Department of Pharmacy[1], ShenYang Pharmaceutical University, Department of Pharmaceutics and Pharmaceutical Engineering[2], Shenyang Pharmaceutical University, Shenyang, China

Galactosylated liposomes as oligodeoxynucleotides carrier for hepatocyte-selective targeting

FENGBO YU[1], TONGYING JIANG[1], JINGHAI ZHANG[2], LIHUA CHENG[1], SILING WANG[1]

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Siling Wang, Department of Pharmacy, Shenyang Pharmaceutical University, 32# post box, 103 Wenhua Road, Shenyang, 110016, China
silingwang@hotmail.com

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18mer oligodeoxynucleotides (ODNs) which can inhibit survivin gene expression were selected as a model gene drug. The glycolipid (5-cholestan-3β-yl)-1-[2-(lactobionyl amido) ethylamido] formate (CHE-LA) which specific target to the cells expressing galactose receptors was synthesized through the reaction of lactone of lactobiono-1,5-lactone (LA) and the amino-group of 2-(cholesteryloxycarbonylamino) ethylamine (CHE). The galactosylated liposome incorporated with CHE-LA containing oligodeoxynucleotides was prepared with SPC, cholesterol, CHE-LA and oligodeoxynucleotides by the thin-film hydration method. 1,1′-Dioctadecyl-3,3,3′,3′tetramethylindocarbocyanine perchlorate (DiI) was used as a marker for all the liposome preparations. Compared with conventional liposomes (CL), the galactosylated liposomes (GL) exhibited a drastically increased distribution to the liver *in vivo* and the galactosylated liposomes containing oligodeoxynucleotides (GLO) can also more efficiently induced an apoptosis of HepG2 cells *in vitro* than the conventional liposome containing oligodeoxynucleotides (CLO). In addition, the GLO represented an improving of the ODNs entrapment efficiency.

1. Introduction

Oligodeoxynucleotides (ODNs) are becoming popular as tools to control the expression of specific genes (Robert et al. 2001; Rolland 1998). In parallel with clinical trials, basic cellular studies on introduction of exogenous oligomers to target cells have been extensively performed. However, the poor cellular uptake and instability limited research and application of ODNs (Juliano and Yoo 2000). As a result, a major pharmaceutical concern is the effective delivery of these molecules *in vitro* and *in vivo*.

Transfection experiments about ODNs were first undertaken in the early 1960s (Fillion et al. 2001) and experimentation with transfecting different cell types and tissues now has become widespread (Rolland 1998). Many different strategies have been attempted for the improved delivery of ODNs with varying degrees of success, among them various nonviral vectors were mostly used. Nonviral vectors employed carriers consisting of lipids, proteins, peptides, polymeric matrixes as well as ligands capable of directing complexes to some cell-surface receptors on target cells (Wu and Wu 1987). Results of those previous studies suggested that liposome are commonly used for the *in vitro* delivery owning to its affording protection to the nucleic acid while enhancing its cellular delivery (Sapra and Allen 1998). As a result, various liposomes for gene delivery have been developed and some exhibited very high transfection efficiency (Mahato et al. 1995). In parallel with the development of liposomes, molecules ranging from synthetic compounds to biomolecules were introduced to liposome-based gene transfer systems to obtain higher efficiency. For example, the introduction of peptides with a NLS-like sequence (Kawakami et al. 2001) or the cationic polymer polyethylenimine (Singh and Ariatti 2003) facilitated nuclear transport, resulting in higher efficiency. Biomolecules such as transferring protein (Rolland 1998) enhanced efficiency by facilitating receptor-mediated endocytosis. It was also reported that various molecules enhanced transfection efficiency, such as inhibitors of microtubules (Degols et al. 1998) including biosurfactants (surface-active compounds produced by microorganisms) and growth factors (Carter et al. 2003) which increased the efficiency of gene delivery when used with cationic liposomes. However, among them, cationic lipids have unfavorable characteristics in application such as cytotoxicity at higher concentrations and interactions with serum components (Mahato et al. 1997).

Recently, it was found that monosialoganglioside enhanced efficiency by facilitating receptor-mediated endocytosis avoiding the toxicity of cationic liposomes (Noguchi et al. 2003). To deliver drugs to specific cell types, receptor-mediated endocytosis is a promising strategy. For selective delivery of drugs to the liver parenchymal cells (PC), galactose modification has been used, because the asialoglycoprotein receptor (ASGP-R) is known to be present only on hepatocytes with high density. Carriers modified with galactose residue are recognized by the ASGP-R on the liver parenchymal cells and are incorporated into cells by endocytosis. Galactose-terminated compounds such as asialofetuin (AF) or synthetic glycolipids have been used to modify liposomes for selective accumulation in the li-

ver (Singh and Ariatti 2003). In addition, it was reported that glycolipids-containing liposomes effectively reduced the splenic uptake and maintained long-term expression of the gene in mice (Sasaki et al. 2001). These findings led us to study gangliosides, especially as transfection agents targeting to hepatocytes.

In the study we aimed to develop a safe, stable and efficient delivery system using galactosylated liposomes to deliver ODNs. Firstly, we synthesized a novel amphiphilic glycolipid, CHE-LA through a simple and feasible procedure. An amide linkage was chosen to couple lactobionic acid to 2-(cholesteryloxycarbonylamino) ethylamine (CHE). Then, the CHE-LA was used to prepare the galactosylated liposomes for hepatocyte-targeting drug delivery. To evaluate the targeting ability of the galactosylated liposomes, the tissue distributions of the GL and CL *in vivo* were investigated. Furthermore, the association between the two kinds of liposomes and the HepG2 cells was investigated and cell apoptosis was analyzed.

2. Investigations and results

2.1. Characterization of CHE-LA

The IR analysis gave the following characteristic absorption bands $\overline{\nu}/cm^{-1}$: 3376 (br, OH); 2935 ($-CH_2-$); 1720 ($-C{=}O$); 1540 ($-NH-$). The results got from ^1H NMR were δ: 7.76 (1 H, t, −NH); 7.06 (1 H, t −NH); 5.33 (2 H, m, 6,3′-H); 5.16 (2 H, m, 1″,2′-H); 4.79 (2 H, m, 3,4′-H); 4.65 (1 H, t, 5′-H); 1.10 (3 H, s, 21-H); 0.97 (3 H, s, 19-H); 0.90 (3 H, d, J = 6 Hz, 27-H); 0.84 (3 H, d, J = 6 Hz, 26-H); 0.65 (3 H, s, 18-H). The molecular weight detected by ESI-MS (m/z) was 835.4 $[M + Na]^+$. The melting point of CHE-LA was also detected by DSC, which gave a single peak at 214 °C. From these results we can conclude that the reaction between LA and CHE was completely and pure CHE-LA was obtained.

2.2. Characterization of liposomes

The drug entrapment efficiency was detected with the gel filtration method by Sephadex G-50. The vesical size and ζ-potential of the GLO and CLO were measured at room temperature by LS Particle Size Analyzer (LS 230 BECKMAN CoaLTER, USA) and Delsa 440SX zeta potential Analyzer (BECKMAN Coulter, USA) respectively. The obtained liposomes had diameters of approximately 200 nm (Table 1) and were useful for improving transfection efficiency. The leakage-rate of ODN from these liposomes was less than 2.7% after 10 h incubation at 37 °C.

Table 1: Characterization of liposomes

Types of liposomes	Drug entrapment	ζ-potential	particle size
CLO	10.5 ± 0.8%	−18.6 ± 0.9 mV	201 ± 13 nm
GLO	16.2 ± 0.6%	−10.6 ± 1.2 mV	195 ± 17 nm

2.3. Evaluation of targetability in vivo

According to the formula $I = \Delta F = F_{GL} - F_{CL}$, ΔF values were calculated. I-time curves of the different tissues of CL and GL are shown Fig. 1. The modified galactosylated liposomes exhibited a decrease in spleen while a significant increase in liver and the galactosylated liposomes can stay in the liver for a longer time which will be very helpful to deliver the ODN drugs to the hepatocytes.

The targetability of the galactosylated liposomes in different tissues was also reflected from the drug targeting index C_e (Gupta and Huang 1989), $C_e = (C_{max})_{GLO}/(C_{max})_{CLO}$. In the equation, C_e (listed in Table 2) represents the delivery

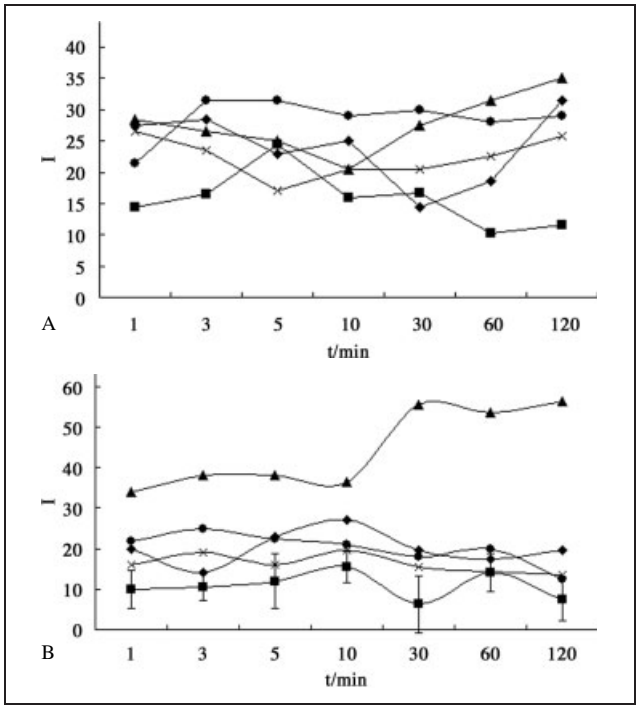
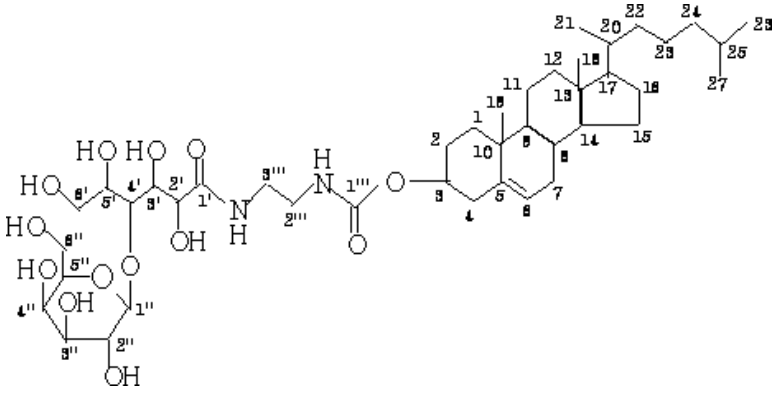


Fig. 1: Tissue accumulation of DiI-labeled CL (A) and GL (B) after iv injection in mice. Fluorescence intensity was determined in the heart (■), liver (▲), spleen (×), lung (□), kidney (●). Each value represents the mean ± SD; n = 5



(5-Cholestan-3β-yl)-1-[2-(lactobionyl amido) ethylamido] formate (CHE-LA)

Table 2: C_e values in different tissues

Tissues	$(C_{max})_{GL}$	$(C_{max})_{CL}$	C_e
Heart	15.5	24.5	0.63
Liver	56.5	35.0	1.6
Spleen	19.5	26.5	0.74
Lung	27.0	31.5	0.86
Kidney	25.0	31.5	0.79

ability of the liposomes to the different tissues *in vivo*, and C_{max} represents the maximum of fluorescence intensity in different tissues. As Table 2 shows, the C_e value of the galactosylated liposomes in liver was 1.6 which was drastically higher than in other tissues.

2.4. Interaction of GLO or CLO with HepG2 cells

We examined the association of the lipid layer components of liposomes with HepG2 cells. DiI was incorporated in GLO and CLO as a lipophilic fluorescent marker. Fig. 2 shows that the association of DiI inserted into the two liposome suspensions when they were incubated for different time periods with HepG2 cells at 37 °C. In the case of GLO, the amount of DiI remaining with the cells was significantly increased with prolonging incubation time, GLO showed sufficient absorption with HepG2 cells incubated for 2 h at 37 °C and the amount of absorption has no change until 3 h. However, CLO labelled with DiI varied little with the incubation time. When GLO was incubated with HepG2 cells for 3 h by the addition of 1 mg/ml AF (a competitor for the ASGP-R), AF significantly ($P < 0.05$) reduced the absorption amount of lipids in GLO. In the absence of AF, the absorption of lipids in GLO was increased highly. The absorption of DiI was not affected by the presence of AF when DiI was loaded in CLO, the results are shown in Fig. 3.

To evaluate the extent of absorption of liposomes on HepG2 cells following the time course, a kinetic study was performed at 0, 0.25, 1.0, 1.5 and 2.0 h. The results showed that, at the beginning, the absorption of lipids values were nearly the same for GLO and CLO, after 0.25 h incubation at 37 °C, the cellular absorption of lipids for GLO increased strongly with time (Fig. 2), and reached at the end of 3 h, almost 1.2 μmol. However, for CLO, the absorption of lipids changed very little in general.

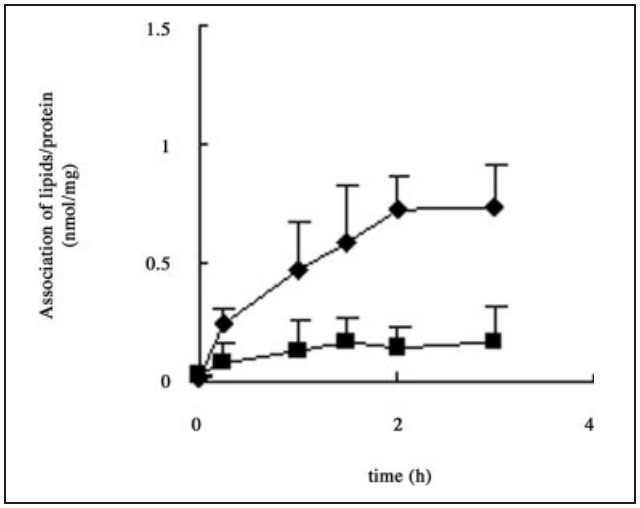


Fig. 2: Association of DiI in GLO or CLO with HepG2 cells. HepG2 cells (3×10^5 cells) were incubated with GLO or CLO labeled with DiI (1.5 μmol lipids/well) for indicated times as shown in A and B at 37 °C. ■■■ represents GLO; ■■■ represents CLO. Each value represents the mean ± S.D. (n = 3). * $P < 0.05$, significantly different

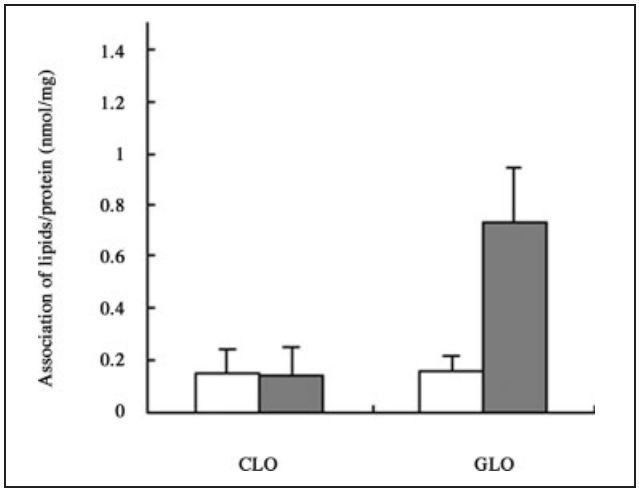


Fig. 3: Association of DiI in GLO or CLO with HepG2 cells (3×10^5 cells). HepG2 cells were incubated with GLO or CLO labeled with DiI (1.5 μmol lipids/well) for 3 h in the presence (open bar) or absence (gray bar) of 1 mg/ml AF at 37 °C. Each value represents the mean ± S.D. (n = 3). * $P < 0.05$, significantly different

2.5. GLO inducing apoptotic death

FACs analysis was performed to determine if galactosylated liposomes could efficiently be delivered to HepG2 cells. Flow cytometric analysis of HepG2 cell distribution showed that exposure to the ODNs incorporated in GLO induced a decrease in the percentage of cells accumulated in the G_1 compartment, and apoptosis in the the left of G1 phase with respect to untreated controls. In parallel, ODNs encapsulated in CLO obviously did not induce change. As shown by data in Fig. 4, apoptotic death assay employing PI staining followed by FACS analysis clearly showed an apoptotic effect of ODNs encapsulated in galactosylated liposomes in HepG2 cells.

3. Discussion

ODNs were regarded as an effective means of lowering the levels of a specific gene product (Carter et al. 2001; Gaus et al. 2005). However, the biological effectiveness of ODNs is hindered by a number of factors, particularly, rapid degradation and poor cellular uptake (Liang et al. 2005). Those factors strongly limit the potential of ODNs for modulating the expression of hepatocyte specific genes. As we know, liver diseases are very epidemic in Asia especially in China, so in the study, ODN ISIS#23665 which can inhibit the survivin protein expression was employed as a model drug to investigate a suitable delivery system for nucleic acid drugs.

It has been reported that a limited flexibility of the ligands is necessary to attain high-affinity binding, and glycosides with high flexible spacer arms (PEG 1000, PEG 2000) were not recognized (Weyermann et al. 2004). An appropriate arrangement as a result of exposure of the carbohydrate ligands and the flexibility of the spacer might lead to high affinity binding, which is different for different receptor proteins (Kang et al. 2005; Sun et al. 2005). In this study, to improve the liposome target specificity and uptake by cells, we synthesized an (5-cholestan-3β-yl)-1-[2-(lactobionyl amido) ethylamido] formate (CHE-LA)

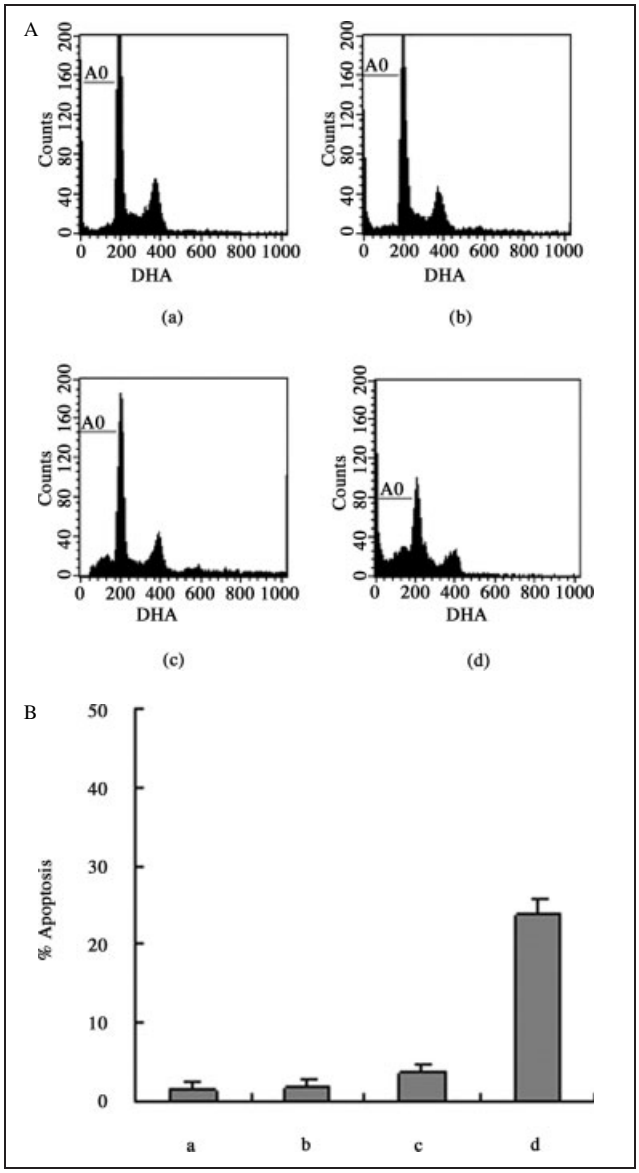


Fig. 4: Apoptotic death assay of the control group (a), ODN solution (b), ODN encapsulated in conventional liposomes (c) and ODN incorporated in galactosylated liposomes (d). In the experiments of A, the number of apoptotic cells was measured by flow cytometry as described in the text. The region designated as A0 was defined as cells undergoing apoptosis-associated DNA degradation. Data represent the mean values of three replications with bars indicating SEM. (* $P < 0.05$ compared with the control groups in which the cells were not treated with ODN encapsulated in liposome without CHE-LA

which possessed bifunctional groups of a lipophilic anchor moiety for stable incorporation to liposomes and a galactose moiety for recognition by the asialoglycoprotein receptors in HepG2 cells. Furthermore, the synthesized CHE-LA possesses monovalent ligands embedded in a fluid phospholipid bilayer that were assumed to be able to delocalize to a certain degree by lateral diffusion and thus, from a certain spacer length onward, to partake in an appropriate arrangement for receptor interaction.

Then galactosylated liposomes were prepared for targeting to hepatocytes in order to increase ODNs pharmacological activity. In addition, we discovered the galactosylated liposomes can also improve the entrapment efficiency of ODNs in liposomes. As previous studies have demonstrated that liposomes containing 50% (mol/mol) galactosides were taken up by Gal/Fuc-recognizing receptor whereas galactosylated liposomes with less than 5% (mol/mol) galactosides were taken up by the asialoglycoprotein receptor on PC (Nishikawa et al. 1995; Kawakami et al. 1998). Based on the finding, the content of galactosides in liposomes was designed to be low (5% (mol/mol)), which allows them to escape from the Gal/Fuc-recognizing receptor.

As the first step towards the understanding of efficiency of galactosylated liposomes as ODNs carrier, we detected the galactosylated liposomes (GL) and conventional liposomes (CL) distribution *in vivo* of the mice, as the results show that the liposomes can quickly reach the liver 1 min after injection and the galactosylated liposomes kept the strongest delivery ability to the liver compared with the conventional liposomes. The galactosylated liposome delivery to the liver was obviously higher than to all the other tissues.

Then the absorption of galactosylated liposomes with a hepatoblastoma cell line HepG2 in culture was studied. In order to demonstrate the ability of the proposed liposomes to bind to the ASGP receptors and to be endocytosed via receptor-mediated endocytosis, we investigated their interaction with hepatic cells. For this, the human hepatoma HepG2, a well characterized cell line, with an abundance of asialoglycoprotein receptor was used. As stated above, GLO and CLO were prepared and used in the cell culture experiments. The quantitative results obtained by the GLO and CLO of DiI marker incubated with HepG2 cells revealed that the length of incubation time had a substantial influence on the uptake of liposomes by the cells which increased in the order: $2\ h < 1\ h < 0.5\ h$ (Fig. 2). However, the uptake of control liposomes varied little.

We can reason that GLO displayed saturable high affinity binding to human hepatocytes after 2 h incubation in comparison to the different time of the control group. Ligand molecules on the vesicles are required for receptor mediated endocytosis to occur.

The present study was undertaken to further investigate the efficacy of ODNs encapsulation into galactosylated liposomes with regard to antitumor activity to prove the effectiveness of the glycolipid. The biological effectiveness of ODN showed that GLO can induce apoptosis in HepG2 cells.

In conclusion we have developed a safe, stable and efficient system using galactosylated liposomes to deliver ODNs in HepG2 cells as target cells. Firstly, we synthesized a novel glycolipid, (5-cholestan-3β-yl)-1-[2-(lactobionyl amido) ethylamido] formate (CHE-LA), which is an amphiphilic molecule. Then, galactosylated liposomes were developed consisting of SPC, cholesterol and CHE-LA. The tissue distribution of galactosylated liposomes (GL) gave a strong evidence that the galactosylated liposomes have a potential for hepatocyte-targeting drug delivery system. Furthermore, the interaction between GLO and the HepG2 cells *in vitro* was also studied, the results showed that the binding absorption of galactosylated liposomes with HepG2 cells reached to 0.72 μmol/mg protein and ODNs encapsulated in the galactosylated liposomes induced an apoptotic effect.

4. Experimental

4.1. Materials

ISIS#23665 (5′cga tgg cac ggc gca ctt 3′) was synthesized by Shanghai Sangon Biological Engineering Technology and Service Co. Itd in China. Cholesteryl chloroformate was purchased from Acros Organics in Germany. Cholesterol, sodium lactobionate, stearylamine and cation-exchange resin (H^{+}) were from Biochemistry Inc. of Changchun in China. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Biotium, Inc. in USA. Methanol was purified by distillation

before use. Other chemicals were of reagent grade and used without further purification. Propidium iodide (PI) was from Sigma in U.S.A. Soy phospholipids (SPC) were gifts from Taiwei Pharmaceutical Co. in China. Sephadex G-50 was purchased from Pharmacia Biotech in Sweden.

4.2. Synthesis of (5-cholestan-3β-yl)-1-[2-(lactobionyl amido) ethylamido] formate (CHE-LA)

4.2.1. Synthesis of 2-(Cholesteryloxycarbonylamino) ethylamine (CHE)

Cholesteryl chloroformate (2.0 g) solved in dichloromethane (6 ml) was added dropwise to a solution of ethylene diamine (2.5 ml) in dichloromethane (6 ml) and pyridine (6 ml). The mixture was stirred for 3 h at room temperature, then the solvent was removed under vaccum and the residue was partitioned between water (90 ml) and dichloromethane (90 ml). The organic layer was washed with water, dried and recrystallized from cyclohexane to give the title compound (Letsinger et al. 1989) ($C_{30}H_{52}N_2O_2$): 1.47 g (70%); R_f on silica (chloroform/methanol, 1 : 1 vol/vol) 0.15; positive ninhydrin test.

4.2.2. Synthesis of lactobiono-1,5-lactone (LA)

A solution of sodium lactobionate was passed through a cation-exchange resin (H^+) to obtain free lactobionic acid. The eluted solution was lyophilized, then the residue was converted to lactobionic lactone by repeated evaporation from methanol and ethanol (Kim et al. 1996; Wang et al. 2006b).

4.2.3. Synthesis of (5-cholestan-3β-yl)-1-[2-(lactobionyl amido) ethylamido] formate (CHE-LA)

The lactobiono-1,5-lactone (1.0 g) was dissolved in methanol (10 ml) under gentle heating, then CHE (1.4 g) in chloroform (10 ml) was added to the LA solution followed by stirring overnight at room temperature. After the solvent was removed under vaccum, the residue was washed with methanol and chloroform followed by drying in the air to give pure CHE-LA (a white solid power): 1.8 g (74%). The chemical structure was confirmed by IR, ^{1}H NMR (300 MHz, DMSO-d_6) and ESI-MS.

4.3. Preparation of ODNs encapsulated in CHE-LA modified liposomes

SPC, cholesterol and CHE-LA at the proportions of 60 : 40 and 60 : 30 : 10 (mg/mg) were individually dissolved in ethanol and dried in a rotary evaporator under reduced pressure at 50 °C to form a thin film on the round-bottom flask wall. The film was hydrated overnight with saline solution with or without 50 μM oligodeoxynucleotides to give a lipid concentration of 30 mg/ml. Multilaminar large vesicles (MLV) were downsized to form oligolamellar vesicles by extrusion at 50 °C in an Extruder device (Lipex Biomembranes, Canada) through polycarbonate membrane filters of variable pore size. Liposomes were extruded in three steps: three consecutive extrusions through a 0.8 μm pore diameter filter, three other consecutive extrusions through 0.4 μm membranes and three consecutive extrusions through a 0.2 μm filter (Wang et al. 2006a). After preparation, liposomes were aliquoted, a nitrogen stream was passed to displace the air, and finally liposomes were stored at 4 ∼ 7 °C in a refrigerator in quiescent conditions. The phospholipids in liposomes avoided the oxidation under these conditions. Before use, they were warmed to 37 °C.

4.4. Evaluation of targetability in vivo

4.4.1. Tissue distribution

Female KM mice (18–22 g) received the galactosylated (10% CHE-LA) liposomes (GL) without oligodeoxynucleotides or conventional liposomes (CL) without oligodeoxynucleotides through the tail vein at the dose of 10 mg/kg. This study used groups of 5 mice per formulation per time point. At different time intervals, blood samples were collected via eye puncture, subsequently, the tissues containing hearts, livers, spleens, lungs and kidneys were recovered and washed with saline after the mice were humanely killed.

4.4.2. Biological samples treatment

DiI, 0.2 ml plasma samples and 0.1 ml methanol were mixed in each 1.0 ml centrifuge tube to make the final concentration of DiI (0.803 μg/ml), the mixture was vortexed (WH-861 Votex Shaker, China) for 30 s and treated in ultrasound for 5 min, then centrifuged at 10000 r/min for 10 min, at last the fluorescence intensity (F) of the supernatant liquid was detected.
The tissue samples (0.1 g, less than 0.1 g were completely used) in saline were homogenated (JJ-2 Tissue Grinder, China), then DiI was added to 0.2 ml tissue homogenate in 0.4 ml methanol and the following steps were same to the plasm sample treatment.

4.5. Cell culture

HepG2 cells were cultured in RPMI1640 (Gibco-BRL) containing 1 mmol/L L-glutamine, 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown at 37 °C in humidified atmosphere containing 5% CO_2/95% air. HepG2 cells are known to express the asialoglycoprotein receptor.

4.6. Determination of cellular absorption of lipids by DiI labeling

In the experiment indicated, CLO and GLO were labeled by the addition of 0.4 mol % DiI to the liposomal suspension for 24 h at 25 °C, all the DiI could be incorporated in liposomes as shown by gel filtration.
The cell association experiments were carried out at 37 °C in 1 ml of serum-free RPMI-1640 medium containing the indicated concentrations of liposomes (Maitani et al. 2001). More than 99% of the cells were viable at the end of the incubation periods as determined by the Trypan blue exclusion test. Incubation was terminated by washing the plates with ice-cold PBS three times. The washed cells were lysed in PBS containing 0.2% Triton X-100. The fluorescence intensity of DiI was assayed using fluorophotometer (Hitachi 650-60, Tokyo, Japan) with excitation and emission wavelengths at 450 and 570 nm respectively, the amounts of lipids were calculated accordingly. The amount of DiI was presented as the associated lipids per 1 mg cellular protein. Protein was measured using a Bradford method.

4.7. Cell cycle analysis

Exponential growth HepG2 cells (8×10^5 cells), were cultured in 25 cm^2 flasks at 37 °C. After 24 h, the medium was removed and replaced with serum-free medium. CLO and GLO containing 50 μmol ODNs were introduced to the cells yielding a final antisense concentration of 10 μM. After 3 h at 37 °C, the medium was replaced with complete medium. Cells were incubated for a further 48 h whereupon cells were trypsinized and washed twice with cold PBS buffer. Then the cells were fixed with 2 ml of ice cold ethanol (70% v/v in water) overnight at −20 °C. After centrifugation, fixed cells were exposed to 500 μl of 180 U/ml RNase for 30 min at 37 °C, then to propidium iodide (PI) staining solution (25 μg/ml PI, 0.1% Triton X-100, and 30 mg/ml polyethylene glycol in 4 mM citrate buffer) for 30 min at 4 °C (Carter et al. 2001). Cell cycle distribution was analyzed using a FACScan flow cytometer and the ModFit LT software.
All experiments in this study were performed at least in triplicate.
Statistical analysis: comparison of cell association reported was done using one-way ANOVA with SPASS software (version 11.5). The turkey post-test was used to compare means. Differences were considered significant at a $p < 0.05$.

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