current study, we further evaluated bcl-2 antisense ODN delivered by HSA-coated liposomes on KB cell growth and chemosensitization with a commonly used anticancer chemotherapy drug, doxorubicin. We used KB oral carcinoma cells as a representative of human carcinoma cells. We found that HSA-coated bcl-2 antisense ODN-liposome complexes could effectively inhibit cell growth and sensitize KB cells treated with doxorubicin. The cytotoxicity was dependent on the ODN concentration, incubation time, doxorubicin concentration, and relatively not on the HSA concentration. In order to exclude unspecified toxic effects, we tested HSA-coated liposomes alone on the cytotoxic effect. We found that HSA-coated liposomes at the molar ratios of HSA from 3:100 to 9:100 slightly inhibited cell growth. HSA-coated liposomes at the molar ratios of HSA from 0.15:100 to 1.5:100 had no effect on the level of cell growth.

Our study showed that HSA-coated liposome–ODN complexes inhibited KB cell growth at an ODN concentration of 1.8, and extensively at 3.6 µM. Doi et al. (2004) showed that the cell viability of H69 small cell lung cancer cells treated with bcl2-ODN in the form of lipoplexes was dose-dependent. Cytotoxicity in B16 (F10) melanoma cells transfected with cationic liposomes loaded with proapoptotic peptide and bcl-2 ODN increased with increasing ODN concentration (Ko et al., 2009).

Our preliminary study on ODN treatment (24 and 48h) following doxorubicin treatment showed that 24 h ODN treatment following doxorubicin treatment was the most effective cytotoxic effect. Therefore, we investigated the chemosensitization of KB cells after 24 h ODN treatment following 24–72 h doxorubicin treatment. Doxorubicin treatment slightly reduced the cell growth. The combination of HSA-coated liposome–ODN complexes with a subsequent treatment with doxorubicin substantially increased the cytotoxicity. Our result showed that treatment of KB cells with low concentration of 0.45 µM by HSA-coated liposome–ODN complexes did not significantly inhibit cell growth following doxorubicin treatment. This suggests that a low concentration of bcl-2 ODN might not be adequate to initiate cell death. Increased ODN concentrations of 0.9–7.2 µM in HSA-coated liposome–ODN complexes were therefore much more effective. The high ODN concentration itself may have potential cytotoxic effect and may not be applicable in clinical use; therefore, the study on the effect of incubation time and doxorubicin concentration was used with ODN concentration of 0.9 µM.

The cytotoxicity of KB cells after pretreatment with HSA-coated liposome–ODN complexes increased with increased incubation time of doxorubicin from 24 to 72 h. This result showed that optimal incubation time after ODN treatment is necessary. Lima et al. (2004) showed that the effective reduction in MCF-7 human breast cancer cell proliferation was 48–96 h cytotoxic drug treatment after siRNA transfection. Growth inhibition of GI-LI-N human neuroblastoma cells treated by c-myc antisense oligodeoxynucleotides encapsulated in anti-disialoganglioside GD liposomes increased with increasing postincubation period and decreasing c-myc protein expression (Pagnan et al., 2000).

The cytotoxicity of KB cells after pretreatment with HSA-coated liposome–ODN complexes increased with increased doxorubicin concentration from 0.1 to 0.75 µM. Hussain et al. (2006) showed that cytotoxicity increased with increasing concentration of doxorubicin in SW2 small cell lung cancer and MCF-7 breast adenocarcinoma cells pretreated with bcl-2 and bcl-xL antisense ODN-loaded immunoliposomes. Cytotoxicity on CD133+ cells after silencing of MDR1 by MDR1 siRNA PEI-lipid complexes increased with increasing paclitaxel concentration (Liu et al., 2009). Apoptosis of U138MG and U251MG human glioblastoma cells treated with taxol was dose-dependent in combination with bcl-2 siRNA duplex (George et al., 2009).

**Conclusion**

In the present study, bcl-2 antisense ODN delivered with HSA-coated liposomes effectively reduced the growth of KB oral carcinoma cells and significantly increased the chemosensitivity to doxorubicin. This study suggests that HSA-coated liposomes are effective delivery vehicles for antisense ODN with potential therapeutic application and can be effectively combined with doxorubicin, and have potential to apply with other antisense ODN and carcinoma cells.

**Declaration of interest**

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**References**


