Targeting of glycosylated lipoplexes in HepG2 cells: Anomeric and C-4 epimeric preference of the asialoglycoprotein receptor

Moganavelli Singh*, Colin B. Rogers† and Mario Ariatti*

This study was conducted to determine the capacity of the asialoglycoprotein receptor on the hepatocyte-derived cell line HepG2 to exhibit an anomeric preference with respect to the D-galactopyranose:chol:O-galactopyranoside (Cho:gal) and cholesteryl-β-D-galactopyranoside (Cho:gal) in cationic liposome/pGL3 plasmid DNA complexes for non-viral, hepatocyte-directed gene transfer. In addition, cholesteryl-α-D-glucopyranoside (Cho:glu) and cholesteryl-β-D-glucopyranoside (Cho:glu) were separately formulated into cationic liposomes at the same molar ratio (11%) to examine the C-4 epimeric selectivity of the asialoglycoprotein lectin for the glucopyranosides derived in derived lipoplexes. Lipoplex formation was examined by gel retardation, ethidium displacement assays, and transmission electron microscopy. Plasmid DNA was shown to be fully liposome associated and maximally compacted at a liposome:DNA ratio of 6:1 (weight ratio), corresponding to a +/– charge ratio of 1.3 with complexes falling in the 80–200 nm size range, whereas at a 5:1 w/w ratio [1.1 (+/–) charge ratio] lipoplexes were somewhat smaller (50–100 nm) but promoted higher transgene activity in HepG2 cells than 6:1 (w/w) lipoplexes, in the following order: Cho:gal > Cho:glu > Cho:glu = Cho:gal. Transgene activity levels in HepG2 cells: Anomeric and C-4 epimeric preference of the asialoglycoprotein receptor were approximately 10% of those achieved in HepG2 cells. Moreover, transgene activity in HepG2 cells was reduced by approximately 90% in the presence of excess asialofetuin, a ligand for the asialoglycoprotein lectin.

Introduction

Among the non-viral approaches being investigated for the cell-specific delivery of corrective genes in vitro and in vivo into mammalian cells, those designed for delivery to liver parenchymal cells are arguably the best characterized. The asialoglycoprotein receptor (ASG-R), an endocytic recycling receptor which is highly represented on the sinusoidal face of the hepatocyte plasma membrane,3 is the lectin of preference for the hepatocyte-specific DNA delivery systems that have been widely studied.4–9 The complexity associated with the assembly of glycoprotein-based delivery systems has provided the impetus for the development of simpler constructs. In several alternative hepatocyte-specific DNA delivery systems, some of which are listed below, the D-galactose moiety located at each non-reducing terminus of the triantennary N-linked heteroglycan structures on asialoorosomucoid, which is required for ASG-R recognition, has been appended to: albumin,10 histones,11 α-helical peptides,12 polylysine,13–15 lysine-serine co-polymers,16 polyethyleneimine,17 polypropyleneimine dendrimers,18 lipopolyamine,19 chitosan,20 LDL and HDL,21 and cationic liposomes.22–27

D-galactopyranosyl moieties in simple glycosidic link with cholesteryl, a component of animal cell membranes, have not hitherto been formulated into liposomes destined for hepatocyte delivery of DNA. The specificity of the ASG-R for the terminal D-galactosyl moieties of glycoproteins is achieved through hydrogen bonding of the sugar 3- and 4-hydroxyl groups with receptor carboxylate and amide side chains.24 D-glucose, an epimer of D-galactose, which differs from this sugar in the configuration of the 4-hydroxyl group (equatorial), would therefore be expected to be more poorly recognized by the ASG-R. Moreover, terminal D-galactosyl moieties on asialoorosomucoid are in a β-glycosidic linkage. This anomeric configuration has also been retained in neoglycoproteins,28,29 peptides,12–16 and other constructs exhibiting D-galactose for ASG-R targeting.19–21

In the study reported here, we prepared the α- and β-anomers of cholesteryl-D-galactopyranoside and cholesteryl-D-glucopyranoside and incorporated them into cationic unilamellar liposomes. Subsequently, lipoplexes were assembled with pGL3 vector DNA and the anomeric and epimeric preferences of the ASG-R on HepG2 cells for sugar moieties displayed on lipoplexes (Fig. 1) was examined by measuring transfection levels attained in each case in the presence or absence of the competing ligand, asialofetuin. Cholesteryl-β-D-galactopyranoside-containing lipoplexes were convincingly more effective than the other glycosylated lipoplexes studied. It is noteworthy that the ASG-R appeared able to discern epimeric and anomeric features of the glycopryanosyl units displayed on lipoplex membrane bilayers, even when in direct glycosidic linkage to the cholesteryl anchor and therefore without an extensive spacer. Luciferase activities achieved by all four lipoplexes in HepG2 cells, which are ASG-R-negative, were very low.

Materials and methods

Chemicals and spectrometry

Dioleoylphosphatidyl ethanolamine (DOPE) was from Sigma (St Louis, MI), β-D-glucose pentaacetate was from Aldrich (Milwaukee, WI) and α, β-D-galactose pentaacetate was from Pfästle (Waukegan, IL). 2-[3-(2-hydroxyethyl)-piperazinyl]-ethanesulphonic acid (HEPES) and cadmium carbonate were from Merck (Darmstadt, Germany). Trypsin-
EDTA and penicillin-streptomycin mixtures were supplied by Whittaker Bioproducts (Walkerville, MD). N,N-dimethylaminopropylamine–cholensterylformylhydrazide (MS09) was prepared as described.25 1H and 13C nuclear magnetic resonance spectra were recorded at 300 MHz and at 75 MHz, respectively, on a Varian Gemini 300 instrument. Chemical shifts (δ) were recorded relative to CDCl3 (7.26 ppm) or CD3OD (2.04 ppm) for 1H and CDCl3 (77 ppm) or CD3OD (135.5 ppm) for 13C. Signal multiplicities are abbreviated as follows: s (singlet), d (doublet), t (triplet) and m (multiplet). Chemical shifts are reflected as parts per million (ppm) from tetramethylsilane (TMS).

**Syntheses**

Cholesteryl-tetra-O-acetyl glycopyranosides. Cholesteryl-tetra-O-acetyl glycopyranosides were prepared by a modified Koenigs–Knorr procedure from the respective tetra-O-acetyl glycopyranosyl bromide and cholesterol in the presence of cadmium carbonate26 followed by deacetylation. Thus, tetra-O-acetyl glycopyranosyl bromides were prepared by passing HBr through a solution of D-glucopyranosyl pentaacetate or 2,3,4,6-tetra-O-acetyl-D-galactopyranosyl bromide or 2,3,4,6-tetra-O-acetyl-D-glucopyranosyl bromide or 2,3,4,6-tetra-O-acetyl-D-galactopyranosyl bromide (20 mmol) in toluene (75 ml) over CdCO3 (4 g, 22.7 mmol) in an apparatus fitted with a Dean–Stark trap. The reaction mixture was heated under reflux (EtBr:conc. H2SO4: ethanol, 5:5:90 volume ratios). The solution was poured onto crushed ice and the bromo sugar was extracted into dichloromethane (130 ml), which was subsequently washed with a 1% sodium bicarbonate solution (100 ml) and water (2 x 200 ml). After drying over CaCl2, solvent was removed in vacuo and the residue co-evaporated with toluene. A solution of 2,3,4,6-tetra-O-acetyl-D-glucopyranosyl bromide or 2,3,4,6-tetra-O-acetyl-D-galactopyranosyl bromide (20 mmol) in toluene (30 ml) was dropped down to a refluxing solution of cholesterol (3.9 g, 10 mmol) in toluene (75 ml) over CdCO3, (4 g, 22.7 mmol) in an apparatus fitted with a Dean–Stark trap. The reaction mixture was heated under anhydrous conditions for a further 2 hours, or until complete glycosylation was confirmed by TLC (method described above). The hot solution was filtered under vacuum (celite, Hartley funnel) to remove the inorganic salts. Solvent was removed by evaporation in vacuo and the residue dissolved in hexane:ethyl acetate (8.3 v/v, 30 ml). TLC revealed one major product (β-anomer) and a minor product (α-anomer). Anomers were separated on a silica gel 60 column (70–230 mesh, Merck), which was equilibrated and eluted with hexane:ethyl acetate (8:3 v/v). Finally, the cholesteryl tetra-O-acetyl glycosylpyranosides were recrystallized from ethanol.

Cholesteryl-ε-amin (β)-D-glycopyranosides. The acetylated cholesteryl glycosides in CHCl3, were treated with excess sodium ethoxide in ethanol for 8 hours at room temperature. Solvent was removed by rotary evaporation (25°C) and the residues triturated with water at 4°C. The powdery precipitates were suspended in water, filtered and dried in vacuo at 60°C (BuchI-TO pistol drier).

**Plasmid DNA**

In this study, the pGL3 control vector (5256 bp), which encodes the Photorhinus pyralis luciferase gene flanked by SV40 promoter and enhancer sequences, was used (Promega, Madison, WI). This arrangement affords strong expression in a variety of mammalian cell types. The pBR322 plasmid (4363 bp) (Roche Diagnostics, Mannheim) was used in the dye displacement assay only.

**Liposome preparation**

Individual cholesteryl glycosides (0.5 µmol) were dissolved in pyridine (200 µl) and added to DOPE (2 µmol) and MS09 (2 µmol) in CHCl3 (1.0 ml). Solution was evaporated in vacuo and components deposited as a thin film. This was dried further for 2 hours under vacuum and the film was re-hydrated overnight in HBS (20 mM HEPES, 150 mM NaCl, pH 7.5, 1 ml). The re-hydration mixture was vortexed briefly and sonicated for 5 minutes (Transsonic bath-type sonicator). Preparations were routinely stored at 4°C for several weeks with no evidence of aggregation or precipitation.

**Transmission electron microscopy**

Size and lamellarity of liposomes were determined by TEM. Thus liposome suspensions (50 µl, 4.5 µmol lipid/ml HBS) on paraffin film were mixed with 0.5% uranyl acetate (50 µl) and left to stand for 3 min, whereupon the mat surfaces of formvar-coated copper grids were brought into contact with the mixtures for 3 min. Thereafter, discs were air-dried overnight and viewed on a JEM-1010 transmission electron microscope (JEOL, Tokyo) operating at 60 kV. Images were photographed at a 2-second exposure on fine-grain release positive film. Lipoplexes formed by the addition of liposomes to plasmid DNA at ratios of 5:1 and 6:1 (w/w) in HBS were treated and viewed as described for liposomes.

**Liposome–DNA interactions**

**Gel retardation assays**

Liposome:DNA mixtures were prepared in HBS (10µl) and incubated at 20°C for 30 min before mixing with 3 µl gel loading buffer (50% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol, 72 mM Tris–HCl, 60 mM NaH2PO4 and 20 mM EDTA at pH 7.5). Samples were loaded onto 1% agarose gels in a Mini-Sub apparatus (Bio-Rad, Richmond, CA). Electrophoresis was carried out for 90 min at 30 V (constant). Ethidium bromide (EtBr) running buffer (36 mM Tris-Cl, 30 mM NaH2PO4, 10 mM EDTA, pH 7.5). Gels were treated for 30 min with ethidium bromide (1 µg/ml running buffer) and viewed under transillumination at 300 nm. Images were captured on a Gene Genius Bioimaging System (Syngene, Cambridge, U.K.).

**Nucleosome digestion assays**

Liposome:DNA complexes were assembled from glycosylated cationic liposomes and pGL3 DNA in HBS (20 µl) and incubated for 30 min (20°C). Fetal bovine serum was added to a final concentration of 10% and samples were incubated at 37°C for 4 hours, whereupon SDS and EDTA were introduced to a final concentration of 0.5% (w/v) and 10 mM, respectively. Mixtures were then maintained at 55°C for 20 min before adding gel loading buffer (3 µl) and subjecting to agarose gel electrophoresis as described above.

**Ethidium displacement assays**

The association of liposomes with plasmid DNA to form lipoplexes was also studied in ethidium bromide (EtBr) displacement assays, which are based on the premise that on intercalation with DNA, the dye exhibits an increase of the fluorescence quantum yield and on condensation of the nucleic acid the dye is displaced with a resultant decrease in fluorescence.27 The assays performed in this study were adapted from that described by Tros de Ildary et al. Fluorescence measurements were recorded on a Shimadzu RF-551 spectrophluorometer detector (Kyoto, Japan) at excitation and emission wavelengths of 520 and 600 nm, respectively. Briefly, baseline fluorescence (0%) was established with an EtBr solution in HBS (1 µg/500 µl buffer). Thereafter, pBR322 DNA (6 µg/ml was introduced and the solution was used to set the instrument to 100% fluorescence. Aliquots (3 µg) of glycosylated liposomes were then introduced and readings taken after mixing at each step, until 45 µg of glycosylated liposome had been added.
Cell culture

The human cervical carcinoma cell line HeLa and human hepatocellular carcinoma line HepG2 were from Highveld Biological, Kelvin, South Africa, and cultured in 25-cm² flasks in 5 ml of MEM (minimum essential medium) with Earle’s salts (Gibco, BRL, Incuinhann, Scotland) containing 10% (v/v) FBS (Delta Bioproducts, Johannesburg), 100 U/ml penicillin G, 100 µg/ml streptomycin sulphate (Whittaker, M.A. Bioproducts, MD), 10 mM NaHCO₃, and HEPES (pH 7.5). Cells were passaged 1:3 (HeLa) and 1:2 (HepG2) every 3–4 days and stored at −80°C in complete medium containing 10% DMSO (v/v).

Cell growth inhibition assays

Cells were trypsinized and seeded into 24-well plates (Bibby-Sterilin, Staffordshire, U.K.) at a density of 2 × 10³ cells per well. These were incubated at 37°C for 24 hours in complete medium (0.5 ml) to allow cell attachment and growth to semi-confluence. Liposome–DNA complexes were formed in HBS (15 µl) and incubated at 20°C for 30 min. Growth medium bathing cells was removed and replaced by serum-free medium (0.5 ml) and liposomes were added. After incubation for 4 hours (37°C), the medium was replaced with complete medium and cells were incubated for an additional 48 hours. Adherent cells were quantified by the method of Schellekens and Sittz.²¹ Briefly, cells were washed with PBS (2 × 1 ml) and stained with 200 µl crystal violet solution (0.8%, w/v sodium chloride; 5% v/v formaldehyde; 0.5% crystal violet and 50% v/v ethanol) for 20 min. After removal of stain, wells were washed exhaustively with water and plates were dried at room temperature (24 hours). Stain from cells was extracted into 2-methoxyethanol (500 µl, 36 hours) and absorbances were determined at 550 nm (Novaspec spectrophotometer, Biochrom, Cambridge, U.K.).

Transfection in vitro

HeLa and hepatocellular carcinoma cells (HepG2) and cervical carcinoma cells (HeLa) were seeded into 24-well plates 24 hours prior to transfection, at a density of 2 × 10³ cells per well in complete medium (0.5 ml). Lipoplexes containing glycosylated cationic liposomes (0–7 µg per well) and pGL3 plasmid DNA (1 µg per well) were prepared in 20 mM Hepes, 150 mM NaCl (15 µl per well) and incubated at 21°C for 30 min prior to transfection. Growth medium was removed from cells which were then washed with PBS (0.5 ml) and bathed in serum-free medium (MEM + antibiotics, 0.5 ml). Lipoplexes were added and after 4 hours at 37°C the medium was removed and replaced with complete medium (MEM + antibiotics + 10% FBS). After 48 hours cells were lysed and supernatants assayed for luciferase activity (Promega, Madison, WI) in a Lumac Biocounter M1500 (Landgraaf, Netherlands). Soluble protein in cell lysates was determined by the BCA assay (Sigma) using BSA as the protein standard. Transfection activities of all four liposome–DNA complexes in HepG2 cells were also evaluated in the presence of 10% FBS and separately in the presence of asialofetuin (300 µg per well).

Results and discussion

Cholesteryl glycosides

Four cholesteryl glycosides (Fig. 2) were synthesized for a study of their asialoglycoprotein receptor-targeting potential when incorporated into the bilayer of cationic liposomes. The syntheses were effected by a Koenigs–Knorr procedure from the appropriately protected bromo-sugar and cholesterol under anhydrous conditions.²⁶ During the course of this project, Wimmer and co-workers²⁶ reported a similar procedure for the preparation of glucoside and galactoside derivatives of cyclic (secondary) alcohols in good yield using cadmium carbonate as promoter under anhydrous conditions. Structures were confirmed by reference to ¹H and ¹³C NMR spectra recorded in CDCl₃, for the intermediate cholesteryl-tetra-O-acetylglucosylpyranosides and in CD₃D₄N (deuteropyridine) for the deprotected final products. ¹³C signal assignments for the cholesteryl moieties were very similar to one another in the eight cholesteryl glycosides generated in the present study and are in agreement with assignments reported previously in an investigation of glycosidation shifts in ¹³C NMR spectra of related sterilyglycosides.²⁷ Chemical shifts of the carbohydrate components in the ¹H and ¹³C NMR spectra of the compounds analysed agreed with those reported by Agrawal²⁸ (¹H) and Seo et al.²⁹ (¹³C). ¹H peak assignments for the tetra-O-acetyl-β-D-galactoside entity were virtually identical to those reported by Ren et al.³⁰ in a study of galactosylated dendrimers containing multiple primary amino groups.

Liposome characterization by TEM

Liposomes prepared here were constituted from MS09, DOPE, and cholesteryl glycosides (4:4:1 mole ratios, respectively) with the glycoside component limited to 9% on a weight ratio basis. Other ratios were not explored. It is apparent, however, that the sugar density in liposomes is an important factor. It was observed that at a ratio of 5% (w/w) liposome/glycolipid particles are efficiently processed by the ASG-R, whereas at a loading ratio of 50% (w/w) uptake is not blocked by excess asialofetuin.³¹ It has also been demonstrated that in the loading range 0–7.5% (w/w) for the galactosyl steryl derivative Gal-C₄-chol, the highest uptake of galactosylated liposomes by parenchymal cells was achieved at 5%.³² A large number of galactose or glucose residues could also interfere with the electrostatic interaction of the liposomes with DNA. Liposomes prepared in this study are bifunctional, with each liposome possessing a cationic component (MS09) for binding to the DNA molecule and a terminal galactose or glucose component for targeting to the galactose or asialoglycoprotein receptor on the HepG2 cells. The co-lipid DOPE was chosen for its known ability to improve intracellular trafficking of lipoplexes³³ and for improving the rate of endosomal escape of the DNA cargo.³⁴ All liposome formulations were stable at 4°C for several weeks and showed no aggregation or precipitation of components. Transmission electron microscopy revealed the formation of predominantly spherical or oval vesicles and confirmed the unilamellar nature of all liposome preparations. Liposome Cholgal sizes varied from 100 nm to 200 nm, liposome Cholßgal from 50 nm to 150 nm, liposome Cholglu from 50 nm to 200 nm, and Cholßglu from 100 to 200 nm (see Fig. A(a)–(d) in online supplement, respectively).

Liposome–DNA interactions

To determine the optimal ratio for binding between liposomes and DNA, gel retardation assays are commonly performed. In Fig. 3(A–D) it can be seen that for all four liposomes no migration of the plasmid DNA was obtained at the liposome:DNA ratio of 6:1 (w/w), equivalent to a (+/-) charge ratio of 1.3:1. At this ratio and higher, all DNA was liposome-associated. In the ethidium displacement assays, liposomes which have been formulated to contain the synthesized glycolipids, readily bring about displacement of the intercalated ethidium bromide upon their stepwise introduction to the plasmid DNA. This process is largely driven by the association of DNA phosphodiester
negative charges forming ion pairs with the cationic head groups of MS09 (Figs 4A and 4B). Fluorescence intensity decreased steadily until a ratio was attained which correlated with total retardation seen for lipoplexes in gel retardation assays (6:1 w/w). Further addition of cationic liposome did not result in a reduction in fluorescence, indicating that no further compacting of the double-stranded nucleic acid had taken place. Cholβgal and Cholβglu liposomes both displayed higher displacement of the dye than their Cholαgal and Cholβgal counterparts, suggesting that β-anomers allow greater accessibility of cationic centres to the DNA than their corresponding α-anomers. On complexing with plasmid DNA, cationic liposomes yielded lipoplexes, which appeared as clusters or aggregates of globules, when viewed by TEM after negative staining, similar to those reported by others and ourselves in unglycosylated MS09-containing lipoplexes. At a liposome:DNA ratio of 5:1 (w/w), (+/–) charge ratio of 1.1, the diameter of all four lipoplexes ranged from 50 nm to 100 nm (see Fig. B(a), (c), (e) and (g) in online supplement), whereas at a higher liposome:DNA ratio of 6:1 (w/w), (+/–) charge ratio of 1.3, lipoplex diameters fell in the 80–200 nm range (Fig. B(b), (d), (f) and (h) online). In all complexes, small (10–20 nm) liposome-like vesicles were discernible.

Nuclease digestion assays

These studies enabled us further to characterize the strong binding interaction between the liposomes and the DNA and to observe what protection is afforded by the cationic liposomes to the bound DNA. The effect of the serum nucleases on two of the four lipoplexes can be seen in Figs 5A and B (Cholβgal and Cholβglu, respectively). Generally good protection was afforded to the DNA by the liposomes at liposome:DNA ratios of 3:1 to 7:1 (w/w). However, the greatest degradation of the DNA was noted for lipoplexes at a liposome:DNA ratio of 1:1 (w/w). This may be attributed to the low liposome:DNA (+/–) charge ratio at which there may be unbound DNA molecules present. Indeed, at such a low ratio it is anticipated that liposome-bound DNA may also be susceptible to serum nuclease digestion as
condensation may be incomplete. At higher charge ratios it can be seen that the DNA was well protected within the lipoplexes. The adsorption of serum proteins can induce a number of effects such as complex destabilization, aggregation or retargeting. The stability of lipoplexes or polyplexes depends on the strength of the electrostatic interaction, the total charge and the charge density of the carrier molecule. In liver tissue a large proportion of acid DNAses is located in the sinusoidal cells, a significant factor to be considered in the design of hepatotropic assemblies. Nuclease protection patterns in Cholgal and Cholglu lipoplexes were similar to those presented in Figs 5A and B (not shown).

Effect of lipoplexes on cell proliferation

To minimize possible perturbations arising from osmotic effects in toxicity assays and transfection experiments, lipoplexes were assembled in HEPES buffered saline, an environment in which the osmolality falls within the range found in MEM containing sodium bicarbonate or in mammalian blood. HepG2 and HeLa cells in 24-well plates were exposed to the same lipoplex concentration range chosen for in vitro transfection studies and for the same time. Growth inhibition effects in the two cell lines were very similar. At the highest liposome:DNA ratio of 7:1 (w/w) the four lipoplex series achieved a growth inhibition in the range 16–18% whilst at the optimal transfection ratio (5:1 w/w) growth inhibition ranged between 7 and 9% except in the case of the Cholglu lipoplex (14%) (Fig. C(A) and (B) in online supplement). Results suggest that the lipoplexes are well tolerated by both cell lines.

Transfection studies

Galactosylated PEGylated polyplexes transfect hepatoma cells with a tenfold higher efficiency than corresponding glucosylated particles but show no difference in cell lines that lack the ASG-R. Interestingly, it has been reported that replacement of galactose by glucose in PEI/DNA complexes (5% galactose) abolished transfection in HepG2 cells. This trend was followed in the present study of the four glycosylated liposome formulations. Thus there was a fourfold increase in targeted transgene activity in HepG2 cells by the Cholβgal lipoplex (Fig. 6B) over its α-anomeric counterpart Cholgal (Fig. 6A) at a liposome:DNA ratio of 5:1 (w/w), corresponding to a (+/-) charge ratio of 1.1. Furthermore, there was a greater than eightfold increase in activity of the Cholβgal lipoplexes over both Cholβglu and Cholgal lipoplexes at the same charge ratios (Fig. 6C and D, respectively). The selectivity demonstrated by the ASG-R with respect to the glycoconjugates may be of practical significance in liver diseases where the asialoglycoprotein receptor is overexpressed. To summarise, all four lipoplexes, which promoted the highest levels of transgene activity, were least affected by serum.

Conclusion

Stable galactosylated and glucosylated cationic liposomes have been prepared from the α- and β-anomers of the cholesteryl-D-galactopyranosides, the cationic cytofectin MS09 and the neutral co-lipid DOPE in a 1:4:4 mole ratio. The asialoglycoprotein receptor on the hepatocyte-like cell line HepG2 showed a clear anomeric preference for lipoplexes incorporating the β-anomer of cholesteryl-D-galactopyranoside (Cholβgal) as measured by the level of transgene activity. Moreover, the hydroxyl group orientation at the C-4 position of the pyranosides strongly influenced levels of transfection. The marked epimeric preference of the lectin for the galacto-
pyranosides over the glucopyranosides was evident even in the absence of a spacer between the carbohydrate moieties and the membrane-anchored cholosteryl component. These results comprehensively indicate that the Cholgal lipoplexes are suitable candidates for further development.

Supplementary material is available in the online version of this paper at www.sajs.co.za

This work was supported, in part, by a University of Durban-Westville research grant. M.S. held a USAID staff development grant.

This work was supported, in part, by a University of Durban-Westville research grant. M.S. held a USAID staff development grant.

Received 23 March. Accepted 22 June 2007.


Fig. 6. Transfection studies of glycosylated cationic liposome-DNA lipoplexes in HepG2 and HeLa cell lines. Experiments were conducted with semi-confluent cells in 24-well plates. Cells were treated with lipoplexes comprising 1 µg pGL3 DNA and varying amounts of liposomes (0.0, 4.0, 5.0, 6.0, 7.0 µg) in 0.5 ml minimum essential medium for four hours. Thereafter, medium was replaced with complete medium. Competition assays in the presence of 300 µg asialofetuin were conducted in the HepG2 cell line. In addition, transfections were conducted in HeLa cells, which are ASG-R negative. The effect of 10% FBS on transfection levels in HepG2 cells was also examined. Control 1 contained only HepG2 cells, while Control 2 contained HepG2 cells and naked pGL3 DNA. (A) Cholgal; (B) Cholgal; (C) Cheloglu; (D) Chologlu. Data are presented as means ± s.d. (n = 4).


Spectral data
Cholesteryl-β-D-tetra-O-acetylglucopyranoside. M.p. 157–159°C; 1H NMR (300 MHz, CDCl₃): δ 0.65 (s, 3H, C-CH₃), 0.83 (d, 6H, J = 6.6 Hz, CH₂CH₃), 0.89 (d, 3H, J = 6.5, CH-CH₂), 0.96 (s, 3H, C-CH₃), 1.96–2.12 (12H, 4 × CO-CH₃), 3.47 (m, 1H, Chol-H₄), 3.86 (t, J = 7.0, H-5'), 4.05–4.19 (m, 2H, H-6'a, H-6'b), 4.52 (dd, 1H, J = 10.4, 3.4 Hz, H-3'), 5.16 (dd, 1H, J = 10.4, 7.9 Hz, H-2'), 5.35 (m, 2H, 4H, Chol-H). ¹³C NMR (75 MHz, CDCl₃): sugar region: δ 100.29 (C-1'), 69.07 (C-2'), 70.75 (C-3'), 67.03 (C-4'), 71.02 (C-5'), 61.30 (C-6'). 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-23), 28.32 (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').

Cholesteryl-α-D-tetra-O-acetylglucopyranoside. M.p. 194–197°C; ¹H NMR (300 MHz, CDCl₃): δ 0.65 (s, 3H, C-CH₃), 0.84 (d, 6H, J = 6.6 Hz, CH₂CH₃), 0.89 (d, 3H, J = 6.5, CH-CH₂), 0.98 (s, 3H, C-CH₃), 1.97–2.12 (12H, 4 × CO-CH₃), 3.43 (m, 1H, Chol-H₄), 4.07 (t, 2H, J = 7.0, 5.4, H-6'a, H-6'b), 4.32 (m, 1H, H-5'), 5.03 (t, 1H, J = 9.6, H-4'). ¹³C NMR (75 MHz, CDCl₃): sugar region: δ 99.64 (C-1'), 71.68 (C-2'), 72.90 (C-3'), 68.50 (C-4'), 71.47 (C-5'), 62.09 (C-6'). Cholesteryl moiety: δ 11.85 (C-18), 18.72 (C-21), 19.36 (C-19), 21.04 (C-11), 22.57 (C-26), 22.83 (C-27), 24.28 (C-15), 28.23 (C-16), 29.44 (C-23), 28.02 (C-25), 31.85 (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), 38.91 (C-4), 42.32 (C-13), 50.14 (C-9), 56.74 (C-14), 56.14 (C-17), 80.09 (C-3'), 122.18 (C-6), 140.34 (C-5').

Cholesteryl-β-D-tetra-O-acetylgalactopyranoside. M.p. 201–203°C (See et al.² reported 202–204°C); ¹H NMR (300 MHz, CDCl₃): δ 0.65 (s,3H,C-CH₃), 0.84 (d, 6H, J = 6.5 Hz, CH₂CH₃), 0.89 (d, 3H, J = 6.5, CH-CH₂), 0.96 (s, 3H, C-CH₃), 1.99–2.06 (12H, 4 × CO-CH₃), 3.43 (m, 1H, Chol-H₄), 4.06-4.21 (m, 2H, H-6'a, H-6'b), 5.32 (m, 1H, H-1'), 4.93 (m, 1H, J = 9.5, 8.0 Hz, H-3'), 4.57 (m, J = 7.9 Hz, H-1'), 5.18 (t, 1H, J = 9.4, H-2'), 5.33 (m, 1H, J = 5.0, Chol-H), 5.05 (t, 1H, J = 9.6, H-4'). ¹³C NMR (75 MHz, CDCl₃): sugar region: δ 104.29 (C-1'), 72.79 (C-2'), 70.22 (C-3'), 68.49 (C-4'), 71.47 (C-5'), 62.09 (C-6'). Cholesteryl moiety: δ 11.85 (C-18), 18.72 (C-21), 19.36 (C-19), 21.04 (C-11), 22.57 (C-26), 22.83 (C-27), 24.28 (C-15), 28.23 (C-16), 29.44 (C-23), 28.02 (C-25), 31.85 (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), 38.91 (C-4), 42.32 (C-13), 50.14 (C-9), 56.74 (C-14), 56.14 (C-17), 80.09 (C-3'), 122.18 (C-6), 140.34 (C-5').

Supplementary material to:
Fig. A. Transmission electron micrographs of unilamellar liposomes prepared from MS09, DOPE and cholesteryl glycosides in 4:4:1 molar ratio. (a) Chol/c34gal; (b) Chol/c36gal; (c) Chol/c34glu; (d) Chol/c36glu. Bar = 100 nm.
Fig. B. Transmission electron micrographs of lipoplexes formed between glycosylated cationic liposomes and pGL3 DNA. (a) and (b) Chol gal; (c) and (d) Chol gal; (e) and (f) Chol glu; (g) and (h) Chol glu. Liposome:DNA ratios were set at 5:1 (w/w) (a), (c), (e) and (g) or 6:1 (w/w) (b), (d), (f) and (h). Bar = 50 nm.
Fig. C. Growth inhibition assays. Semi-confluent cells in 24-well plates were treated with lipoplexes in which glycosylated cationic liposomes were varied in quantity (4.0, 5.0, 6.0, 7.0 µg/well) while pGL3 was kept constant (1 µg/well) in a total volume of 0.5 ml of minimum essential medium. Incubation for the first four hours was in the absence of FBS. Thereafter, the medium was replaced by complete medium. Control wells (no liposomes or DNA) were assumed to show 100% survival. (a) HepG2 cells; (b) HeLa cells. Data are presented as means ± s.d. (n = 4).