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Novel Targeted Liposomes Deliver siRNA to Hepatocellular Carcinoma Cells *in vitro*

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Liposomes form a major class of non-viral vectors for short interfering RNA delivery, however tissue and cell-specific targeting are additional requirements in the design of short interfering RNA delivery systems with a therapeutic potential. Selective delivery of liposomes to hepatocytes may be achieved by directing complexes to the asialoglycoprotein receptor, which is expressed on hepatocytes, and which displays high affinity for the β-D-galactopyranosyl moiety. We aimed to show that the D-galactopyranosyl ring in direct β-glycosidic link to cholesterol, when formulated into liposomes with 3β[*N*-(*N*′,*N*′-dimethylaminopropane) carbamoyl] cholesterol (Chol-T) or its quaternary trimethylammonium analogue (Chol-Q), may promote targeted delivery of cytotoxic short interfering RNA to the human hepatoma cell line HepG2 via the asialoglycoprotein receptor. Liposome-short interfering RNA interactions were characterized by electron microscopy, dye displacement, gel retardation and nuclease assays. Stable short interfering RNA-protective lipoplexes were formed at N/P ratios in the range 5:1–7:1. Targeted lipoplex 4 achieved high transfection efficiencies at 50 nM short interfering RNA (70%) and <10% in a competition assay, whilst untargeted complexes reached low levels at the same concentration (<25%). Transfection efficiencies of all lipoplexes in the asialoglycoprotein receptor-negative cell line HEK293 under the same conditions were low. Lipoplexes containing cholesteryl-β-D-galactopyranoside may therefore form the basis for the development of useful hepatotropic short interfering RNA delivery vectors.

Key words: hepatocyte delivery, HepG2, lipoplex, RNA interference, steryl glycoside

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RNA interference (RNAi) is an innate eukaryotic mechanism for the modulation of gene expression, which operates at the mRNA level in the cytoplasm of eukaryotic cells. Thus, exogenous and endogenous long double-stranded RNA (dsRNA) is cleaved by the enzyme DICER into short interfering RNA (siRNA) duplexes with two nucleotide 3′-overhangs, which become incorporated into RNA-induced silencing complexes. These ribonucleoprotein complexes are activated by guide strand (antisense) selection whereupon they bind to and degrade complementary mRNA sequences (1). This mechanism may be harnessed to silence disease-promoting gene expression through appropriate design of tailored siRNA duplexes. However, cellular uptake of the polyanionic siRNA is poor as the duplexes are hydrophilic and are also repelled by the negatively charged cell membrane. Moreover, if the nucleic acid remains unprotected, it is rapidly degraded by serum nucleases (2) and/or removed by renal glomeruli. Efficient delivery of siRNA still remains a challenge for RNAi-based therapeutics, and the design of appropriate vehicles is an ongoing process. Cationic liposomes form a major class of non-viral vectors, which are being explored for siRNA delivery as they exhibit relatively low toxicity and elicit weak immune responses (3). In essence, the polyanionic nucleic acids form condensed electrostatic complexes with the positively charged cationic liposomes that are subsequently taken into cells by endocytosis. In the design of siRNA delivery systems with a therapeutic potential, consideration must also be given to tissue- and cell-specific targeting.

The liver, which is the largest visceral organ in mammals, is composed largely of hepatocytes. These parenchymal cells perform important functions in lipid and glucose homoeostasis and are responsible for the biosynthesis of several serum proteins. In addition, a number of pathogenic viruses including hepatitis B and C viruses (HBV, HCV) infect hepatocytes. Hence, the silencing of genes associated with viral replication and metabolic disorders by RNAi in hepatocytes is an emerging area of research. Although the selective delivery of siRNA lipoplexes to hepatocytes may be achieved by directing complexes to the asialoglycoprotein receptor (ASGP-R), which is expressed abundantly and almost exclusively on the sinusoidal face of the plasma membrane of liver parenchymal cells, few examples have been reported. Thus, ASGP-R-mediated uptake of siRNA has been achieved using liposomes that include cholesten-5-yloxy-*N*-{4-[(1-imino-2-D-thiogalactosyl-ethyl) amino] butyl} formamide (Gal-C4-Chol) (4) or lactosylated phosphatidylethanolamine (5) in their formulations. In both cases, it is the appropriate display of the D-galactopyranosyl moiety on lipoplexes, which is recognized by the mammalian lectin (6). The receptor shows high affinity for several asialoglycoproteins (ASGPs) in blood circulation by recognition of this hexose, which is found in a β-glycosidic link at the non-reducing

termini of the heteroglycan components of the ASGPs (7). Phytosteryl glucosides have for some time been included in liposome formulations intended for hepatocyte-targeted DNA delivery (8,9). More recently, however, Cho *et al.* (10) have constructed cationic liposomes that contain the plant sterol glucoside, β-sitosterol glucoside, for liver targeting of siRNA via the ASGP-R; however, this receptor is known to exhibit high affinity for ligands displaying *N*-acetyl-D-galactosamine or D-galactose. The specificity is achieved, in part, by interactions between the sugar 3-OH and 4-OH groups with receptor carboxylate and amide groups. The crystal structure of the H_1 subunit of the receptor reveals that these two hydroxyl groups co-ordinate to a Ca^{2+} ion. The conformational requirement for this interaction is for an equatorial 3-OH and axial 4-OH (11). An equatorial 4-OH, as is found in glucose, would be expected to result in a weaker binding interaction as had been demonstrated in an earlier investigation (12). Moreover cholesterol, a natural component of animal cell membranes, which is biodegradable endogenously, may be a more appropriate anchor for the hexopyranoside in liposomal formulations intended for the transfection of nucleic acids in mammalian systems.

We had previously reported that cationic liposomes containing a cationic cholesteryl cytofectin, the co-lipid dioleoylphosphatidylethanolamine (DOPE) and cholesteryl-β-D-galactopyranoside (Chol-β-Gal, Figure 1A) formed stable lipoplexes with pGL3 plasmid DNA and promoted high levels of transfection activity in HepG2 cells by ASGP-R-mediation (13). To explore the possibility that similarly constituted liposomes may be used for hepatocyte-targeted siRNA delivery, we have selected two closely related cholesteryl cytofectins that are higher homologues of the popular DC-Chol and that differ from each other only in the degree of cationic head group methylation. Thus, 3β[*N*-(*N*′,*N*′-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T, Figure 1B), which bears a terminal tertiary dimethylamino function and 3β[*N*-(*N*′,*N*′,*N*′-trimethylammoniumpropane) carbamoyl] cholesterol iodide (Chol-Q, Figure 1C), which displays a quaternary trimethylammonium head group have been shown to be very effective DNA transfecting agents in liposome formulations *in vitro* (14,15). Chol-T, however, exhibited higher transfection activity in the cell lines tested viz. HeLa and HepG2 (15). This is in agreement with the findings of an earlier report on related ester-linked cholesteryl cytofectins where the dimethylamino head group afforded a more active molecule than did the trimethylammonium functionality (16). We show in the present siFection study that Chol-β-Gal, when formulated into cationic liposomes with the cytofectin Chol-T, or its quaternary analogue Chol-Q, and DOPE, promotes efficient transfection of siCONTROL Tox siRNA (Dharmacon) into the hepatocyte-derived human hepatocellular carcinoma cell line HepG2 by ASGP-R-mediation. Non-galactosylated siRNA lipoplexes were, however, delivered less efficiently at the same siRNA concentration (50 nM). Moreover, transfection efficiencies achieved by all siCONTROL Tox lipoplex preparations in the ASGP-R-negative human embryonic kidney cell line HEK293 were low. Lipoplexes constituted with siGENOME non-targeting siRNA were only very weakly cytotoxic in both cell lines at the same lipoplex concentrations.

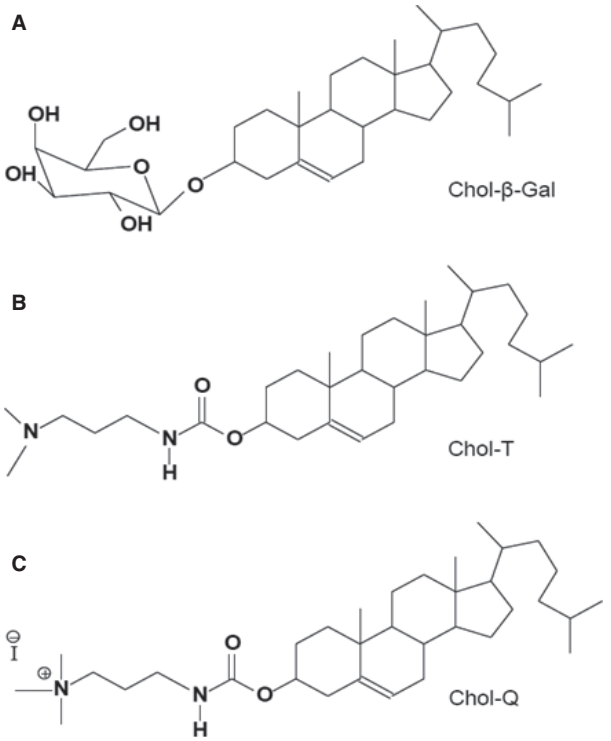

Figure 1: (A) Cholesteryl-β-D-galactopyranoside (Chol-β-Gal); (B) 3β[*N*-(*N*′,*N*′-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T): (C) 3β[*N*-(*N*′,*N*′,*N*′-trimethylammoniumpropane) carbamoyl] cholesterol iodide (Chol-Q).

Methods and Materials

Reagents and analysis

Dioleoyl-L-α-phosphatidylethanolamine (DOPE) was obtained from Sigma-Aldrich (St Louis, MI, USA). SYBR Green II RNA gel stain (10 000× concentrate in DMSO) was purchased from Cambrex Bio Science Rockland Inc. (Rockland, ME, USA). The α,β-D-galactopyranosyl pentaacetate was supplied by Pfanstiehl Laboratories Inc. (Waukegan, IL, USA). Silica gel 60 (70–230 mesh), silica gel $60F_{254}$ TLC plates, $CdCO_3$ and 2-[-(2-hydroxyethyl)-piperazinyl]-ethanesulphonic acid (HEPES) were from Merck (Darmstadt, Germany). HepG2 cells and irradiated foetal bovine serum (FBS) were obtained from Highveld Biological (PTY) Ltd. (Lyndhurst, South Africa). HEK293 cells were obtained from Professor P Arbuthnot (Antiviral Gene Therapy Research Unit, University of the Witwatersrand, South Africa). Minimum Essential Medium (MEM) containing Earle's salts and L-glutamine, penicillin (10 000 U/mL)-streptomycin (10 000 μg/mL) mixtures and trypsin-EDTA were purchased from Lonza BioWhittaker (Walkerville, MD, USA). The CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay Solution containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and the electron coupling reagent phenazine ethosulphate was supplied by Promega (Madison, WI, USA). Eighteen megaohms water was used throughout. All other reagents used in this study were of analytical grade. ^{1}H and ^{13}C NMR spectra were obtained on a Varian Gemini 300 instrument (Varian Inc., Palo Alto, CA, USA) at 300 and 75 MHz, respectively. ^{1}H chemical shifts were recorded relative to C_5D_5N (8.57 ppm) or $CDCl_3$ (7.24 ppm) and ^{13}C chemical shifts relative to C_5D_5N (135.5 ppm) or $CDCl_3$ (77.0 ppm). Abbreviations for signal multiplicities are as

follows: s(singlet), d(doublet), t(triplet), q(quartet) and m(multiplet). Chemical shifts are reported for ^{1}H spectra as shifts (multiplicity, integration, coupling assignment).

Synthesis

3β[*N*-(*N*′,*N*′-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) and 3β[*N*-(*N*′,*N*′,*N*′-trimethylammoniumpropane) carbamoyl] cholesterol iodide (Chol-Q) were prepared as described elsewhere (14,15).

3β[*N*-(*N*′,*N*′-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T)

The desired product was obtained as a crystalline white compound (yield: 82%). Mp 103–105 °C. ^{1}H NMR ($CDCl_3$) includes the following: δ = 0.65 (s, 3H, C-C$\underline{H}_3$), 0.83 (d, 6H, J = 5.2, CH(C$\underline{H}_3$)$_2$), 0.89 (d, 3H, J = 6.5 Hz, CHC$\underline{H}_3$), 2.19 (s, 6H, (C$\underline{H}_3$)$_2$NCH$_2$CH$_2$)), 2.30 (t, 2H, J = 6.6 Hz,(CH$_3$)$_2$NC$\underline{H}_2$CH$_2$CH$_2$), 3.21(q, 2H, J = 6.1, (CH$_3$)$_2$NCH$_2$CH$_2$C$\underline{H}_2$NH), 4.46 (m, Chol-$\underline{H}_{3\alpha}$), 5.35 (d, 1H, J = 5.3, $\underline{H}_6$). M/z = 514.4490 (M^+) ($C_{33}H_{58}N_2O_2$ = 514.4498).

3β[*N*-(*N*′,*N*′,*N*′-trimethylammoniumpropane) carbamoyl] cholesterol iodide (Chol-Q)

Chol-Q was obtained as a white crystalline compound. Yield: 85%. Mp 168–170 °C (decomp). ^{1}H NMR ($CDCl_3$): δ = 0.64 (s, 3H, C-CH$_3$), 0.83 (d, 6H, J = 6.6 Hz, CH(C$\underline{H}_3$)$_2$), 0.88 (d, 3H, J = 6.6 Hz, CHC$\underline{H}_3$), 0.97 (s, 3H, C-CH$_3$), 2.28 (t,2H, J = 4.6 Hz, (CH$_3$)$_3$NC$\underline{H}_2$CH$_2$), 3.33 (q, 2H, J = 6.3 Hz, (CH$_3$)$_3$NCH$_2$CH$_2$C$\underline{H}_2$NH), 3.4 (s, 9H, (C$\underline{H}_3$)$_3$NCH$_2$CH$_2$), 3.72 (t, 2H, J = 7.3, 8.2, (CH$_3$)$_3$NC$\underline{H}_2$CH$_2$CH$_2$), 4.46 (m, 1H, Chol-$\underline{H}_{3\alpha}$), 5.32 (d, 1H, J = 4.4 Hz, $\underline{H}_{6)}$ 5.59 (bs, 1H, CH$_2$CH$_2$N$\underline{H}$). M/z = 530 ($M\text{-}I^+$, 100%).

Cholesteryl-β-D-galactopyranoside (Chol-β-Gal)

Chol-β-Gal was prepared by a Koenigs–Knorr procedure from the appropriately protected bromo sugar and cholesterol under anhydrous conditions with $CdCO_3$ as promoter, by an adaptation of methods reported elsewhere for the syntheses of related glycosides (Figure S1) (17,18). Briefly, HBr was passed through a solution of α,β-D-galactopyranosyl pentaacetate (7.8 g, 20 mmol) in glacial acetic acid (100 mL) at 4 °C to afford 2,3,4,6-tetra-*O*-acetyl-$\alpha(\beta)$-D-galactopyranosyl bromide. The reaction was monitored by TLC in hexane:ethyl acetate (8:3 v/v) followed by visualization with p-anisaldehyde spray (p-anisaldehyde:conc. H_2SO_4:ethanol, 5:5:90 v/v/v). The reaction mixture was mixed with crushed ice and the product extracted into dichloromethane. This extract was washed with 1% sodium bicarbonate (100 mL) and water (2 × 100 mL) before drying over $CaCl_2$. Solvent was removed *in vacuo*, and the product was obtained in quantitative yield. A solution of cholesterol (3.9 g, 10 mmol) in toluene (75 mL) over $CdCO_3$ (4 g, 22.7 mmol) was brought to reflux in an apparatus fitted with a Dean-Stark trap. To this was added gradually a solution of the bromo sugar in toluene (30 mL). Complete glycosylation was observed after an additional 2 h under reflux (TLC). The solution was filtered and the solvent removed *in vacuo*. The β-D-tetra-*O*-acetylgalactopyranoside was separated from the α-anomer by silica gel 60 (70–230 mesh) column chromatography (hexane:ethyl acetate, 8:3 v/v).

Mp 157–159 °C. ^{1}H NMR ($CDCl_3$): δ 0.65 (s,3H,C-C$\underline{H}_3$), 0.83 (d, 6H, J = 6.6Hz, CHC$\underline{H}_3$), 0.89 (d, 3H, J = 6.5, CHC$\underline{H}_3$), 0.96 (s, 3H, C-C$\underline{H}_3$), 1.96–2.12 (12H, 4 × CO-C$\underline{H}_3$), 3.47 (m, 1H, Chol-$\underline{H}_{3\alpha}$), 3.86 (t, J = 7.0, $\underline{H}$-5′), 4.05–4.19 (m, 2H, $\underline{H}$-6′a, $\underline{H}$-6′b), 4.52 (d, 1H, J = 8.0 Hz, $\underline{H}$-1′), 4.99 (dd, 1H, J = 10.4, 3.4 Hz, $\underline{H}$-3′), 5.16 (dd, 1H, J = 10.4, 7.9 Hz, $\underline{H}$-2′), 5.35 (m, 2H, H-4′, Chol-$\underline{H}_6$). ^{13}C NMR ($CDCl_3$): sugar region: δ 100.29 (C-1′), 69.07 (C-2′), 70.55 (C-3′), 67.03 (C-4′), 71.02 (C-5′), 61.30 (C-6′) ppm. Cholesteryl moiety: δ 11.86 (C-18), 18.72 (C-21), 19.36 (C-19), 21.05 (C-11), 22.57 (C-26), 22.83 (C-27), 24.29 (C-15), 28.23 (C-16), 29.52 (C-2), 23.82 (C-23), 28.02 (C-25), 31.86 (C-8), 31.94 (C-7), 35.78 (C-20), 36.18 (C-22), 36.71 (C-10), 39.52 (C-24), 39.74 (C-12), 37.19 (C-1), 38.96 (C-4), 42.32 (C-13), 50.14 (C-9), 56.74 (C-14), 56.14 (C-17), 80.35 (C-3), 122.19 (C-6), 140.33 (C-5) ppm.

To a solution of cholesteryl-β-D-tetra-*O*-acetylgalactopyranoside (72 mg, 0.1 mmol) in $CHCl_3$ (1.5 mL) was added a solution of sodium ethoxide (0.35 mL, 0.76 mmol). After 12 h at 21 °C, solvent was removed *in vacuo* (25 °C) and the residue was triturated in water (4 °C) to remove sodium acetate and excess sodium ethoxide. The powdery white precipitate was resuspended in water and stored at 4 °C overnight. The product was obtained by filtration and was dried exhaustively *in vacuo* in a Büchi-TO pistol drier at 60 °C. Yield: 48 mg (87%). Mp 270–273 °C. ^{1}H NMR ($CDCl_3$, C_5D_5N): δ 0.58 (s, 3H, C-C$\underline{H}_3$), 0.84 (d, 6H, J = 4.0 Hz, CHC$\underline{H}_3$), 0.88 (d, 3H, J = 6.5, CHC$\underline{H}_3$), 4.35 (d, 1H, $\underline{H}$-1′). ^{13}C NMR ($CDCl_3$, C_5D_5N): sugar region: δ 102.26 (C-1′), 74.84 (C-2′), 78.63 (C-3′), 71.43 (C-4′), 78.09 (C-5′), 62.61 (C-6′) ppm. Cholesteryl moiety: δ 11.88 (C-18), 18.80 (C-21), 19.31 (C-19), 21.11 (C-11), 22.60 (C-26), 22.86 (C-27), 23.95 (C-23), 24.35 (C-15), 28.08 (C-25), 28.35 (C-16), 31.89 (C-8), 37.30 (C-1), 39.57 (C-24), 39.81 (C-12), 42.33 (C-13), 50.18 (C-9), 56.18 (C-17), 56.69(C-14), 121.75 (C-6), 140.65 (C-5) ppm.

siRNA duplexes

siGENOME non-targeting siRNA (D-001210-01) and siCONTROL Tox siRNA (D-001500-01) were obtained from Thermo Scientific Dharmacon Products (Lafayette, CO, USA). The non-targeting duplex has at least four mismatches with all known human genes and is used here to assess non-sequence-specific effects of siRNA and siRNA lipoplexes on cell lines *in vitro*. The recognition sequence for siGENOME non-targeting siRNA is 5′-UAG CGA CUA AAC ACA UCA A-3′. The sequence and physical properties of siCONTROL Tox siRNA are proprietary. Cells that have been successfully transfected with siCONTROL Tox siRNA undergo apoptosis and cell death shortly thereafter (24–48 h). Duplexes were dissolved in RNAse-free 18 MOhm H_2O at a final concentration of 20 μM and stored in 100 μL aliquots at −80 °C.

Preparation of liposomes

A mixture of Chol-T or Chol-Q (2 μmol) and DOPE (2 μmol) in $CHCl_3$ (1.0 mL) was deposited as a thin film on the inner wall of a round bottomed tube by rotary evaporation of the solvent *in vacuo* at 22 °C. Traces of solvent were removed by additional vacuum desiccation at 200 mTorr for 2 h. Thereafter, the film was rehydrated in HBS (20 mM HEPES, 150 mM NaCl, pH 7.5, 1 mL) overnight. The mixtures were vortexed (5 min) and sonicated for 5 min in a

Transsonic-bath-type sonicator (T 460/H) at 22 °C to afford unilamellar liposome suspensions, which were routinely stored at 4 °C without aggregation for several weeks. Targeted liposomes were prepared as described earlier with the inclusion of Chol-β-Gal (0.5 μmol) in pyridine (200 μL) in the initial lipid mixtures. The compositions of the inner and outer membrane leaflets of the liposome bilayer are assumed to be very similar. Small differences may arise from curvature effects.

Liposome-siRNA band shift assay

To siGENOME non-targeting siRNA samples (0.5 μg, 2 μL) were added varying amounts of cationic liposomes (0–16 μg). Mixtures were adjusted to 10 μL with HBS and set aside for 30 min at 22 °C. Thereafter, gel loading buffer (50% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanol, 2 μL) was added to the siRNA lipoplex suspensions. Samples were then subjected to electrophoresis on 2% agarose gels in TPE buffer (36 mM Tris–HCl, 30 mM sodium phosphate, 10 mM EDTA, pH 7.5) for 40 min at 50 volts. Gels were stained with SYBR Green II solution (1:10 000 dilution) in TBE buffer (89 mM Tris base, 89 mM boric acid, 1 mM EDTA pH 8.0) for 20 min and viewed under transillumination in a G:BOX gel documentation apparatus (Syngene, Frederick, MD, USA) with a short pass filter.

SYBR Green II displacement assay

Liposome-siRNA interactions may also be studied by monitoring the decay in sample fluorescence that accompanies displacement of siRNA-bound SYBR Green II upon association of the nucleic acid with the liposome vesicles. Thus, the fluorescence of SYBR Green II (10 000× diluted) in HBS (0.5 mL) was measured in a Shimadzu RF-551 spectrofluorometric detector at excitation and emission wavelengths of 497 and 520 nm, respectively. This value was set to represent 0% relative fluorescence. Thereafter, siGENOME non-targeting siRNA (1.3 μg) was added. The fluorescence reading of this solution was set at 100% relative fluorescence. Subsequently, 1 μL (2.5–3.1 μg) aliquots of the liposome suspensions were added in a stepwise manner to the solution and readings taken until no further drop in relative fluorescence was observed with further addition of liposomes.

Nuclease digestion assay

Lipoplexes were pre-formed by incubating a mixture of siCONTROL Tox siRNA (0.5 μg) and varying amounts of liposomes in HBS (13 μL) for 30 min at 22 °C. FBS was then added to a final concentration of 10% (v/v), and the resulting mixture was maintained at 37 °C for 4 h. EDTA was then added to final concentrations of 10 mM. The siRNA was then released from liposomes by addition of SDS to a final concentration of 0.5% (w/v). Finally, mixtures were incubated at 55 °C for 20 min, cooled and subjected to 2% agarose gel electrophoresis as described earlier. Comparisons were then made between the siRNA in individual digest mixtures and control untreated siRNA.

Cryo transmission electron microscopy of liposomes and lipoplexes

Liposome and lipoplex (siGENOME non-targeting siRNA) suspensions (1 μL) were mixed with aqueous 1% (w/v) uranyl acetate solution (1 μL) and placed on Formvar-coated copper grids. Grids were blotted with filter paper after 3 min and plunged into liquid ethane at −183 °C using a Leica CPC system (Vienna, Austria). The vitrified samples were viewed using a GATAN cryotransfer device at −150 °C in a JEOL-1010 (Tokyo, Japan), transmission electron microscope operating at 100 kV. Images were captured digitally using an Olympus MegaView III camera and SIS iTEM software (Tokyo, Japan), which also facilitated measurements of liposomes on calibrated images.

Preparation of siRNA lipoplexes for transfection and cytotoxicity studies

Lipoplexes were prepared 30 min prior to exposure to cells by addition of liposome suspensions (2.5–3.1 μg/mL) to siRNA (0.2 or 0.4 μg) in HBS. Incubation mixtures were diluted to 13 μL with HBS). Lipoplex suspensions were prepared at two siRNA concentrations viz. 0.48 and 0.96 μM corresponding to final concentrations of 25 and 50 nM when dispersed in 250 μL of medium during incubation with cells.

Cell cytotoxicity and transfection studies

HEK293 and HepG2 cells were seeded in 48-well plates at a density of 2×10^4 cells/well and incubated in MEM containing 10% (v/v) FBS, 100 U/mL penicillin G, 100 μg/mL streptomycin sulphate, 10 mM $NaHCO_3$ and 20 mM HEPES (pH 7.5) at 37 °C for 24 h to permit growth to about 60% confluence. Medium was then removed and replaced with 250 μL serum-free MEM. Lipoplexes were then added, and cells were incubated for 4 h whereupon the medium was replaced with complete medium (containing 10% FBS). Cells were incubated for a further 48 h before the addition of 50 μL of Cell Titre 96® AQ_{ueous} One Solution Cell Proliferation Assay Solution to each well. The MTS tetrazolium compound is reduced by dehydrogenases in cells that are metabolically active into a coloured formazan, which is soluble in the cell culture medium. After a further 4 h at 37 °C, optical absorbances were recorded at 490 nm on a Thermo Electron Corporation Biomate 3 UV/visible spectrophotometer. In assays to determine cytotoxicities, siGENOME non-targeting siRNA was used, while siCONTROL Tox siRNA was used in transfection efficiency assays. Untreated cells and controls with siRNA at 25 and 50 nM in the absence of liposomes were also included in the assays.

Statistical analysis

All transfections and cytotoxicity assays were performed in triplicate, and results are presented as mean ± SD. The data were compared by one-way ANOVA, and the unpaired Student's *t*-test was performed when comparing two individual groups. Differences achieving $p < 0.05$ were considered to be statistically significant.

Results and Discussion

Cholesteryl-β-D-galactopyranoside (Chol-β-Gal)

Structures of cholesteryl tetra-*O*-acetyl-β-D-galactopyranoside and Chol-β-Gal and their anomeric purity were confirmed by reference to their ^{1}H and ^{13}C NMR spectra. ^{13}C signal assignments for the

cholesteryl ring system in cholesteryl tetra-*O*-acetyl-β-D-galactopyranoside and Chol-β-Gal were in accordance with those previously reported for related steryl glycosides (19) and cholesteryl polyamine carbamates (20), while ^{1}H peak assignments for the tetra-*O*-acetyl-β-D-galactopyranosyl entity were in agreement with those reported elsewhere (21). Assignments for the D-galactosyl moiety chemical shifts in the ^{13}C spectrum of Chol-β-Gal were consistent with those reported by Agrawal (22). The C-1′ signal at 102.26 ppm in the ^{13}C spectrum and the H-1′ signal in the ^{1}H spectrum at 4.35 ppm for the galactopyranosyl ring in Chol-β-Gal were characteristic of the β-configuration at C-1′ and differ markedly from those of the α-anomer, Chol-α-Gal (98.29 and 5.29 ppm, respectively) (13).

Liposomes and lipoplexes

Cationic cholesterol amphiphiles in combination with the neutral co-lipid DOPE afford stable, generally non-toxic liposomes that are suitable for non-viral gene delivery (15,23–26). Chol-T has been shown to have comparable DNA transfection activity with the commercially available DC-Chol *in vitro*, in three different cell lines (24), while Chol-Q exhibited greater activity than DC-Chol both *in vitro* and *in vivo* (27). The phospholipid DOPE plays an important role in membrane fusion and destabilization. This property is attributed to its ability to switch from the L_α phase into the hexagonal H_{II} phase (28). Moreover, in the acidic environment of the endosomes, it interacts with the anionic lipids of the membrane, promoting disruption of the bilayer and release of the nucleic acid. Standard negative staining procedures for the visualization of liposomes by TEM have been widely used, but may result in the formation of artefacts through vesicle dehydration. While cryo TEM is generally more reliable, it offers images with low contrast. A cryo TEM protocol that includes a brief negative staining step with dilute uranyl acetate was therefore adopted in this study. Images revealed untargeted vesicles Chol-T:DOPE (**1**) and Chol-Q:DOPE (**3**) to be in the 80–120 nm size range, while the targeted liposomes Chol-T:DOPE:Chol-β-Gal (**2**) and Chol-Q:DOPE:Chol-β-Gal (**4**), were predominantly in the 30–80 nm range (Figure 2, Liposomes, A–D). Galactosylation may have partially inhibited vesicle fusion during liposome formation to afford smaller liposomes. Lipoplexes were prepared for cryo TEM at liposome:siRNA ratios corresponding to the minimum amount of liposome suspension required to fully bind 0.5 μg siRNA in 10 μL HBS (end ratio, Table 1). In micrographs, lipoplexes appeared as 300–500 nm aggregates of smaller vesicles (Figure 2, Lipoplexes, A–D) and resembled complexes reported by Cao *et al.* (25) for related liposomes.

siRNA liposome binding studies

The stoichiometry of siRNA-liposome complexes may be conveniently studied by band retardation analysis on agarose gels (29). Thus, liposome preparations **1**–**4** were shown to fully bind siRNA at siRNA/liposome weight ratios ranging from 1:18 to 1:28 corresponding to cytofectin positive/siRNA negative charge ratios (N/P ratios) in the range 5:1–7:1 (Figure 3, Figure S2 and Table 1). These values are in close agreement with those obtained in a study of siRNA interaction with liposomes containing DOPE and 3β[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), a lower homologue of Chol-T (30). Notably, inclusion of the targeting component Chol-β-Gal in liposome preparations **2** and **4** resulted in higher N/P end-point ratios than those for preparations **1** and **3** (Table 1), indicating that the β-D-galactopyranosyl moieties may partially adumbrate adjacent positive charges on the bilayer. The siRNA-liposome binding interaction may also be examined under the more dilute conditions employed in transfection experiments through a dye displacement assay (31). SYBR Green II was selected for the assay as the fluorescence quantum yield of RNA complexes with this dye is more than seven times greater than that of ethidium bromide/RNA complexes (32). The dye is believed to associate with DNA by base pair intercalation and minor groove binding (33). The assay hinges on the displacement of the dye by the cationic

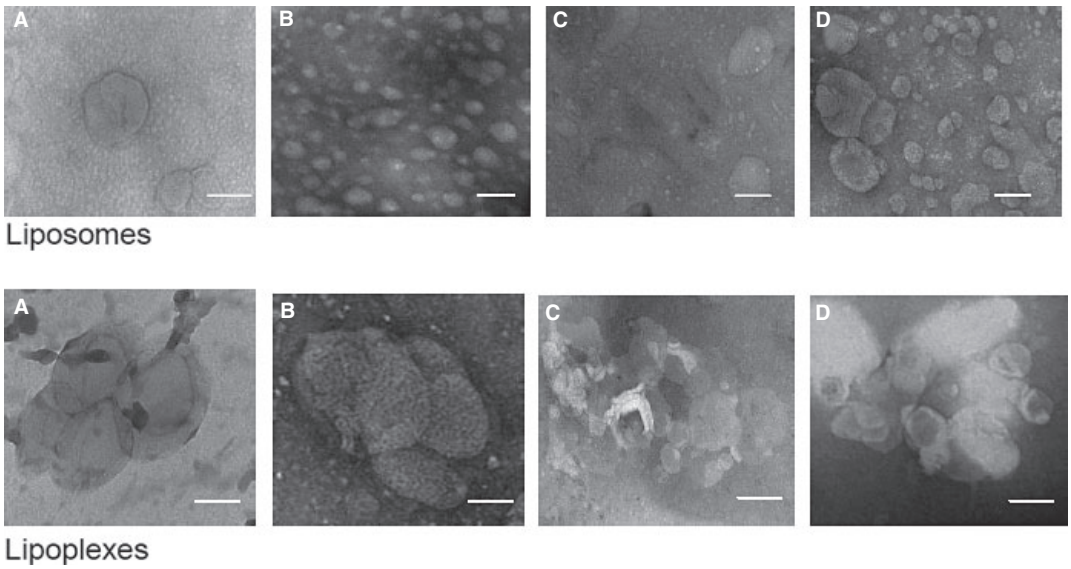

Figure 2: Cryo electron microscopy of liposomes and short interfering RNA (siRNA) lipoplexes. Chol-T:DOPE (A); Chol-T:DOPE:Chol-β-Gal (B); Chol-Q:DOPE (C); Chol-Q:DOPE:Chol-β-Gal (D). Bar = 100 nm. DOPE, dioleoylphosphatidylethanolamine.

Table 1: Liposome composition and lipoplex end-point ratios

Liposome	Composition (molar ratio)	Gel retardation end-point (liposome/siRNA)		SYBR Green assay end-point (liposome/siRNA)	
		N/P[a]	w/w	N/P[a]	w/w
1	Chol-T:DOPE (1:1)	5.5:1	18:1	5:1	17:1
2	Chol-T:DOPE: Chol-β-Gal (4:4:1)	6:1	24:1	6.5:1	26:1
3	Chol-Q:DOPE (1:1)	5.5:1	22:1	6.5:1	23:1
4	Chol-Q:DOPE: Chol-β-Gal (4:4:1)	6.5:1	28:1	7:1	30:1

DOPE, dioleoylphosphatidylethanolamine; siRNA, short interfering RNA.
[a]In calculating liposome (N)/DNA (P) charge ratios, an average molecular mass of 350 per nucleotide was assumed and one (–) charge per nucleic acid nucleotide. Cytofectins Chol-T and Chol-Q were each assumed to carry one (+) charge per molecule at pH7.5.

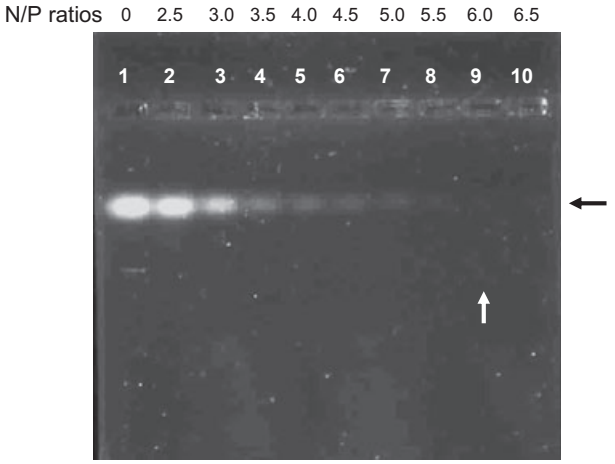

Figure 3: Representative band retardation analysis of short interfering RNA (siRNA) lipoplexes on 2% agarose gels (complete set in Supporting Information). Incubation mixtures contained siGENOME non-targeting siRNA (0.5 μg) in HBS (10 μL) and varying amounts of liposome suspension. Lane: (1) naked siRNA; (2–10), Chol-T:DOPE:Chol-β-Gal complexes with siRNA at N/P ratios of 2.5:1, 3.0:1, 3.5:1, 4.0:1, 4.5:1, 5:1, 5.5:1, 6.0:1, 6.5:1. End-points and unbound siRNA bands are indicated with white and black arrows, respectively. Complete set in Figure S2. DOPE, dioleoylphosphatidylethanolamine.

liposomes and the attendant reduction in SYBR Green II fluorescence. The decay in fluorescence on stepwise addition of the cationic liposomes to preformed dye/siRNA complexes may then be followed on a spectrofluorometer. End-point ratios obtained here were in close agreement with those observed for corresponding liposomes in band shift assays (Figure 4, Table 1). Although based on different underlying principles, results from the two assays were corroborative (Figures 3 and 4) and suggest that the 20-fold difference in the concentration of reactants encountered in the two assays did not affect liposome/siRNA end-point ratios.

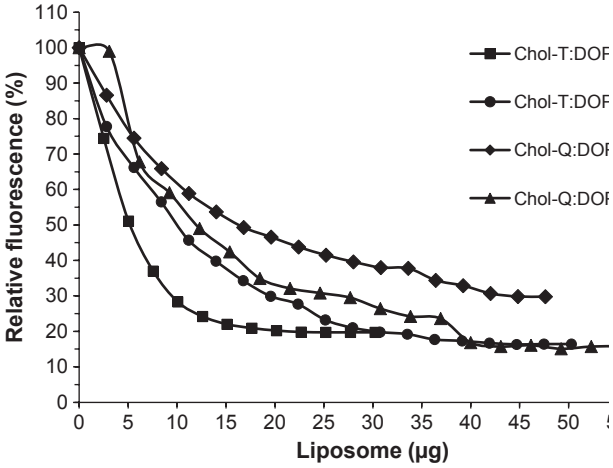

Figure 4: SYBR Green intercalation assays. Incubation mixtures, in HBS (0.5 mL), contained siGENOME non-targeting short interfering RNA (siRNA) (1.3 μg). Liposome suspensions were added in stepwise increments (1 μL, 0.25–0.31 μg). Liposome preparations **1** (Chol-T:DOPE); **2** (Chol-T:DOPE:Gal); **3** (Chol-Q:DOPE) and **4** (Chol-Q:DOPE:CholGal), respectively.

Serum stability of liposome-bound siRNA

Unmodified siRNA is rapidly degraded in serum by the action of nucleases (34). However, siRNA is not commonly delivered to target cells or tissue in its naked state. Non-viral vectors, which form non-covalent complexes with siRNA and which may be given a cell or tissue targeting aspect, are more commonly used for delivery. The susceptibility of the nucleic acid, in such complexes, to serum nuclease-catalysed breakdown is therefore of some importance. Untargeted and targeted siRNA lipoplexes generated in the present study were exposed to 10% FBS at 37 °C for 4 h, and the siRNA was thereafter examined by electrophoresis on 2% agarose gels. Results presented in Figure 5 confirm that free (uncomplexed) siRNA is completely degraded under these conditions, while liposome preparations **1**–**4** afforded their siRNA cargos varying degrees of protection. It is interesting to note that human serum has been reported to contain considerably less ribonuclease activity than FBS (35).

siRNA lipoplex transfections (RNA interference)

The efficiency of siRNA delivery into HEK293 and HepG2 cells by liposomes **1**–**4** was determined using siCONTROL Tox siRNA, which induces cell death 24–48 h post-transfection. Cell viability was established by the MTS assay and then correlated to the transfection efficiency (36). The % transfection may be readily obtained by subtracting the % surviving cells from 100. These results are presented in Table 2 with the cytotoxicities of siGENOME non-targeting siRNA lipoplexes. Lipoplexes were prepared at N/P ratios from 5:1 to 7:1 Results presented in Figure 6 represent transfection values obtained within this range (6:1). Results obtained in the ASGP-R-negative HEK293 cell line confirmed that on exposure to siCONTROL Tox siRNA lipoplexes, transfection efficiencies were low and in the order of 20%. Moreover, no targeting effect was observed with lipoplexes **2** and **4** (Figure 6A). However, in the human hepatoma

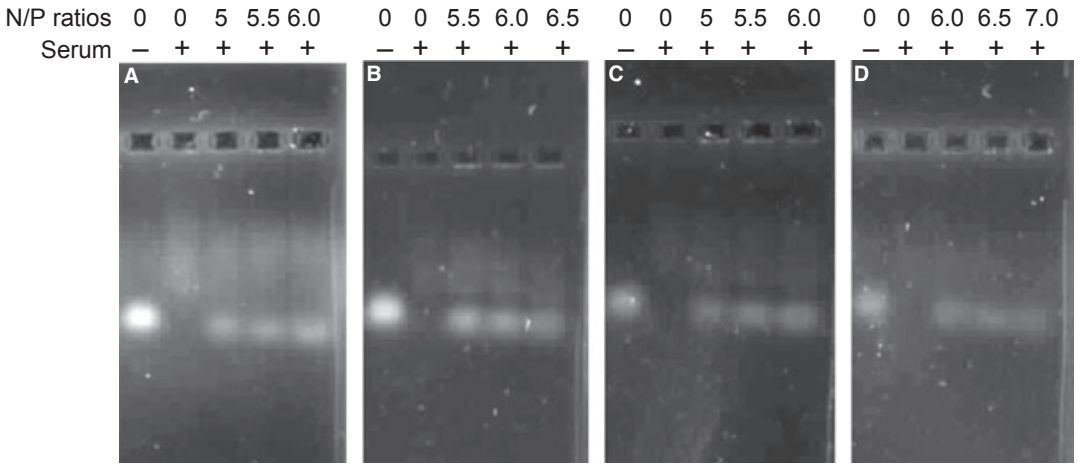

Figure 5: Serum nuclease protection assays. Incubation mixtures in HBS contained siCONTROL Tox short interfering RNA (siRNA) (0.5 μg), FBS (10%, v/v) and varying amounts of liposomes **1**–**4**. Lane: (1) siRNA alone without FBS; (2) siRNA with FBS; (3–5) N/P ratios from below to above the end-point ratios determined by band shift assays. A, Chol-T:DOPE (**1**); B, Chol-T:DOPE:Chol-β-Gal (**2**); C, Chol-Q:DOPE (**3**); D, Chol-Q:DOPE:Chol-β-Gal (**4**).

Table 2: Transfection efficiencies and cytotoxicities of lipoplexes at 25 and 50 nM siRNA

	Transfection efficiency (%)[a]				Cytotoxicity (% survival)[a]			
	HEK293 (nM)		HepG2 (nM)		HEK293 (nM)		HepG2 (nM)	
Lipoplexes	25	50	25	50	25	50	25	50
siRNA (alone)[b]	22.0	25.2	13.4	9.6	91.8	93.7	89.3	88.1
Chol-T	11.8	27.9	20.0	24.7	92.9	83.2	98.0	98.0
Chol-T-Gal	20.0	21.2	22.4	57.0	92.9	97.1	83.2	92.1
Chol-Q	23.9	24.7	9.9	25.4	92.0	81.7	96.8	82.8
Chol-Q-Gal	21.3	26.0	40.0	70.0	84.6	92.7	90.5	90.6

siRNA, short interfering RNA.
[a]Mean values (see Figures 6 and 8, $n = 3$).
[b]siCONTROL Tox siRNA in transfection studies and siGENOME non-targeting siRNA in cytotoxicity assays.

cell line HepG2, lipoplexes containing liposome preparations **2** and **4** exhibited a marked targeting effect. Thus, siRNA lipoplex **2** at 50 nM siRNA achieved 58% transfection efficiency, a level that was significantly higher than that of the untargeted Chol-T lipoplex **1** at the same charge ratio and siRNA concentration (Figure 6B). The galactosylated Chol-Q lipoplex **4** achieved transfection efficiencies of 40% and 70% at 25 and 50 nM siRNA, respectively. These values were also significantly higher than those obtained with their ungalactosylated counterpart, lipoplex **3** at equivalent charge ratios and nucleic acid concentrations (Figure 6B). The extent of ASGP-R-mediated lipoplex cell entry into HepG2 cells was determined in a competition assay. Therefore, siCONTROL Tox siRNA complexes were incubated with cells in the presence or absence of asialofetuin (1 mg/mL). As shown in Figure 7, loss of cell viability induced by targeted siRNA lipoplexes containing Chol-T (**2**) and Chol-Q (**4**) was

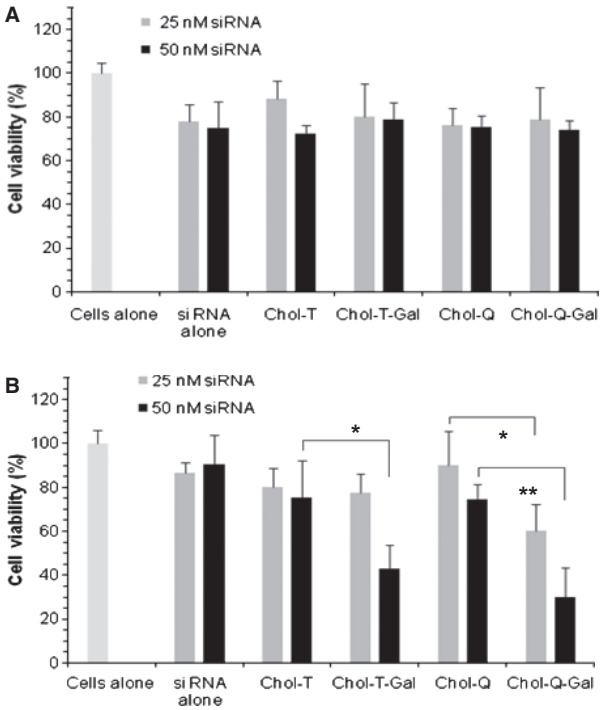

Figure 6: Transfection assays. Cells (2.0×10^4 per well) in 48-well plates were incubated with siCONTROL Tox short interfering RNA (siRNA) lipoplexes (N/P = 6:1) for 4 h in serum-free medium. Thereafter, medium was replaced with complete medium (10% FBS) and incubation extended for a further 48 h before determining cell viability by an MTS assay. (A), HEK293; (B), HepG2 cells. Data are presented as means ± SD ($n = 3$). Significant differences were found between targeted and untargeted lipoplexes at the same N/P ratio (*$p < 0.05$, **$p < 0.01$).

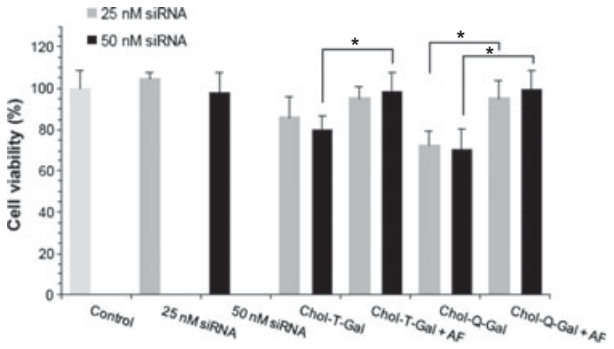

Figure 7: Inhibitory effect of asialofetuin on the transfection activity of short interfering RNA (siRNA) lipoplexes in HepG2 cells. SiCONTROL Tox siRNA was complexed with Chol-T targeted liposomes (**2**) or Chol-Q targeted liposomes (**4**) at a N/P ratio of 6:1. Cells in 48-well plates were transfected in the presence or absence of asialofetuin (AF) (1 mg/mL) at 25 and 50 nM siRNA. Control cells were untreated. Data are presented as means ± SD (n = 3). *Significant differences were found ($p < 0.05$).

significantly reduced by the inclusion of the competing ligand, thus strongly supporting the notion that cell entry of targeted lipoplexes is largely via ASGP-R mediation.

Liposome, lipoplex and siRNA non-specific cytotoxicity

Non-specific cytotoxicity attributable to siRNA sequence-independent effects and siRNA delivery method are frequently overlooked in siRNA transfection studies. We therefore assessed the cytotoxicity induced in HEK293 and HepG2 cells by siGENOME non-targeting siRNA at 25 nM (0.2 μg/250 μL) and 50 nM (0.4 μg/250 μL) both in free form and in complex with liposomes **1**–**4** at the same N/P ratio used in the transfection assay. It is evident that the siRNA alone at the 25 and 50 nM levels is essentially without effect in HEK293 cells, with cell viabilities >90% (Figure 8A). siRNA lipoplexes at both siRNA concentrations gave viabilities >80%. In a comparable experiment with HepG2 cells (Figure 8B), uncomplexed siCONTROL Tox siRNA was essentially non-toxic at concentrations of 25 and 50 nM. Complexes were again well tolerated with all siRNA lipoplexes giving cell viabilities >80%.

Conclusion

In clinical trials, cationic liposomes comprise the leading non-viral DNA delivery system (37). Drawbacks that may arise include the possibility of lipoplex opsinization, complement activation and accumulation of complexes in the cells of the mononuclear phagocyte system (38,39). In an attempt to confront these issues polymer-coated sterically stabilized liposomes are being developed (40). The similarity in physicochemical properties between DNA and RNA has facilitated the development and adaptation of cationic liposomes for siRNA delivery (29,30,41). Here, we report on the hepatocyte-specific siRNA delivery potential of two liposome formulations containing cytofectins Chol-T or Chol-Q, DOPE and Chol-β-Gal in a 4:4:1

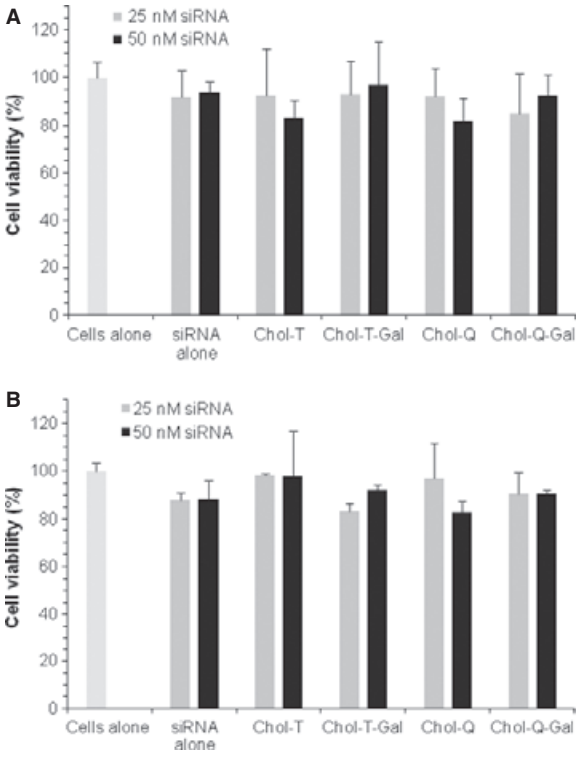

Figure 8: Cytotoxicity assays. Cells (2.0×10^4 per well) in 48-well plates were incubated with siGENOME non-targeting short interfering RNA (siRNA) lipoplexes (N/P = 6:1) for 4 h in serum-free medium. Thereafter, medium was replaced with complete medium (10% FBS) and incubation extended for a further 48 h before determining cell viability by an MTS assay. (A), HEK293 cells; (B), HepG2 cells. Data are presented as means ± SD (n = 3).

mole ratio (**2** and **4**). Two untargeted liposome preparations without Chol-β-Gal (**1** and **3**) were also included in the study. All four liposome suspensions fully bound siRNA at N/P ratios in the range 5:1–7:1 as determined by gel retardation and SYBR Green displacement analysis. The 6 Å spacer between the cholesteryl anchors and the cationic head groups of the cytofectins (15) permit the formation of stable electrostatic lipoplexes between the siRNA and the liposomes. In both assays, N/P ratios were higher for galactosylated liposomes indicating that the galactosyl ligand may be exerting a partial masking effect on neighbouring cationic headgroups in the liposome bilayer. Lipoplexes with N/P ratios of 2:1 and above have positive zeta potentials (1) favoring dispersion stability of complexes in suspension and an interaction with the negatively charged cell membranes. All lipoplexes prepared in this study were only mildly cytotoxic in HEK293 and HepG2 cell lines with no discernable differences between targeted and untargeted complexes. Complexes formed with siCONTROL Tox siRNA failed to achieve significant protein knockdown in the ASGP-R negative HEK293 cells, and transfection activities were low. The galactosylated lipoplexes, however, showed significantly higher transfection efficiencies than their ungalactosylated counterparts in the HepG2 cell line. These differences cannot be attributed to differences in cytotoxicities as none was observed. The proposal that the targeted complexes were

entering the hepatocyte-derived cell line by receptor mediation was supported by results obtained in a competition assay. Thus, loss of HepG2 cell viability induced by siCONTROL Tox siRNA-containing Chol-T and Chol-Q lipoplexes was abolished by inclusion of the competing ligand asialofetuin in transfection assays. It is noteworthy that the galactopyranosyl moiety in direct β-glycosidic link to the liposomal membrane-embedded rigid cholesteryl ring system is capable of strong binding to the ASGP-R when present at 11 mole% in the lipid bilayer of the liposomes. Further, Chol-Q-containing liposomes (**4**) were particularly effective in targeting siRNA to HepG2 cells. These formulations therefore form the basis for the development of useful hepatotropic siRNA delivery systems. Studies to optimize transfection efficiencies by variation of the Chol-β-Gal and cytofectin content in targeted cationic liposomes are underway in our laboratory.

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Declaration of interest

The authors report no declaration of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Reaction scheme depicting synthesis of Chol-β-Gal from cholesterol and 2,3,4,6-tetra-*O*-acetyl D-galactopyranosyl bromide.

Figure S2. Gel retardation analysis of siRNA lipoplexes.

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