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Preparation of a TK/GCV administration system mediated by transferrin modified pro-cationic liposomes

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Transferrin modified pro-cationic liposomes were prepared and used to investigate the effect of targeting therapeutic genes to human hepatoma carcinoma cells *in vitro*. The main lipid CHETA, cholest-5-en-3β-yl[2-[[4-[(carboxymethyl)dithio]-1-iminobutyl]amino]ethyl] carbamate ($C_{36}H_{61}N_3O_4S_2$), was synthesized and used to prepare pro-cationic liposomes. The thymidine kinase (TK) gene loaded pro-cationic liposomes were prepared by first mixing the plasmid DNA and protamine together, and then incubating the resulted polyplexes with blank pro-cationic liposomes preformed by the thin film dispersion-sonication method. Transferrin (Tf) was adsorbed on the surface of pro-cationic liposomes via electrostatic interactions to form transferrin modified pro-cationic liposomes. Cellular association was measured by fluorimetry at excitation and emission wavelengths of 490 and 520 nm, respectively. The viability of TK gene infected cells following administration of ganciclovir (GCV) was investigated by MTT assay. The transferrin modified TK gene pro-cationic liposomes had a mean diameter of 240 ± 12 nm and zeta potential of -24.10 ± 2.5 mV ($n = 3$). The transmission electron microscopy image indicated that most of the liposomes were relatively regular and spherical with a condensed core inside. Cell-associated fluorescence of Tf-liposomes and unmodified liposomes (without transferrin) was 7.8×10^6, and 3.2×10^6 per milligram protein, respectively. Compared to Lipofectamine™ 2000 (Invitrogen, USA) the pro-cationic liposomes and transferrin modified pro-cationic liposomes had less cytotoxicity to cells. The transduced TK gene HepG2 cells were more sensitive to GCV than the un-transduced TK gene ones and the human normal Chang liver cells were not affected by the TK/GCV system mediated by procationic liposomes.

1. Introduction

Gene therapy has become increasingly important as a new therapeutic approach for treating a variety of human diseases (Feldman 1996; Hargest and Williamson 1996; Mahato 1997), in which the HSV-TK "suicide gene" system is a common gene therapy strategy. In this system, HSV-TK phosphorylates the prodrug GCV leading to disruption of DNA synthesis and inhibition of cell proliferation (He et al. 2004).

On the other hand, a safe and efficient gene transfer system is necessary for successful tumor gene therapy. Viral vectors can facilitate uptake of nucleic acids by target cells and also promote transport of the genetic material into the nucleus, but problems with immunogenicity and target tissue penetration have limited the realisation of this strategy's full potential. Cationic liposomes have been widely applied to the delivery of plasmid and antisense DNA into eukaryotic cells (Zhou and Huang 1994; da Cruz 2001) and some clinical trials are currently in progress (Shand et al. 1999) because of their low immunogenicity and high capacity to deliver plasmids across cell membranes. Unfortunately, medical applications of this methodology are also compromised by the inherent toxicities of most cationic lipids and the incompatibility of positively charged vectors with the serum environment. Anionic liposomes have been extensively studied as vehicles for the delivery of pharmaceutical loadings (Amin et al. 2001). They may be used as a drug reservoir for controlled release, to reduce toxicities associated with drugs exhibiting narrow therapeutic indices, or to target drugs directly to cells (Wasan et al. 1993).

In this work, we used CHETA to prepare a novel gene-transferring vector, pro-cationic liposomes, which are combine properties of cationic liposomes and anionic liposomes. Pro-cationic liposomes are negatively charged in a physiological environment. However, when brought into contact with cellular membranes or internalized by cells, the surface charge becomes positive (Leamon 2002).

For "suicide gene" therapy which lacks specificity, another important factor is the targeting function. Receptor-mediated gene delivery methods have also been explored. The expression of transferrin receptors is relatively higher on human hepatoma carcinoma cells than on the corresponding normal ones, making this receptor an attractive target for cancer gene therapy (Xuo et al. 1999; Kircheis et al. 2001). Transferrin can be conjugated with liposomes by covalent bonding or may be adsorbed on the surface of

liposomes via electrostatic interactions. The isoelectric point of lactoferrin (one member of the transferrin family) is 8.70 and as a result, lactoferrin has a net positive charge at physiological pH, making it possible to be adsorbed on the surface of pro-cationic liposomes via electrostatic interactions (da Cruz et al. 2004).
In the present study, we prepared transferrin modified pro-cationic liposomes which could mediate the TK/GCV administration system to human heptoma cells.

2. Investigations and results

2.1. Characteristics of pro-cationic liposomes

The size distribution and surface charge (known as zeta potential) of the liposomes were determined by a Malvern Zetasizer Nano ZS90 (Malvern instruments Ltd., UK) instrument. The average size of the pro-cationic liposomes without transferrin was 228.9 ± 8 nm with a polydispersity index of 0.122 ± 0.02 ($n = 3$) and the zeta potential was -25.08 ± 2.5 mV ($n = 3$). When TK gene pro-cationic liposomes were mixed with transferrin in a transferrin/CHETA (w/w) ratio of 1 : 1, the resultant lipoplexes had a mean diameter of 240 ± 12 nm with a polydispersity index of 0.150 ± 0.03 ($n = 3$), and a zeta potential of -24.10 ± 2.5 mV ($n = 3$). The morphology of the pro-cationic liposomes was observed under transmission electron microscopy (TEM) on a JEM-100SX electron microscope (Japan). The TEM image (not shown here) indicated that most of the liposomes were relatively regular and spherical with a condensed core inside.

2.2. Changing zeta potential of liposomes

To check whether the reductive agent could change the zeta potential of procationic liposomes from negative to positive, the preformed procationic liposomes were diluted with distilled water to give a final concentration of 1 mmol/ml total lipid. One milliliter of the diluted liquid was placed in each of four tubes. While vortexing the test tubes, 0 µl, 10 µl, 20 µl and 30 µl of the disulfide reducing agent dithiothreitol (0.5 mM DTT, 6.85 mM NaCl, 0.13 mM KCl, 0.32 mM Na_2HPO_4 and 0.07 mM KH_2PO_4, pH 7.4) was added. The mixtures were then incubated for 30 min at room temperature and the zeta potential was measured. When 0 µl DTT was added, the zeta potential was -33.83 ± 1.23 mV, but when 20 µl DTT was added, it was $+22.74 \pm 1.08$ mV. On the other hand, there was not very much difference between the addition of 20 µl and 30 µl DTT, which indicated that the disulfide bonds had been almost cleaved by DTT.

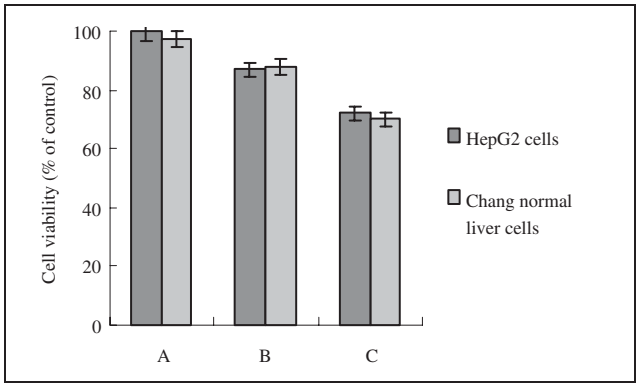


Fig. 1: Viability of cells determined by MTT assay with different liposome formulations: (A) Pro-cationic liposomes; (B) Transferrin modified pro-cationic liposomes; (C) Cationic liposomes: Lipofectamine™ 2000

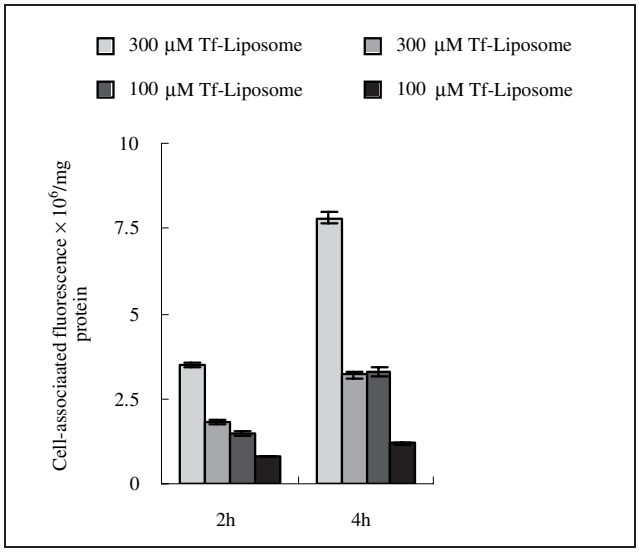


Fig. 2: Effect of incubation time and lipid concentration on the extent of cell association of different liposome formulations

2.3. Cell viability

When the cells had grown to 60–80% confluence at 37 °C under 5% CO_2, they were incubated with pro-cationic liposomes, transferrin modified pro-cationic liposomes and cationic liposomes (Lipofectamine™ 2000, Invitrogen USA). Cell viability was determined by the MTT assay (Fig. 2). Fig. 2 shows that the cell viabilities of HepG2 and Chang normal liver cells display a similar trend. Compared to Lipofectamine™ 2000, the pro-cationic liposomes and transferrin modified pro-cationic liposomes had much less cytotoxicity to these cells under the same experimental conditions.

2.4. Association of pro-cationic liposomes with HepG2 cells and Chang liver cells

The results of fluorimetric studies (Fig. 2) indicate that an increase both in the incubation time and in the phospholipid concentration resulted in a higher degree of liposome-cell association. Competitive inhibition studies (Fig. 3,

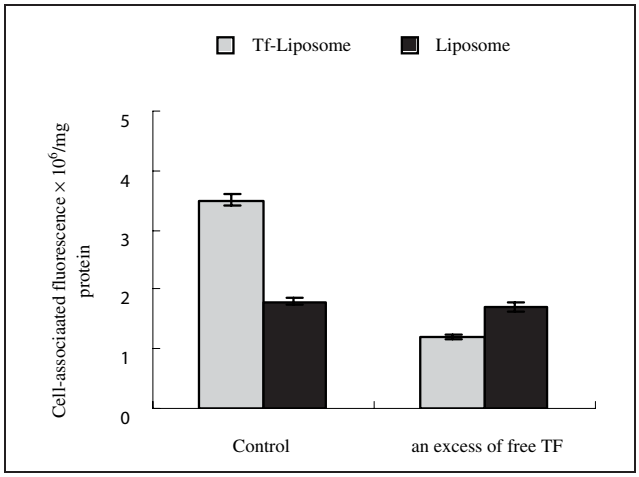


Fig. 3: Effect on the extent of cell association of the presence of excess free Tf (4 mg/well) (300 mM, 2 h)

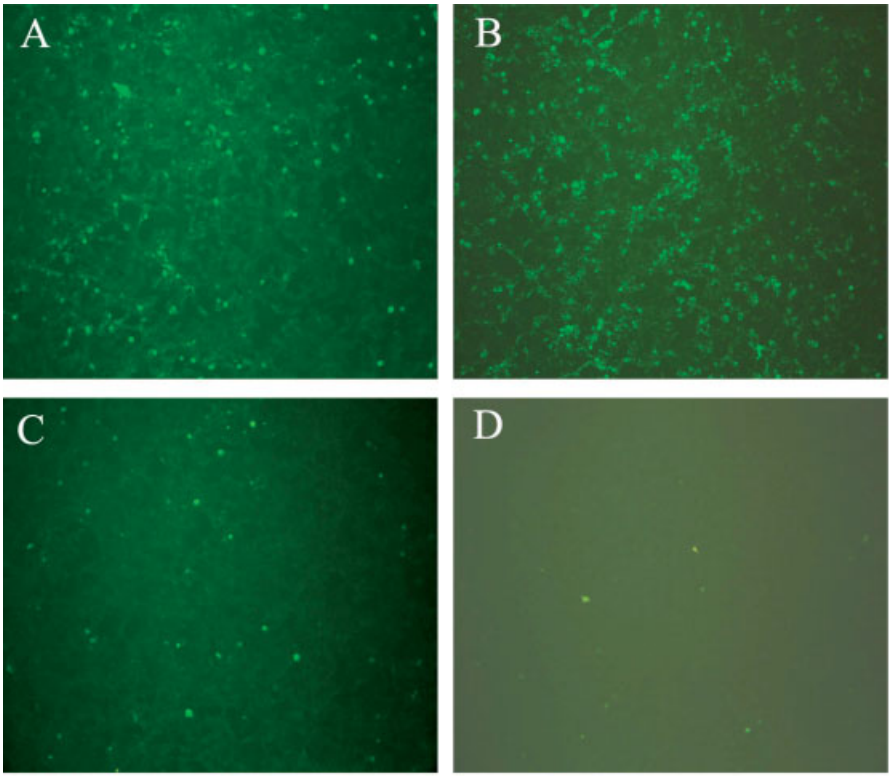


Fig. 4:
Fluorescence micrographs of HepG2 cells incubated with different formulations of liposomes (final phospholipid concentration 100 or 300 mM). Cells were incubated with (A) 300 mM Tf-Liposome (2 h) or (B) 100 mM Tf-Liposome (4 h) or (C) 300 mM Tf-Liposome (cells were preincubated with excess free Tf, 2 h) (D) 100 mM Liposome (2 h) for 4 h at 37 °C

Fig. 4A and C) indicated that pre-treatment of HepG2 cells with an excess of free transferrin (at non-toxic concentrations) resulted in a decrease in the extent of cell association of the targeted liposomes but had no effect on the association of the non-targeted formulations. The extent of cell association of the different liposome formulations with HepG2 cells was further evaluated by fluorescence microscopy (Fig. 4). As can be observed in Fig. 4, the fluorescence intensity of cells mediated by 300 mM Tf-Liposome for 2 h was almost the same than that of cells mediated by 100 mM Tf-Liposome for 4 h, which is consistent with values obtained above from the fluorimetric studies (Fig. 2). Following incubation of HepG2 cells with Tf-liposomes, an intense fluorescence was observed (Fig. 4A and B), while for non-targeted formulations the fluorescence intensity was much lower (Fig. 4D).
Fluorimetric studies and fluorescence microscopy analysis demonstrated that association of targeted liposomes to HepG2 cells occurs to a significantly larger extent than with non-targeted formulations.

2.5. Effect on cell growth inhibition and bystander effect mediated by TK/GCV

Fig. 5 shows that when 20% TK gene positive were added, 92% of cells remained active, but when 40% TK gene positive cells were added it was 43%. This result indicates that the bystander effect mediated by transferrin modified pro-cationic liposomes was apparent in this study.
Fig. 6A shows the cell viability of HepG2 cells and Chang normal liver cells cultured in various concentrations of GCV for 72 h, and it illustrates a significant dose-dependent and time-dependent inhibition of cell growth. From Fig. 6A, we can see that in this study concentrations of GCV above 0.5 μM were toxic to the HepG2 cells and Chang normal liver cells. Therefore, 0.5 μM GCV was used in all subsequent studies. Fig. 6B shows the cell viability of the above cells cultured in 0.5 μM GCV for different times (from 12 h to 96 h), and it indicates that the TK/GCV system mediated by transferrin modified procationic liposomes did not affect the cell growth of Chang normal liver cells, which may be due to there being very few (or even no) transferrin receptors on Chang normal liver cells. Fig. 6B also illustrates that the growth of untransduced TK gene HepG2 cells was not affected by the administration of GCV, but that of transduced TK gene HepG2 cells was efficiently inhibited by the TK/GCV administration system. When the time reached 96 h, very few cells remained alive.

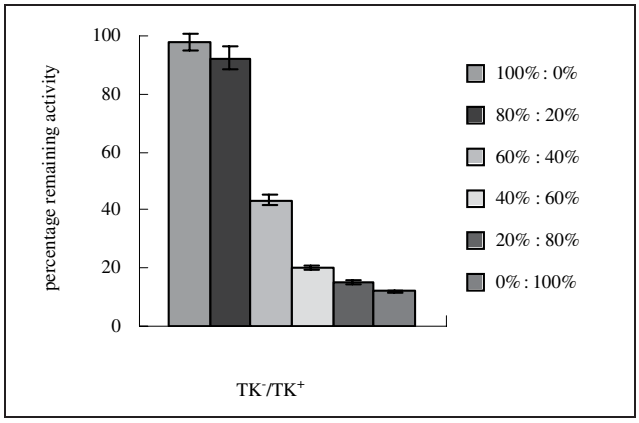


Fig. 5: Influence of bystander effect on assay (%)

3. Discussion

The pro-cationic liposomes mainly comprised CHETA. CHETA is a cholesterol derivative possessing a hydrophobic cholesterol tail group and a hydrophilic head group. The head group incorporates both positively and negatively charged regions connected by a disulfide bond (Leamon 2002). The disulfide bond is susceptible to clea-

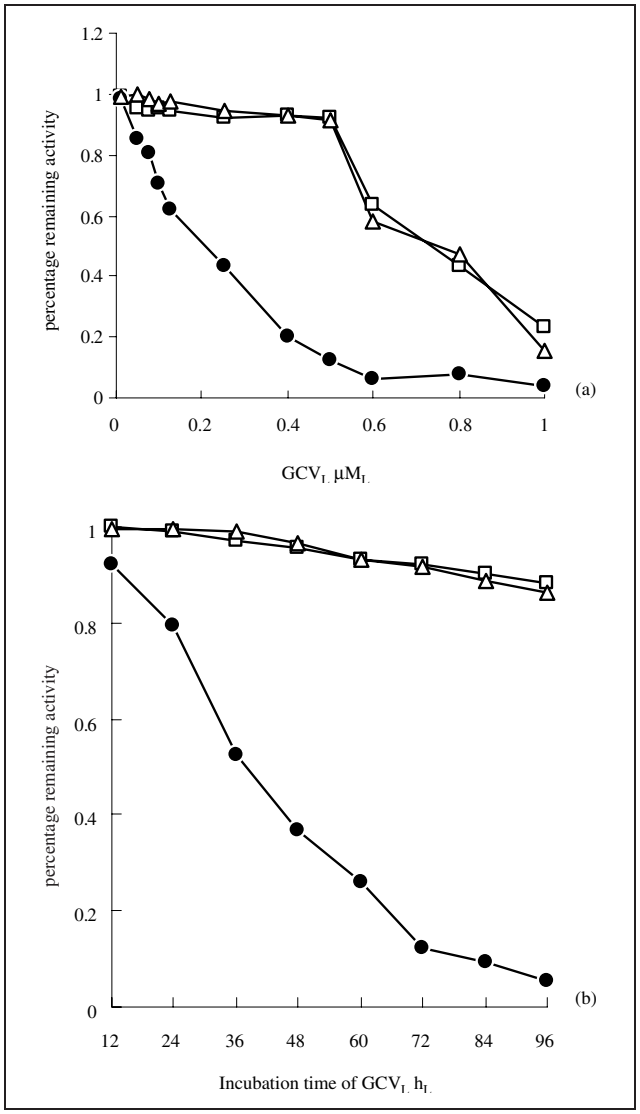


Fig. 6: Sensitivity of cells to GCV. (A) Different concentrations of GCV (72 h) (B) Different incubation times (0.5 μM GCV); (HepG2 + TK (●), (HepG2 + plain-vector (□), Chang normal liver cells + TK (□))

vage by components of intracellular membrane such as protein disulfide isomerase (PDI) or thioredoxin reductase (TrxR). Since the content of TrxR in tumor cells is 10 times higher than in normal tissues (Turunen et al. 2004; Lincoln et al. 2003) when the pro-cationic liposomes reach the surface of tumor cells, the disulfide bond will be cleaved, resulting in the removal of the negatively charged region from the head group and the formation of cationic liposomes.

The fact that the transfection efficiency of Tf-PLPD was nearly twice that of PLPD indicated that the positively charged transferrin may have been adsorbed on the surface of PLPD. The average size of PLPD was smaller than that of Tf-PLPD and a comparison of zeta potential between PLPD and Tf-PLPD also gave similar results. In the cell association test, an intense fluorescence was observed following incubation of HepG2 cells with Tf-liposomes, while for non-targeted formulations the fluorescence intensity was much lower. This result also supports a similar conclusion.

Fig. 1 shows that both procationic liposomes and transferrin modified procationic liposomes had less cytotoxicity to cells than Lipofectamine^{TM} 2000. This may be due to the fact that when procationic liposomes or transferrin modified procationic liposomes came into contact with cells, their surface charge was negative at first, but with the help of reductant factors gradually became positive. However, the surface charge of Lipofectamine^{TM} 2000 was originally positive, and therefore, there was no gradual change process such as with procationic liposomes. It seems that direct contact with a positive charge would not be beneficial to cell viability, which requires further study to elucidate.

In this study we used protamine to condense plasmid DNA for the reason that when DNA is administered to the whole body it can be attacked by nucleases in the blood and/or cellular lysosomes, and finally degraded (Sorgi et al. 1997). Therefore, the gene delivery carrier should function as a protector of the DNA during *in vivo* administration. Some research indicates that the addition of protamine as co-polymer to condense DNA can markedly enhance the transfection efficiency of several types of cationic liposomes by 2 ∼ 28-fold in a number of cell lines *in vitro* and *in vivo* (Gao and Huang 1996). Liposomes will interact with condensed DNA resulting in lipid rearrangement and the formation of a compact liposome/DNA complex.

Treatment with GCV also causes the death of cells that do not express the HSV-TK gene, which are adjacent to the TK positive cells. This has been termed the "bystander effect." To maximize the efficacy of gene delivery vectors currently used in clinical trials, the bystander effect is considered to be a major prerequisite for employing HSV-TK/GCV therapies (Freeman et al. 1996; Denning and Pitts 1997). As shown in Fig. 7, when 40% TK gene positive cells were added, the bystander effect was highlighted.

In summary, we achieved the preparation of transferrin modified pro-cationic liposomes which showed less cytotoxicity to cells than cationic liposomes. This vector could target TK genes to HepG2 cells and the TK/GCV system mediated by it showed a cell growth inhibitory effect. It may have potential for transferring vectors in *in vivo* studies.

4. Experimental

4.1. Chemicals and reagents

Recombinant plasmid pEGFP-TKAFP was donated by Dr. Liu Ji (Sichuan University). It was transformed into *Escherichia coli* DH5α and purified following amplification using the Qiagen Giga Endo-free plasmid purification kit (CA, USA). Protamine sulfate (derived from salmon) and lactoferrin of bovine origin were purchased from Sigma (USA). The BCA Protein Assay Kit was purchased from Pierce (USA), and cell culture medium DMEM was obtained from Gibo Co. (USA). Chang normal liver cells and HepG2 human hepatoma carcinoma cells were obtained from Shanghai Cell Institute, China Academy of Sciences.

4.2. Synthesis of CHETA (Fig. 1)

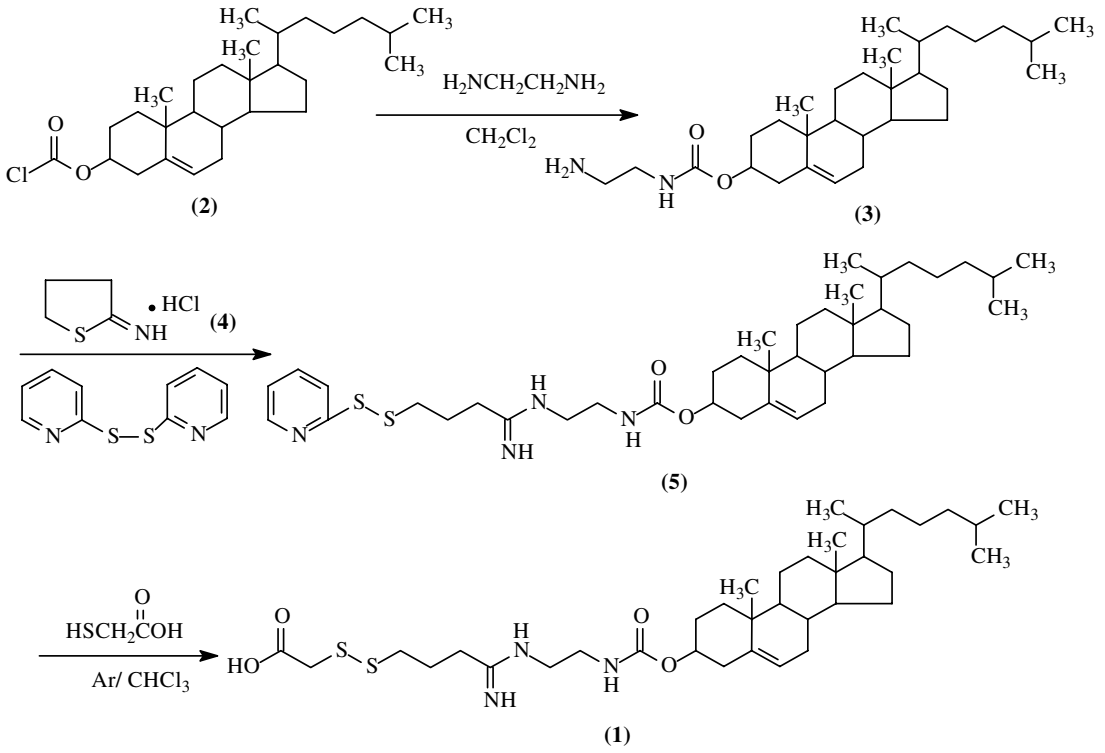
4.2.1. Synthesis of cholest-5-en-3β-yl[2-[2,3-diamino-1-oxopentyl]amino]-ethyl]carbamate (**3**)

A solution of 10.8 g (0.02 mol) cholesteryl chloroformate in 40 ml dry dichloromethane was added dropwise to a solution of 40 ml (0.5 mol) ethylenediamine in 40 ml dry dichloromethane at 0 ∼ 5 °C. The mixture was stirred for 5 h, then the solvent was removed under vacuum and the residue was partitioned between 60 ml water and 70 ml dichloromethane. The organic layer was washed with water, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. The resulting residue was recrystallized from ethanol to give 9.8 g (86.2% yield) of compound **3** as a white crystalline product. m.p. 164–167 °C. ESI$^+$-MS (Da/e): 474 ($M^+ + H$).

4.2.2. Synthesis of cholest-5-en-3β-yl[2-[[1-imino-4-(2-pyridinyldithio)butyl]amino]ethyl]carbamate (**5**)

A sample of 7.5 g (0.016 mol) of compound **3** was dissolved in 120 ml dry dichloromethane, and 10.1 g (0.045 mol) of 2,2′-dipyridyl disulfide and 4.32 ml (0.031 mol) of triethylamine were added. Then a solution of 3.15 g (0.023 mol) 2-iminothiolane hydrochloride (**4**) dissolved in 30 ml of methanol was added dropwise under argon atmosphere. After stirring at room temperature overnight, the mixture was concentrated *in vacuo*. To the

Scheme



residue, 50 ml of acetonitrile was added to precipitate the crude product. The product was purified on silica gel, eluting with $CHCl_3$/MeOH = 8 : 1, affording 6.7 g (62.0% yield) of pure intermediate **5** as a white powder product. ^{1}H NMR ($CDCl_3$, 400 MHz), δ 9.81 (brs, 1 H), 9.21 (brs, 1 H), 8.52 (m, 1 H), 7.73 (m, 2 H), 7.20 (m, 1 H), 6.45 (s, 1 H), 5.32 (m, 1 H), 4.40 (m, 1 H), 3.60 (m, 2 H), 3.45 (m, 3 H), 2.90 (m, 3 H), 2.82 (m, 2 H), 2.26 (m, 2 H), 2.01–1.91 (m, 3 H), 1.82 (m, 4 H), 1.65–1.03 (m, 19 H), 0.98 (s, 3 H), 0.90 (d, 3 H, J = 6.4 Hz), 0.86 (d, 6 H, J = 6.8 Hz), 0.66 (s, 3 H); ESI^+-MS (Da/e): 685 ($M^+ + H$).

4.2.3. Synthesis of cholest-5-en-3β-yl[2-[[4-[(carboxymethyl)dithio]-1-imino-butyl]amino]ethyl]carbamate (**1**)

Under argon atmosphere, a sample of 6.2 g (8.6 mmol) of **5** was dissolved in 150 ml dry dichloromethane, and a solution of 1.24 ml (17.8 mmol) of mercaptoacetic acid, and 4.7 ml (33.8 mmol) of triethylamine in 20 ml of dry dichloromethane were added dropwise over 1 h. After stirring at room temperature for 6 h, the reaction mixture was evaporated *in vacuo* to a gum and 45 ml of acetonitrile was added to give crude product **1**. The solid was purified by chromatography on silica gel in $CHCl_3$/MeOH = 3 : 1, affording 3.31 g (55.0% yield) of **1** as a white powder product. ^{1}H NMR ($CDCl_3$, 400 MHz), δ 10.04 (brs, 1 H), 9.53 (brs, 1 H), 8.87 (brs, 1 H), 6.92 (brs, 1 H), 5.35 (m, 1 H), 4.39 (m, 1 H), 3.49 (m, 3 H), 3.46 (m, 4 H), 2.78 (m, 4 H), 2.30 (m, 2 H), 2.10 (m, 2 H), 2.00 (m, 2 H), 1.95–1.84 (m, 4 H), 1.68–1.12 (m, 19 H), 0.99 (s, 3 H), 0.93 (d, 3 H, J = 6.4 Hz), 0.87 (d, 6 H, J = 6.8 Hz), 0.67 (s, 3 H); ESI^+-MS (Da/e): 664 (M^+).

4.3. Preparation of pro-cationic liposomes

4.3.1. Plain pro-cationic liposomes

Pro-cationic liposomes consisting of CHETA/phosphatidylcholine/cholesterol were prepared with a molar ratio of 9 : 10 : 1 according to the central composite method. The lipid mixture was dissolved in an appropriate quantity of chloroform and a thin lipid film was formed in a round-bottomed flask by removing the solvent using a rotary evaporator while heating to 35 °C under moderate vacuum. Then, the dried thin film was hydrated with phosphate-buffered saline (PBS, pH 7.4) and the resuspended lipids were sonicated in a bath sonicator for 2 min and in a probe sonicator for 100 s intermittently to form plain pro-cationic liposomes.

4.3.2. Fluorescent material loaded pro-cationic liposomes

One ml fluorescein saturated acetone solution was added to the lipid mixture and 1 mg of fluorescein sodium was added together with the phosphate buffer to prepare the fluorescent material loaded pro-cationic liposomes, the rest of the procedure followed being similar to that for the preparation of plain pro-cationic liposomes. The free fluorescein and fluorescein sodium was removed by subsequent dialysis in PBS (pH 7.4).

4.3.3. TK gene entrapped pro-cationic liposomes

TK gene entrapped pro-cationic liposomes were formed by first mixing together equal volumes of DNA and protamine. DNA and protamine were both diluted from stock with HEPES-buffered saline solution (10 mM HEPES, 100 mM NaCl, pH 7.4, Sigma). After mixing, the solution was briefly vortexed, and the resulting polyplexes were incubated for 10 min at room temperature. Preformed plain pro-cationic liposomes were subsequently added to the DNA/protamine mixture to achieve the desired final component concentrations and ratios.

4.3.4. Transferrin modified pro-cationic liposomes

Transferrin was dissolved in HEPES buffer to give 420 μg/ml. The transferrin modified pro-cationic liposomes were obtained by gently mixing transferrin solution with a predetermined volume of pro-cationic liposomes in various ratios and further incubating for 15 min at room temperature.

4.4. MTT Assay

HepG2 and Chang normal liver cells were seeded at a density of 20,000 cells/well in 96-well plates and grown to 60–80% confluence at 37 °C under 5% CO_2 following by 5 h of incubation with different compositions of transfection complexes. The MTT [3-(4,5-dimethylthiazol)-2,5- diphenyl tetrazolium bromide] assay (Mosmann 1983) in which cell viability is proportional to the absorbance at the test wavelength (570 nm) was conducted essentially according to the manufacturer's protocol. Briefly, MTT was dissolved in PBS at 5 mg/ml followed by addition of 20 μl to each well and incubation for 3.5 h. Each well was washed with 100 μl PBS followed by the addition of 20 μl PBS and 180 μl DMSO to dissolve the MTT formazan crystals. The absorbance was read at 570 nm using a model-550 microplate reader. The percentage of cell activity remaining was calculated according to the formula: $(A_{treated} - A_{background}) \times 100/(A_{control} - A_{background})$, in which the background wells contained no cells.

4.5. Cell association assay

Pro-cationic liposomes loaded with fluorescein and fluorescein sodium (final phospholipid concentration 100 or 300 mM) were incubated with HepG2 cells in DMEM for 2 h or 4 h at 37 °C. Cells were pre-incubated for 30 min at 37 °C in DMEM in the absence (control) or presence of an excess of free Tf (4 mg/well), and then further incubated for the indicated

time with different liposome formulations. After incubation, every well was washed twice with ice-cold PBS and lysed with 100 µl mammalian cell lysis buffer (0.25 M Tris, pH 8.0) at room temperature for 10 min, followed by alternating freeze-thaw cycles. The cell lysate was sonicated in a bath sonicator and centrifuged for 5 min at 10,000 g to compact debris. The total protein content of the lysates was measured with the BCA assay (Pierce, USA) using bovine serum albumin as the standard. Fluorescence measurements were performed in a fluorospectrophotometer (model RF-5301, Shimadzu Japan) at excitation and emission wavelengths of 490 and 520 nm, respectively. The level of cell association was expressed as cell-associated fluorescence intensity per mg protein. Statistical analysis values are presented as mean ± S.D.

4.6. Effect on cell growth inhibition and bystander effect assay

After further 48 h incubation following transfection, the cells were cultured in the absence or presence of various concentrations of GCV for different times as indicated. The cell viability was determined by MTT assay.

For the bystander effect assay, a TK gene expressing cell line (TK^+) was seeded in 96-well plates with parental cell lines (TK^-) (total 2.0×10^3/well) in the following proportions: (TK^-/TK^+) 100%: 0%, 80%: 20%, 60%: 40%, 40%: 60%, 20%: 80% and 0%: 100%. After 1 d, they were cultured in fresh medium containing 0.5 µM GCV. Cell cultures were terminated at 72 h and cell viability was also determined by MTT assay.

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References

Amin K, Heath TD (2001) LDL-induced association of anionic liposomes with cells and delivery of contents II. Interaction of liposomes with cells in serum-containing medium. J Control Release 73: 49–57.

da Cruz MTG, Simões S, de Lima MCP (2004) Improving lipoplex-mediated gene transfer into C6 glioma cells and primary neurons. Exp Neurol 187: 65–75.

da Cruz MTG, Simões S, Pires PPC, Nir S, de Lima MCP (2001) Kinetic analysis of the initial steps involved in lipoplex-cell interactions: effect of various factors that influence transfection activity. Biochim et Biophys Acta 1510: 136–151.

Denning C, Pitts JD (1997) Bystander effects of different enzyme-prodrug systems for cancer gene therapy depend on different pathways for intercellular transfer of toxic metabolites, a factor that will govern clinical choice of appropriate regimes. Hum Gene Ther 8: 1825–1835.

Feldman LJ, Tahlil O, Steg G (1996) Perspectives of arterial gene therapy for the prevention of restenosis. Cardiovasc Res 32: 194–207.

Freeman SM, Whartenby KA, Freeman JL, Abboud CN, Marrogi AJ (1996) In situ use of suicide genes for cancer therapy. Semin Oncol 23: 31–45.

Gao X, Huang L (1996) Potentiation of cationic liposome-mediated gene delivery by polycations. Biochemistry 35: 1027–1036.

Hargest R, Williamson R (1996) Prophylactic gene therapy for cancer. Gene Ther 3: 97–102.

He Q, Liu J, Sun X, Zhang ZR (2004) Preparation and characteristics of DNA-nanoparticles targeting to hepatocarcinoma cells. World J Gastroenterol 10: 660–663.

Kircheis R, Wightman L, Schreiber A, Robitza B, Rossler V, Kursa M, Wagner E (2001) Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application. Gene Ther 8: 28–40.

Leamon CP. Fusogenic lipids and vesicles for delivery of pharmaceutical agents. US Patent 6,379,698, 30 April 2002.

Lincoln DT, Aliemadi EM, Tonissen KF (2003) The thioredoxin-thioredoxin reductase system: over expression in human cancer. Anticancer Res 23: 2425–2433.

Mahato RI, Takakura Y, Hashida M (1997) Nonviral vectors for in vivo gene delivery-physicochemical and pharmacokinetic considerations. Crit Rev Ther Drug Carrier Syst 14: 133–172.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55–63.

Shand N, Weber F, Mariani L, Bernstein M, Gianella-Borradori A, Long Z, Sorensen AG, Barbier N (1999) A phase 1–2 clinical trial of gene therapy for recurrent glioblastoma multiforme by tumor transduction with the herpes simplex thymidine kinase gene followed by ganciclovir. Hum Gene Ther 10: 2325–2335.

Sorgi FL, Bhattacharya S, Huang L (1997) Protamine sulfate enhances lipid-mediated gene transfer. Gene Ther 4: 961–968.

Turunen N, Karihtala P, Mantyniemi A (2004) Thioredoxin is associated with proliferation, p53 expression and negative estrogen and progesterone receptor status in breast carcinoma. APMIS 112: 123–132.

Wasan KM, Brazeau GA, Keyhani A, Hayman AC, Lopez-Berestein G (1993) Roles of liposome composition and temperature in distribution of amphotericin B in serum lipoproteins. Antimicrob Agents Chemother 37: 246–250.

Xuo L, Pirollo KF, Tang WH, Rait A, Chang, EH (1999) Transferrin-liposome-mediated systemic p53 gene therapy in combination with radiation results in regression of human head and neck cancer xenografts. Hum Gene Ther 10: 2941–2952.

Zhou X, Huang L (1994) DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action. Biochim Biophys Acta 1189: 195–203.