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Growth inhibition of combined treatment of cationic liposome/p53 complexes and cisplatin on human carcinoma cells

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ABSTRACT

A combination of cationic liposome/green fluorescence protein (GFP)-p53 complexes and a chemotherapeutic drug, cisplatin, was evaluated for therapeutic activity in human carcinoma cells. Cationic liposome/GFP-p53 complexes and cationic liposome/PEI (polyethylenimine) 0.8k/GFP-p53 complexes were synthesized and evaluated in HeLa and in A549 cells for uptake and cytotoxicity, alone and in combination with cisplatin. The particle size of cationic liposome/GFP-p53 complexes and cationic liposome/PEI 0.8K/GFP-p53 complexes was 318 ± 18 to 754 ± 108 nm, and zeta potentials were -15.7 ± 2.8–$+27 \pm 08$ mV. The GFP expression on the delivery by cationic liposome/pEGFP (enhanced green fluorescence protein) complexes and cationic liposome/PEI 0.8K/pEGFP complexes was demonstrated. Treatment of the cells with either cationic liposome/GFP-p53 complexes or cationic liposome/PEI 0.8K/GFP-p53 complexes inhibited the cell growth. On post treatment with cisplatin, the growth inhibition of the complexes was further increased in HeLa cells and significantly increased in A549 cells on the lipid-to-DNA ratio. This study concluded that the sensitivity to the cancer cells to cisplatin was dependent on the cell line and the ratio of cationic liposome and GFP-p53. GFP-p53 expression delivered by cationic liposome/GFP-p53 complexes would be useful to increase the effect of cisplatin on the treatment of cancer cells.

INTRODUCTION

Cancer is a major disease in the world (Bray *et al.*, 2018). The number of new cases of lung cancer occurred 11.8% and other cancer 53.9%. Lung cancer is the first rank of incidence, mortality, and prevalence by cancer site (WHO, 2018).

Several attempts including p53 gene-based therapy, antisense and siRNA, p53 vaccine, small molecule activating p53, inhibition of p53-mfm2 interaction, and combination of drugs have been tried to activate p53 (Lane *et al.*, 2010). P53 has an important role in the chemotherapeutic drug response of cancer cells. Stabilizing p53, thus increasing in Bax and decreasing in bcl-2, by Vitamin C sensitized cervical carcinoma cells to cisplatin (Reddy *et al.*, 2001). An introduction of p53 cDNA into ovarian cancer cells increased sensitivity to cisplatin (Jin *et al.*, 2002).

Cisplatin has been shown to have efficacy in cancer treatment. A single high-dose cisplatin was more effective than the fractionated doses; however, side effects, such as vomiting and leukopenia still occurred (Li *et al.*, 2019). Synergist treatment has been demonstrated to be an effective alternative to inhibit cancer. Rh-endostatin combined with vinorelbine plus cisplatin improved the survival rate of patients with advanced non-small-cell cancer (An *et al.*, 2018). A combination of cyclophilin inhibitor and cisplatin has shown synergistic cytotoxicity in human hepatocellular carcinoma cells (Lee, 2010). In addition, a combination of cisplatin and photodynamic therapy has shown a synergistic cytotoxicity in HeLa cells (Wei *et al.*, 2013).

Gene therapy has been depicted to synergize with chemotherapy. Cationic liposome-iNOS gene delivery enhanced the anticancer effect of cisplatin in human lung cancer xenograft mouse models (Ye *et al.*, 2013). Cationic liposome bcl-2 antisense-coated human serum albumin increased the anticancer effect in oral carcinoma cells by the chemotherapeutic drug, doxorubicin (Weecharangsan *et al.*, 2012).

The present study investigated a combined treatment of cationic liposome/p53 complexes and a chemotherapeutic drug,

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cisplatin on human carcinoma cell growth, HeLa, and A549 cells. This study had a novelty on the combined treatment of cationic liposome/p53 complexes and chemotherapeutic drug on human carcinoma cell growth.

MATERIALS AND METHODS

Materials

Dimethyldioctadecyl ammonium bromide (DDAB), polyethylenimine (PEI 0.8KDa, branched), egg phosphatidylcholine, and α-tocopherol polyethylene glycol 1000 succinate were purchased from Sigma-Aldrich (St. Louis, MO). pEGFP (enhanced green fluorescence protein)-C2 plasmid DNA was obtained from Clontech (Palo Alto, CA). Plasmid GFP-p53 encoding p53 protein was obtained from Addgene (plasmid # 12091, Tyler Jacks) (Boyd et al., 2000). Six- and 96-well plates were purchased from SPL Life Sciences (Gyeonggi-do, Korea). Cisplatin was purchased from Roche (Basel, Switzerland). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Bio Basic Inc. (Ontario, Canada). Fetal bovine serum, Minimum Essential Medium (MEM) and OptiMEM were purchased from Invitrogen (Grand Island, NY). Human cervical carcinoma (HeLa) and human lung adenocarcinoma (A549) cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD).

Cationic liposome/GFP-p53 complexes and characterization

Cationic liposomes were prepared with cationic lipid, DDAB as previously described (Maurer et al., 2001; Weecharangsan et al., 2014). Briefly, DDAB, egg phosphatidylcholine, and α-tocopherol polyethylene glycol 1000 succinate were dissolved in absolute ethanol at a molar ratio of 58:40:2, diluted into (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES)-buffered solution at pH 7.4, and mixed for 30 minutes by vortexing at room temperature. Cationic liposomes/GFP-p53 complexes were prepared at weight ratios of 1.5, 3.1, 6.2, 12.5, and 25, and the complexes were initiated by mixing for 3–5 seconds and incubated at 25°C for 15 min. The amount of GFP-p53 used was 0.125–0.5 μg. Cationic liposome/PEI 0.8K/ GFP-p53 complexes were prepared by mixing PEI0.8K with GFP-p53 solution at a PEI:DNA ratio of 1.1:1 and then mixed with cationic liposomes. The formation of cationic liposome/GFP-p53 complexes and cationic liposome/PEI0.8K/GFP-p53 complexes were checked using gel electrophoresis (Weecharangsan et al., 2014).

Particle size and zeta potential measurements

Particle size and zeta potential of cationic liposome/GFP-p53 complexes and cationic liposome/PEI0.8K/GFP-p53 complexes were measured by photon correlation spectroscopy using a Zetasizer (Malvern). The complexes were prepared in DI water, diluted to 1 ml, and measured at 25°C using aqueous flow cell. The amount of cationic liposome/PEI0.8K/GFP-p53 used was 6.25–12.5/1.1/1 μg.

Cell culture

Human cervical carcinoma (HeLa) and human adenocarcinoma (A549) cell lines were used in this study. The cells were grown in MEM with 100 μg/ml streptomycin and 100 U/ml penicillin, 1% amphotericin B, and 10% fetal bovine serum. Cells were incubated in 95% atmosphere and 5% CO_2 at 37°C.

Intracellular delivery and gene expression

pEGFP was transferred into HeLa and A549 cells by cationic liposomes/pEGFP complexes and cationic liposome/PEI0.8K/pEGFP complexes. Cells were seeded in a 6-well plate at 1.5×10^5 cells/well for HeLa and 9×10^4 cells/well for A549. After 24 hours, the cells were treated with OptiMEM having cationic liposomes/pEGFP complexes and cationic liposome/PEI0.8K/pDNA complexes for 4 hours. Cells incubated with pEGFP and without complexes were used as controls. After 4-hour incubation, the cells were rinsed and incubated with the growth media for 24 hours. Following this, cells were rinsed and trypsinized. The number of GFP expressing cells was measured by flow cytometer InCyte software (Guava, Merck, Germany).

Cell growth inhibition

Cell growth inhibition by cationic liposomes/GFP-p53 complexes and cationic liposome/PEI0.8K/GFP-p53 complexes was determined using MTT assay. HeLa cells were cultured in growth media and seeded into 96-well plate (5×10^3 cells/well), and let them grown. After 24 hours, the cells were rinsed, and the cationic liposomes/GFP-p53 complexes and cationic liposome/PEI0.8K/GFP-p53 complexes prepared in 62.5 μl OptiMEM at a weight ratio of 6.25:1 and 6.25:1.1:1, respectively, were transferred to the cells. After 4-hour incubation, the cells were rinsed, growth medium was added, and the cells were further incubated for 24 hours. The cell growth inhibition was measured by MTT assay using a microplate reader (SpectraMax M3, Molecular Devices, San Jose, CA). The cell growth inhibition (%) was calculated by the absorbency of the treated cells relative to the untreated cells.

Chemosensitization of cisplatin to HeLa and A549 cells

Cells were seeded into 96-well plate at 5×10^3 cells/well for HeLa cells and 3×10^3 cells/well for A549 in growth media. After 24-hour incubation, cells were rinsed and incubated with cationic liposomes/GFP-p53 complexes and cationic liposome/PEI0.8K/GFP-p53 complexes prepared in 62.5 μl OptiMEM at a weight ratio of 6.25:1 and 6.25:1.1:1, respectively, for 4 hours. After incubation, cells were rinsed and incubated with growth media for 24 hour under 95% atmosphere and 5% CO_2. After 24-hour incubation, the growth media were replaced with growth media having 4 μM cisplatin and further incubated for 24 hours. The cell growth inhibition was measured by MTT assay as described in cell growth inhibition.

Statistical analysis

The statistical analysis was determined by one-way analysis of variance following with Fisher's Least Significant Difference (LSD) post hoc test. A comparison between means was determined using t-test. The significant level was set at $p < 0.05$.

RESULTS AND DISCUSSION

Cationic liposomes/pDNA complexes and cationic liposome/PEI0.8K/pDNA complexes

Figure 1 shows the analysis of DPT liposome/GFP-p53 and DPT liposome/PEI 0.8 kDa/GFP-p53 complexes on 1% agarose gel. DPT liposome/GFP-p53 formed complete complexes at a lipid-to-DNA ratio of 6.25:1 (Fig. 1A). DPT liposome/PEI

0.8 kDa/GFP-p53 complexes formed complete complexes at a lipid-to-DNA ratio of 1.5:1.1:1. PEI 0.8 kDa/GFP-p53 complexes formed complete complexes at the PEI-to-DNA ratio of 1.1:1 (Fig. 1B). DPT liposome/GFP-p53 complexes at lipid-to-DNA ratios of 6.25:1 and 12.5:1 had a particle size of 319 ± 19–352 ± 27 nm. DPT liposome/PEI 0.8 kDa/GFP-p53 complexes at a lipid-to-PEI-to-DNA ratio of 6.25:1.1:1 had a particle size of 754 ± 109 nm. PEI 0.8 kDa/GFP-p53 complexes at PEI-to-DNA ratios of 1.1:1 had a particle size of 2,072 ± 142 nm. DPT liposome/GFP-p53 complexes at a lipid-to-DNA ratio of 6.25:1 had a particle size which is not different from that of 12.5:1. PEI 0.8 kDa/GFP-p53 complexes had particle size bigger than DPT liposome/PEI 0.8 kDa/GFP-p53 complexes (Fig. 2A). DPT liposome/GFP-p53 complexes at lipid-to-DNA ratios of 6.25:1 and 12.5:1 had a zeta potential of −15.7 ± 2.8–+27.0 ± 0.9 mV. DPT liposome/PEI 0.8 kDa/GFP-p53 complexes at a lipid-to-PEI-to-DNA ratio of 6.25:1.1:1 had a zeta potential of +3.8 ± 0.7 mV nm. PEI 0.8 kDa/

GFP-p53 complexes at the PEI-to-DNA ratio of 1.1:1 had a zeta potential of −1.0±0.1. Lipid-to-DNA ratios of 6.25:1 to 12.5:1 and the presence of PEI 0.8 kDa increased the zeta potential of DPT liposome/GFP-p53 complexes (Fig. 2B).

Intracellular delivery and gene expression

An intracellular delivery of DPT liposome/PEI 0.8 kDa/pEGFP complexes was examined by GFP gene expression. Plasmid DNA delivered into HeLa cells. Figure 3 shows the flow cytometric analysis and GFP expression of HeLa cells by DPT liposome/PEI 0.8 kDa/GFP-p53 complexes. HeLa cells treated with pEGFP had GFP+ = 0.6%. Cells treated with DPT liposome/PEI 0.8 kDa/pEGFP complexes had GFP+ = 6.1%. Cells treated with DPT liposome/pEGFP complexes had GFP+ = 5.4%. HeLa cells treated without pEGFP had GFP+ = 0.2%. GFP expression of HeLa cells treated with DPT liposome/PEI 0.8 kDa/pEGFP and DPT liposome/pEGFP complexes was significantly higher
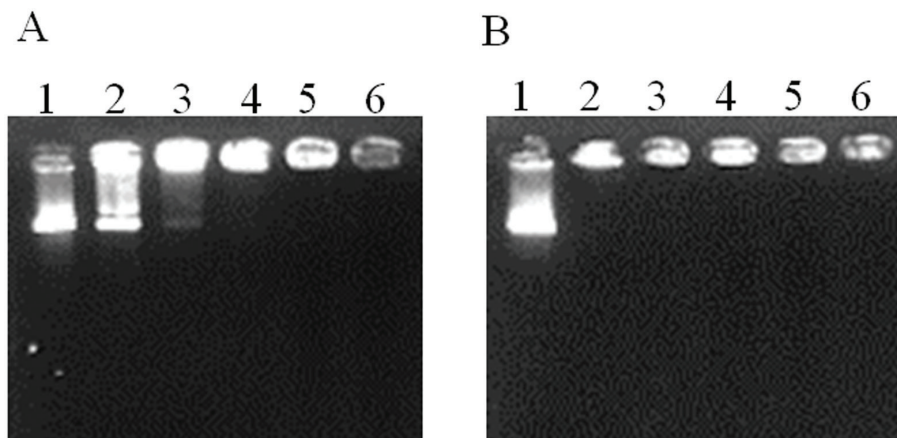

Figure 1. Gel electrophoresis analysis of (A) DPT liposome/GFP-p53. Lane 1: free GFP-p53 0.25 μg. Lanes 2-: DPT liposome/GFP-p53 at lipid-to-DNA ratios of 1.5:1, 3.125, 6.25:1, 12.5:1 and 25:1, respectively, and (B) DPT liposome/PEI 0.8 kDa/GFP-p53. Lane 1: free GFP-p53 (0.25 μg). Lanes 2: PEI 0.8 kDa/GFP-p53 at PEI-to-DNA ratio of 1.1. Lanes 3-7: DPT liposome/PEI 0.8 kDa/GFP-p53 at lipid-to-PEI-to-DNA ratios of 1.5:1.1:1, 3.125:1.1:1, 6.25:1.1:1, 12.5:1.1:1 and 25:1.1:1, respectively.
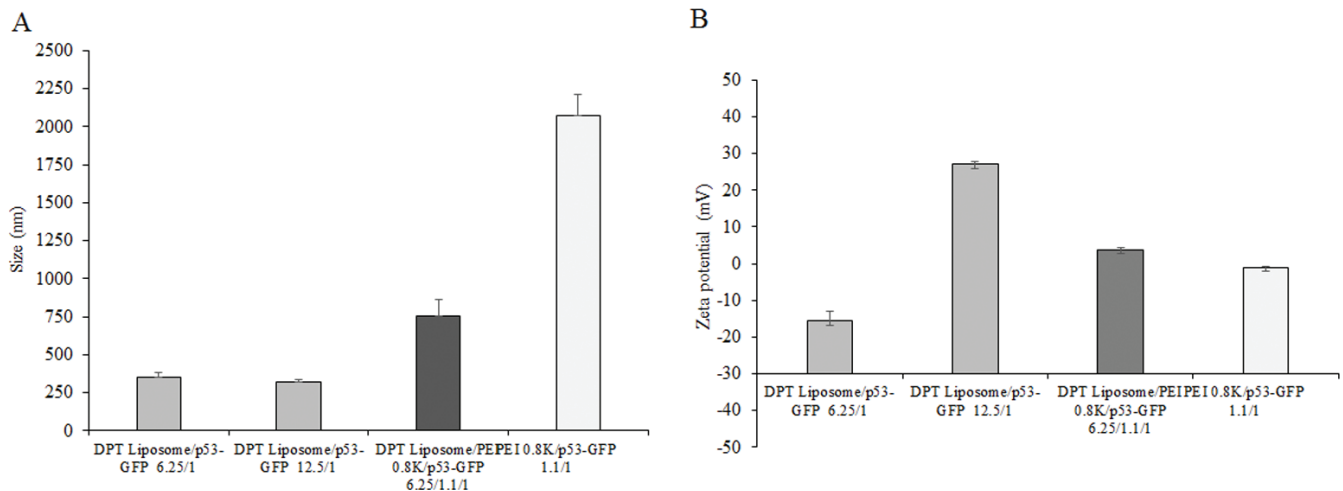

Figure 2. Particle size (A) and zeta potential (B) of DPT liposome/GFP-p53 at lipid-to-DNA ratios of 6.25:1 and 12.5:1, DPT liposome/PEI 0.8 kDa/GFP-p53 at lipid-to-PEI-to-DNA ratio of 6.25:1.1:1, and PEI 0.8 kDa/GFP-p53 at PEI-to-DNA ratio of 1.1:1.
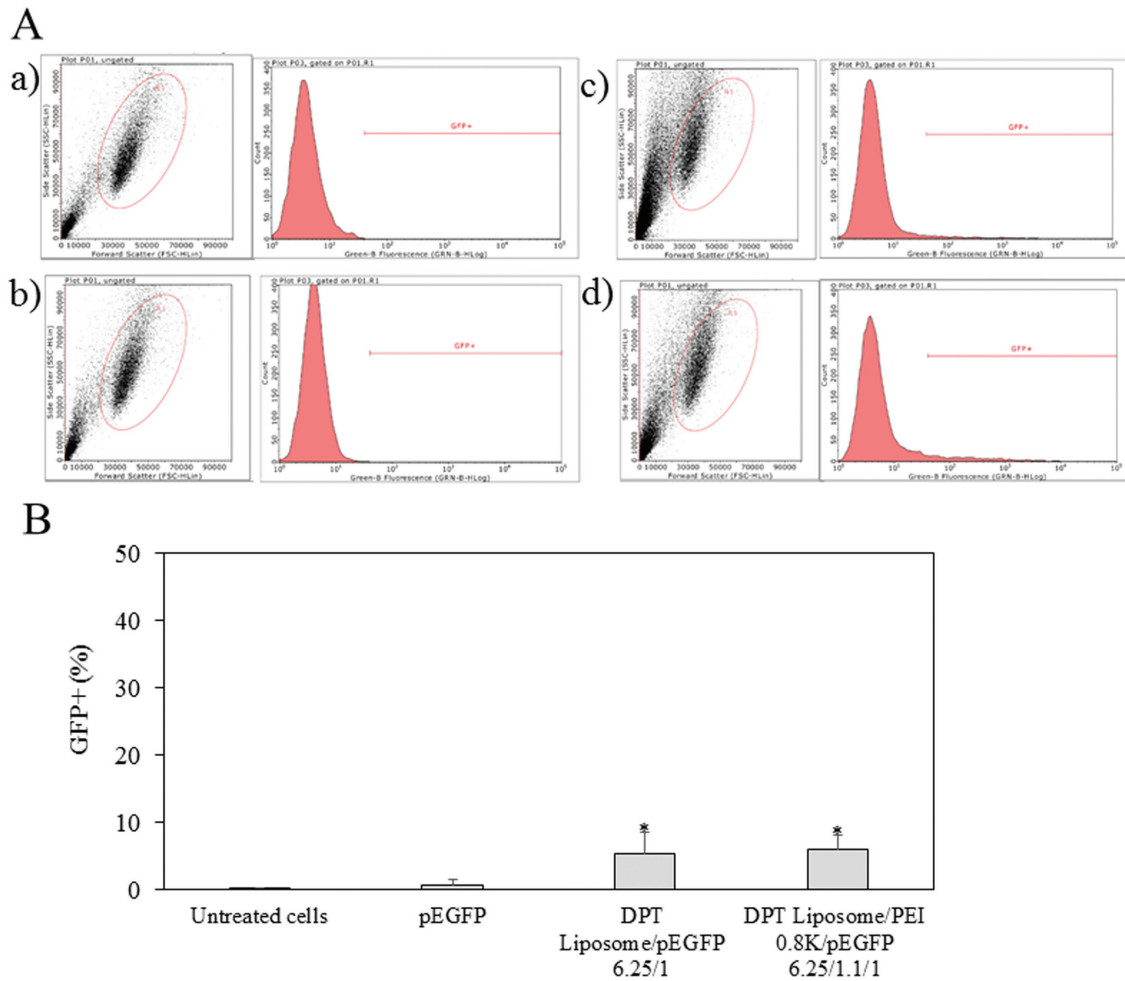
Figure 3. Flow cytometry analysis (A) and GFP$^+$ (%) (B) of HeLa cells treated with media (a) pEGFP (b) DPT liposome/pEGFP at lipid-to-DNA ratio of 6.25:1 (c), and DPT liposome/PEI 0.8 kDa/pEGFP at lipid-to-PEI-to-DNA ratio of 6.25:1.1:1 (d).

than HeLa cells treated with pEGFP ($p < 0.05$). GFP expression of HeLa cells treated with DPT liposome/PEI 0.8 kDa/pEGFP and DPT liposome/pEGFP complexes was not significantly different. Plasmid DNA delivered into A549 cells. Figure 4 shows the flow cytometric analysis and GFP expression of A549 cells by DPT liposome/PEI 0.8 kDa/GFP-p53 complexes. The A549 cells treated with and without pEGFP had GFP$^+$ = 0.3%. Cells treated with DPT liposome/PEI 0.8 kDa/pEGFP complexes had GFP$^+$ = 5.5%, whereas cells treated with DPT liposome/pEGFP complexes had GFP$^+$ = 8.5%. GFP expression of A549 cells treated with DPT liposome/pEGFP complexes was significantly higher than A549 cells treated with DPT liposome/PEI 0.8 kDa/pEGFP complexes and cells treated with pEGFP ($p < 0.05$).

DPT liposome/GFP-p53 complexes and DPT liposome/PEI 0.8 kDa/GFP-p53 complexes were formulated and delivered into HeLa cells and A549 cells. This study showed that DPT liposome and DPT liposome/PEI 0.8 kDa could deliver plasmid DNA into cells. The intracellular delivery and cytotoxicity of the carrier/nucleic acid complexes have been demonstrated to be dependent on the characteristic of carrier and carrier/plasmid DNA ratio and the amount of nucleic acid (Weecharangsan *et al.*, 2017; Zhang *et al.*, 2018)

Growth inhibition of HeLa cells by cisplatin

Figure 5 shows the growth inhibition of HeLa cells by cisplatin at a concentration of 0–50 µM. The growth inhibition of HeLa cells increased when the concentration of cisplatin increased from 2.5 to 50 µM. At a cisplatin concentration of 4 µM, growth inhibition of HeLa cells was 8%–10%. Therefore, cisplatin at a concentration of 4 µM was used to evaluate the sensitivity of cancer cells by DPT liposome/PEI 0.8 kDa/GFP-p53 complexes.

Growth inhibition of HeLa cells by DPT liposome/PEI 0.8 kDa/ GFP-p53 complexes and DPT liposome/GFP-p53 complexes

The growth inhibition of HeLa cells by DPT liposome and GFP-p53 was not different from cells treated with a medium. DPT liposome/GFP-p53 complexes significantly inhibited HeLa cell growth ($p < 0.05$) (Fig. 6). An increasing amount of GFP-p53 from 0.125, 0.25, and 0.5 µg increased the growth inhibition of HeLa cells by DPT liposome/PEI 0.8 kDa/GFP-p53 complexes and significantly at the amount of GFP-p53 of 0.5 µg ($p < 0.05$) (Fig. 7). Using PEI 0.8 kDa in DPT liposome/PEI 0.8 kDa/ GFP-p53 complexes did not increase the growth inhibition of HeLa cells. The growth inhibition of HeLa cells by DPT liposome/GFP-p53 complexes was higher than that of by DPT
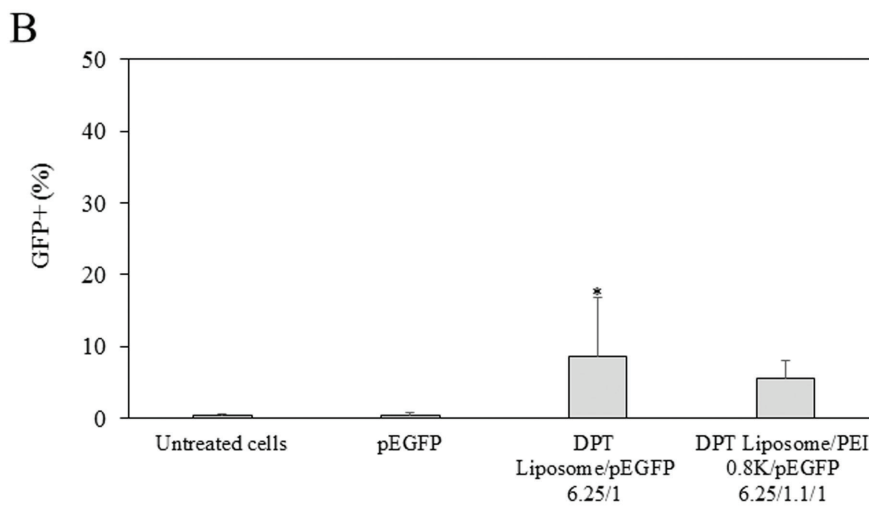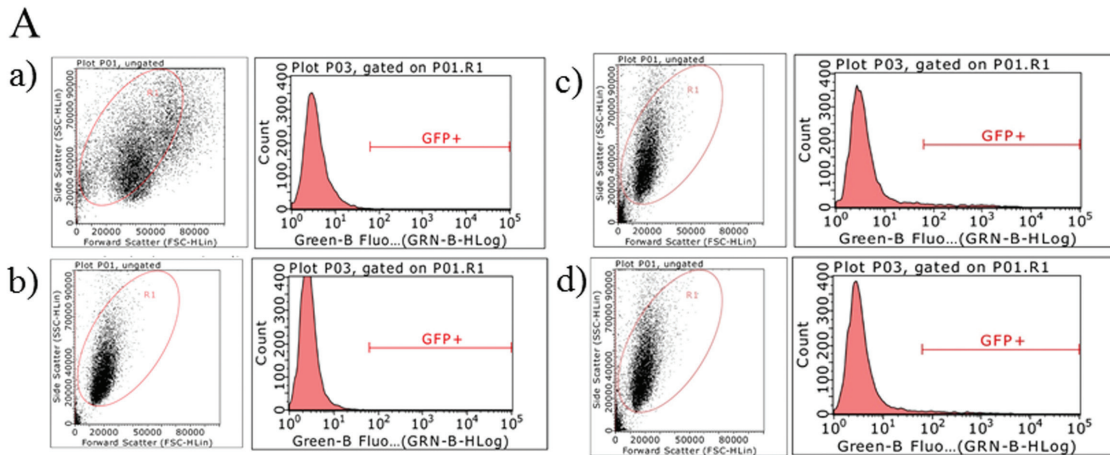
Figure 4. Flow cytometry analysis (A) and GFP⁺ (%) (B) of A549 cells treated with media (a) pEGFP (b) DPT liposome/pEGFP at lipid-to-DNA ratio of 6.25:1 (c), and DPT liposome/PEI 0.8 kDa/pEGFP at lipid-to-PEI-to-DNA ratio of 6.25:1.1:1 (d).
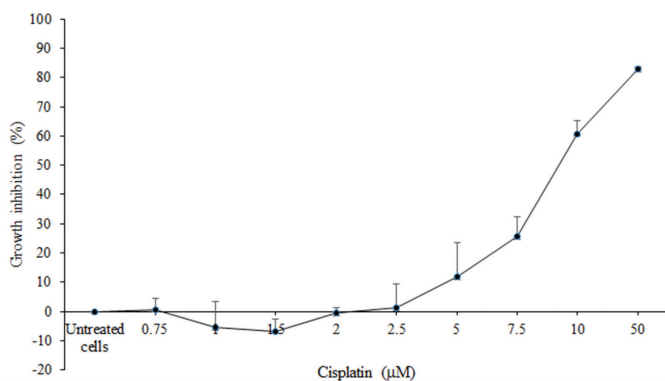

Figure 5. Growth inhibition of HeLa cells by cisplatin at a concentration of 0-50 μM.

Figure 6. Growth inhibition of HeLa cells by DPT liposome/GFP-p53; □ = DPT liposome, ■ = DPT liposome/GFP-p53 at lipid-to-DNA ratio of 6.25:1; *$p < 0.05$ when compared with cells treated with medium, cells treated with GFP-p53, and cells treated with DPT liposome.

liposome/PEI 0.8 kDa/GFP-p53 complexes and by only GFP-p53 ($p < 0.05$) (Fig. 8).

DPT liposome/GFP-p53 complexes and DPT liposome/PEI 0.8 kDa/GFP-p53 complexes could inhibit HeLa and A549 cell growth. The growth inhibition of DPT liposome/PEI 0.8 kDa/GFP-p53 complexes was dependent on the concentration of
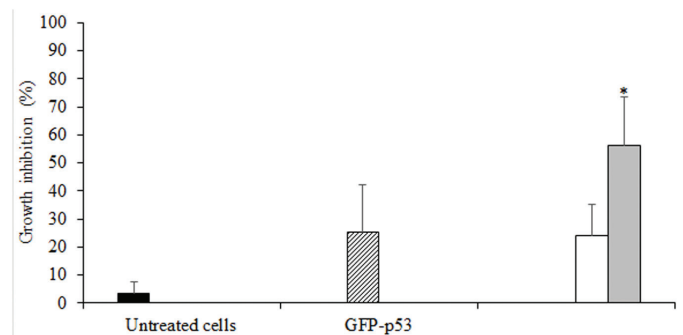
GFP-p53. Song *et al.* (2012) revealed that cationic lipid-coated PEI/DNA polyplex increased the transfection efficiency and reduced the cytotoxicity in mesenchymal stem cells. However, effective delivery of plasmid DNA was dependent on the characteristic of PEI and the polymer and plasmid DNA ratio (Costa *et al.*, 2018). This study showed that DPT liposome/PEI 0.8 kDa/GFP-p53
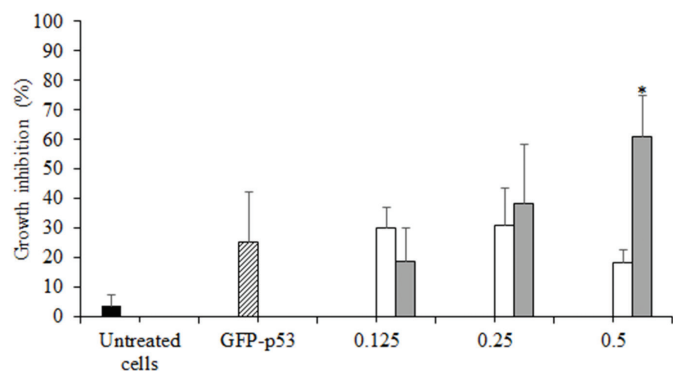
Figure 7. Growth inhibition of HeLa cells by DPT liposome/PEI 0.8 kDa/GFP-p53; □ = DPT liposome, ■ = DPT liposome/PEI 0.8 kDa/GFP-p53 at lipid-to-DNA ratio of 6.25:1.1:1; *p<0.05 when compared with cells treated with medium, cells treated with GFP-p53, and cells treated with DPT liposome/PEI 0.8 kDa.
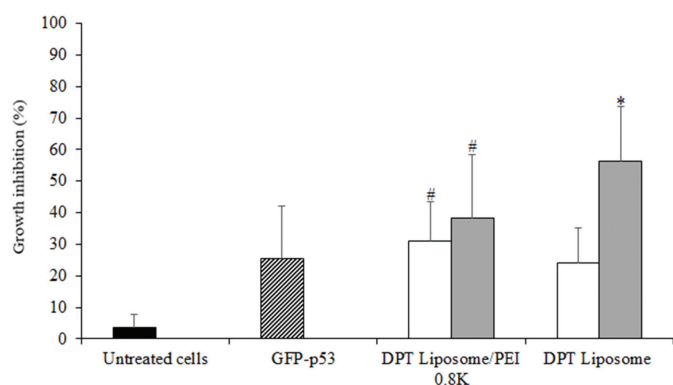

Figure 9. Growth inhibition of HeLa cells by DPT liposome/PEI 0.8 kDa/GFP-p53 at lipid-to-PEI-to-DNA ratio of 6.25:1.1:1 and DPT liposome/GFP-p53 at lipid-to-DNA ratio of 6.25:1; □ = no cisplatin, ■ = plus cisplatin.

Figure 8. Growth inhibition of HeLa cells by DPT liposome/PEI 0.8 kDa/GFP-p53 at lipid-to-PEI-to-DNA ratio of 6.25:1.1:1 and DPT liposome/GFP-p53 at lipid-to-DNA ratio of 6.25:1; □ = liposomes, ■ = liposome/GFP-p53 ; *p<0.05 when compared with cells treated with medium, cells treated with GFP-p53, and cells treated with DPT Liposome; # $p < 0.05$ when compared with untreated cells.

Figure 10. Growth inhibition of A549 cells by DPT liposome/GFP-p53 at lipid-to-DNA ratio of 6.25:1; □ = no cisplatin, ■ = plus cisplatin; *p<0.05 when compared with cells treated with DPT liposome/GFP-p53 at the same lipid-to-DNA ratio and no cisplatin.

complexes did not show more effective intracellular delivery and gene expression and growth inhibition than DPT liposome/GFP-p53 complexes in HeLa and A549 cells. In this study, PEI 0.8 kDa resulted in a large particle size of DPT liposome/PEI 0.8 kDa/GFP-p53 complexes and did not improve intracellular delivery of GFP-p53 from DPT liposome/GFP-p53 complexes, thus not increasing the growth inhibition of cationic liposome/GFP-p53 complexes in HeLa and A549 cells. The particle size of liposome/PEI/DNA complexes could be dependent on the molecular weight and the structure of PEI.

Growth inhibition of HeLa and A549 cells by DPT liposome/PEI 0.8 kDa/GFP-p53 complexes in combination with cisplatin

Further treatment with cisplatin, growth inhibition of HeLa cells was slightly increased by DPT liposome/PEI 0.8 kDa/GFP-p53 complexes at a lipid-to-PEI-to-DNA ratio of 6.25:1.1:1 and DPT liposome/GFP-p53 complexes at a lipid-to-DNA ratio of 6.25:1 (Fig. 9). In A549 cells, growth inhibition was significantly different by DPT liposome/GFP-p53 complexes at lipid-to-DNA ratios of 6.25:1 treated with cisplatin as compared to cells treated
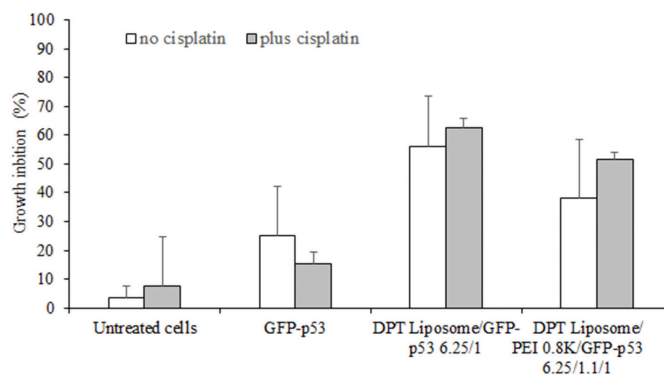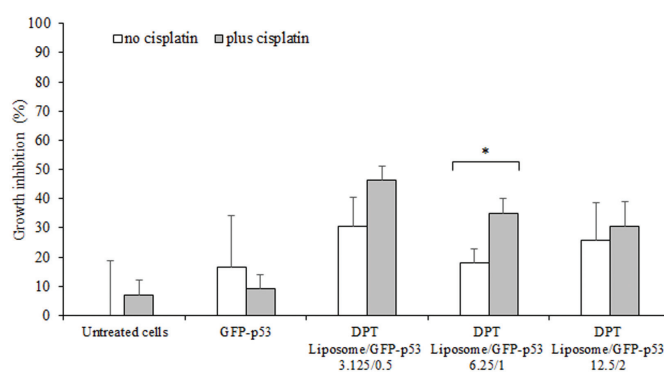
with DPT liposome/GFP-p53 complexes at the same lipid-to-DNA ratio and no cisplatin (*$p < 0.05$) (Fig. 10).

DPT liposome/GFP-p53 complexes and DPT liposome/PEI 0.8 kDa/GFP-p53 complexes inhibit HeLa cell growth in combination with cisplatin. DPT liposome/GFP-p53 complexes in combination with cisplatin significantly inhibit the A549 cell growth. The combination of DPT liposome/GFP-p53 complexes and cisplatin significantly inhibits A549 cell growth. Liu *et al.* (2017) depicted that the survival rate of human lung adenocarcinoma cell line was reduced by p53 alpha gene and increased the sensitivity to cisplatin treatment. Ye *et al.* (2013) showed that cationic liposome/pVAX-iNOS complex enhanced the cisplatin to inhibit cell proliferation of A549 human lung cancer cells and suppression of subcutaneous tumor growth. Liu *et al.* (2013) demonstrated that cisplatin synergized the TRAIL-induced apoptosis in breast cancer cell lines. Li *et al.* (2014) showed that the downregulation of p53 by Vasohibin 2 decreased the cisplatin sensitivity to hepatocarcinoma cells.

CONCLUSION

This study concluded that the sensitivity to the cancer cells of cisplatin was based on the cell line and the ratio of cationic liposome and GFP-p53. This study suggests that GFP-p53 expression delivered by cationic liposome/GFP-p53 complexes

would be useful to increase the effect of cisplatin on the treatment of cancer cells.

CONFLICT OF INTEREST

FUNDING

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