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Synthesis of Galactosyl Compounds for Targeted Gene Delivery

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Abstract—Cell-specific DNA delivery offers a great potential for targeted gene therapy. Toward this end, we have synthesized a series of compounds carrying galactose residues as a targeting ligand for asialoglycoprotein receptors of hepatocytes and primary amine groups as a functional domain for DNA binding. Biological activity of these galactosyl compounds in DNA delivery was evaluated in HepG2 and BL-6 cells and compared with respect to the number of galactose residues as well as primary amine groups in each molecule. Transfection experiments using a firefly luciferase gene as a reporter revealed that compounds with multivalent binding properties were more active in DNA delivery. An optimal transfection activity in HepG2 cells requires seven primary amine groups and a minimum of two galactose residues in each molecule. The transfection activity of compounds carrying multi-galactose residues can be inhibited by asialofetuin, a natural substrate for asialoglycoprotein receptors of hepatocytes, suggesting that gene transfer by these galactosyl compounds is asialoglycoprotein receptor-mediated. These results provide direct evidence in support of our new strategy for the use of small and synthetic compounds for cell specific and targeted gene delivery.

Introduction

It is a well known biological process that the hepatic asialoglycoprotein receptor could recognize and bind to β-D-galactoside terminated glycoproteins.[1] Previous efforts in understanding this recognition event using synthetic glycopeptides revealed that the binding affinity of these glycopeptides for asialoglycoprotein receptor is highly dependent on the valency of Gal/GalNAc, as well as on the three-dimensional arrangement of the galactoside residues.[2] Recognition of the importance of multivalency of carbohydrate in carbohydrate–protein interaction has led to the successful synthesis of galactosyl ligand for this receptor.[3]

Recently, asialoglycoprotein receptor has been studied as a target for gene delivery into hepatocytes.[4] Research in this area was pioneered by Wu and Wu, who first demonstrated that DNA containing a reporter gene could be delivered to hepatocytes through a receptor-mediated endocytosis by using an asialoorosomucoid-poly-L-lysine conjugate as a DNA carrier.[5] In their design, the cationic poly-L-lysine portion condenses negatively charged DNA through charge–charge interactions and the asialoorosomucoid moiety recognizes the receptor on hepatocytes. Thus, through a receptor-mediated endocytosis, the reporter gene was delivered into the hepatocytes. However, the preparation of this multi-component conjugate was proven to be technically difficult and requires sophisticated procedure,[6] thereby hampering its application for targeted gene delivery.[7] To simplify the preparation of asialoorosomucoid-poly-L-lysine conjugate, Merwin et al. have used synthetic galactoside ligand YEE(GalNacAH)$_3$ in place of asialoorosomucoid for covalent conjugation to poly-L-lysine.[8] This more recent work has stimulated intensive synthetic efforts, including the synthesis of several galactose containing polymers and peptides.[9]

Additional approaches for gene delivery to hepatocytes include recent work of Behr,[10] who showed that a targeted gene delivery could be achieved when an amphiphile bearing galactosyl ligand was used in the preparation of DNA–lipospermine complexes. In this event, lipospermine serving as the DNA binding domain and neo-galactolipid acting as the cell surface targeting ligand were designed on two separated components. In a rare approach, DNA intercalating agent bisacridine was coupled with galactose ligand to yield a targeting vector.[11] The weak binding and thermal instability of DNA–target ligand complexes, however, render this approach impractical for targeted gene delivery, and therefore, leaving room for improvement.

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We are interested in using small cationic molecules for targeted gene delivery into hepatocytes. Our previous work demonstrated that cationic lipids could effectively bind with DNA and deliver reporter gene into different cell lines in a nonspecific manner.[12] For targeted gene delivery, we extended our initial study by conjugating galactoside residue at the hydrophobic terminal of a quaternary ammonium lipid. Unfortunately, the resulted galactosides linked aromatic ring-based quaternary ammonium compound **1** (Chart 1) gave a poor binding with DNA,[13] presumably due to the influence of hydrophilic galactoside ligands, which dramatically decrease the hydrophobic interaction of the aromatic ring-based cationic lipids. A similar phenomenon was also observed when hydrophilic poly(ethylene glycols) (PEG) modified quaternary ammonium compounds **2** and **3** were used for DNA delivery (Chart 1). Compounds **2** and **3** gave a low transfection activity.[14] To overcome this problem, we reasoned that a multi-cationic group may be essential for DNA binding. Toward this end, the mono quaternary ammonium head group has been substituted by DAB-dendr–$(NH_2)_8$ in compound **Tri-Gal**[15] (Chart 1). The multi-primary amines on the out-rim of a dendrimer, positively charged at the neutral pH, indeed improved binding to the phosphate group on the DNA back bone by electrostatic interactions and formed thermal stable complexes in the presence of clustered galactosyl ligand. The promising in vitro transfection activity on HepG2 cell line, a human hepatoma cell line, inspired us to extend this strategy to the synthesis of structurally diverse compounds with mono- and di-galactose ligand. Furthermore, combined with our previously reported compound **Tri-Gal** (Chart 1), we evaluated the transfection activity of these three types of compounds in a control cell line (BL-6, murine melanoma cells) and HepG2 cells.

Chemistry on DNA binding domain

The impetus to synthesis of compounds **7**, **8**, and **9** is to search for the minimum number of primary amines that could effectively bind with DNA in the presence of galactose residue on a galactosyl compound. Treatment of the known α-galactose trichloroacetimidate **4**[16] with primary alcohol in the presence of AgOTf[17] gave the corresponding β-glycoside derivative **5** in 90% yield (Scheme 1). Desilylation of **5** with tetrabutylammonium fluoride by a standard method released the primary alcohol, which was activated with 1,1′-carbonyldiimidazole (CDI)[18] under 4-(dimethylamino)pyridine (DMAP)[19] to afford the imidazole carboxylic ester **6**. Compound **6** was treated with excess amounts of tris(2-aminoethyl)amine to give the corresponding carbamate. The excess amounts of tris(2-aminoethyl)amine and the byproduct imidazole were removed by workup with 5% potassium hydroxide aqueous solution. Deprotection of peracetate in the carbamate was achieved under Zemplén conditions while leaving the carbamate bond intact. Neutralization with acidic resins followed by concentration, dialysis, and lyophilization gave the final compound **7**. Similarly, treatment of the imidazole carboxylic ester **6** with excess amount of DAB-dendr–$(NH_2)_4$ or DAB-dendr–$(NH_2)_8$ followed by deprotection of acetate yielded cationic compounds **8** and **9**, respectively.

With these cationic compounds in hand, the transfection activity was tested in BL-6 cells. We learned from previous work that the existence of the hydrophilic galactose or PEG moiety (Chart 1) in the compounds decreases their binding to DNA and results in a low transfection activity. Herein, through adjusting the number of primary amine groups, we expected an

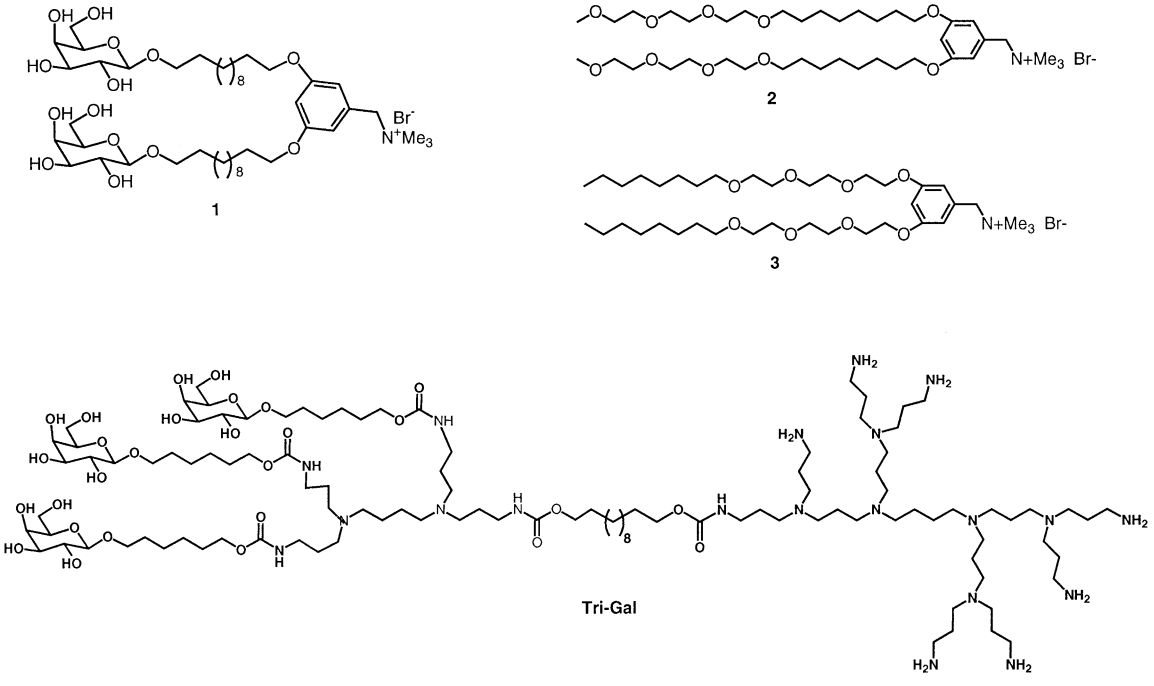
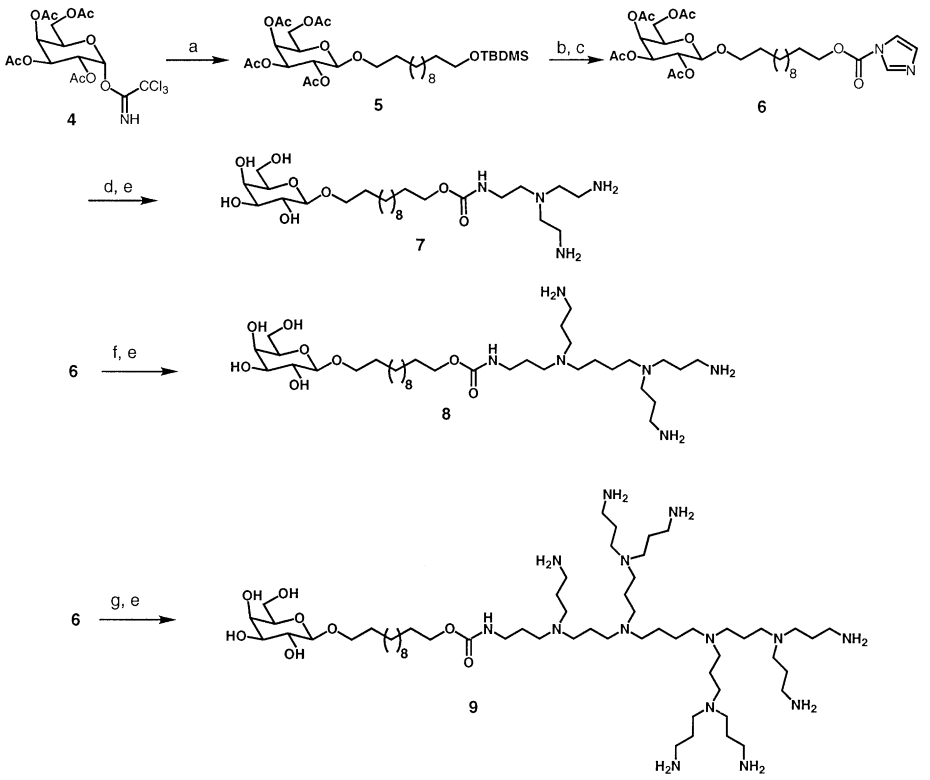


Chart 1.



Scheme 1. Reactions and conditions: (a) $HO(CH_2)_{12}OTBDMS$ (0.5 equiv), 4 Å MS, AgOTf (0.25 equiv), 0 °C, 30 min; then rt, 2 h, 90%; (b) Bu_4NF (1 equiv), THF, rt, 12 h, 100%; (c) CDI (1.25 equiv), DMAP (0.2 equiv), dry CH_2Cl_2, rt, 30 min, 90%; (d) $N(CH_2CH_2NH_2)_3$ (4 equiv), dry THF, reflux, 2 h; (e) 0.04 M NaOMe–MeOH, rt, 1 h; then Dowex 50WX4-100; (f) DAB-dendr–$(NH_2)_4$ (4 equiv), dry THF, reflux, 1.5 h; (g) DAB-dendr–$(NH_2)_8$ (4 equiv), dry THF, reflux, 1.5 h. CDI = 1,1′-carbonyldiimidazoe.

increased binding affinity. Indeed, while compounds **7** and **8** could not effectively bind with DNA and therefore exhibited a low transfection activity in BL-6 cells, compound **9** with 7 primary amine groups could effectively bind with DNA and showed a much higher transfection activity in the same type of cells.

Being successful in synthesizing compound **9** bearing cationic polyamine which could effectively bind with DNA and transfect BL-6 cells, we turned our attention to the galactose residues of the compounds to mimic structural ensembles present in asialoglycoprotein. Here we manipulated the chemistry to prepare the galactosyl vector bearing mono- or di-galactose residue.

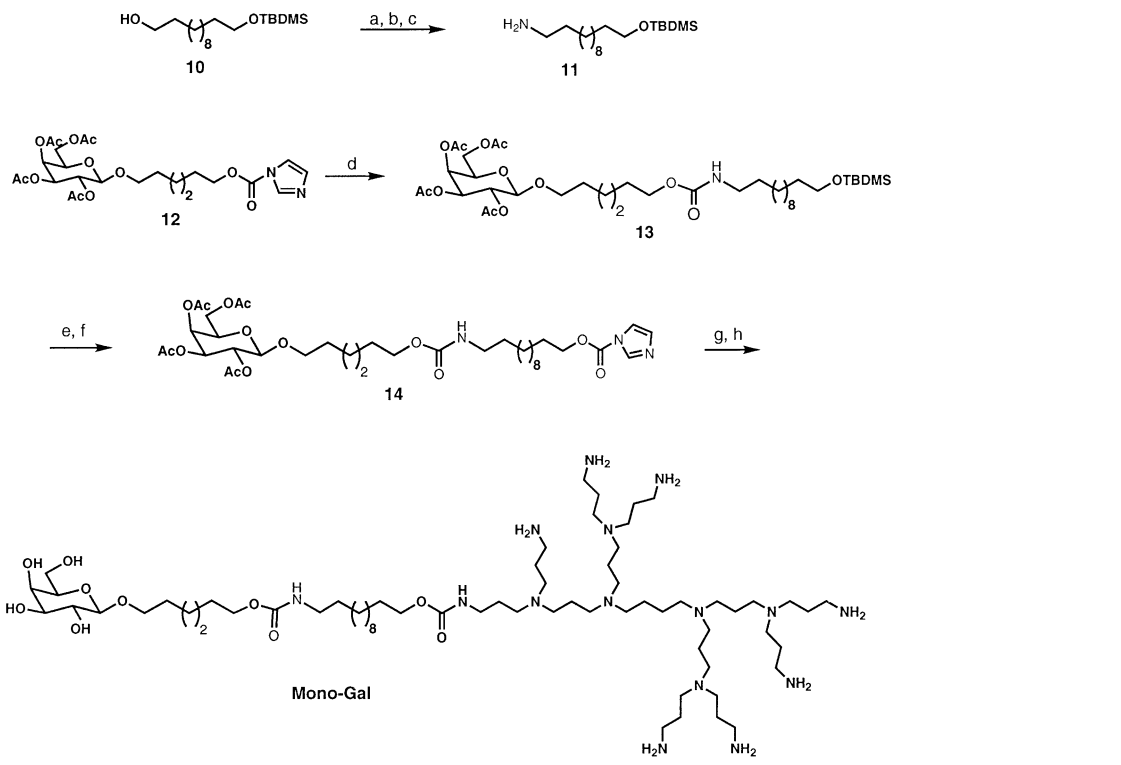
Synthesis of compounds Mono-Gal and Di-Gal

Compound **Mono-Gal** was synthesized according to Scheme 2. Treatment of the TBDMS protected primary alcohol **10** with Appel regent afforded the bromide. Azidation and Staudinger reduction[20] yielded the primary amine **11,** which was treated with our known imidazole carboxylic acid ester **12**[15] to generate the carbamate **13**. Removal of the TBDMS protection group under TBAF released the primary alcohol, which was further activated with CDI chemistry. The resulted imidazole carboxylic acid ester **14** was treated with excess amounts of DAB-dendr–$(NH_2)_8$ to form the final carbamate bond. After deprotection and neutralization, the reaction residues were dialyzed against water in a membrane with the molecular weight cutoff 500. Finally, lyophilization of the dialyzed compound afforded the desired product **Mono-Gal** in ca. 59% yield for the last two steps.

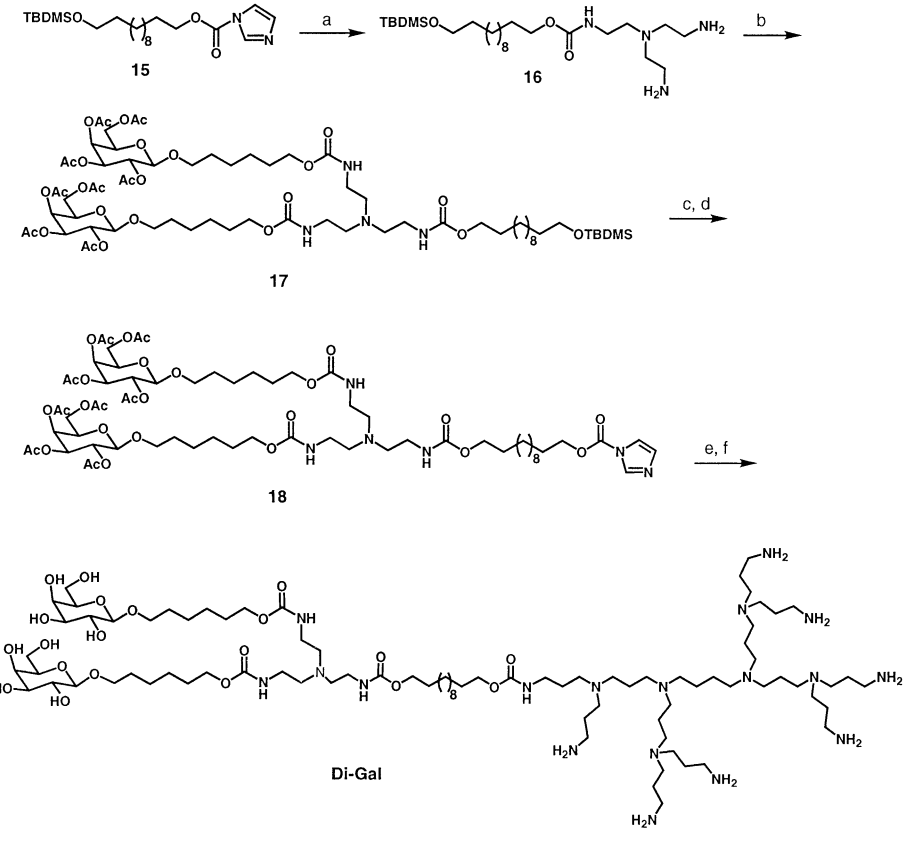
Compound **Di-Gal** was synthesized according to synthetic approach in Scheme 3. Treatment of our known imidazolide **15**[15] with excess amounts of tris(2-aminoethyl)amine afforded the carbamate **16**. The primary amine groups in carbamate **16** were expected to react with the known terminally activated galactoside compound **12** via a S_N2 manner. Indeed, when compound **16** was reacted with an excess amount of imidazolide **12** in refluxing THF for 3 h, the clustered disaccharide derivative **17** was obtained in 69% separated yield. Exposure of clustered disaccharide silyl ether **17** to tetrabutylammonium fluoride in THF slowly liberated the primary alcohol, which was further activated with CDI to give the imidazole carboxylic ester **18**. Treatment of **18** with excess amounts of DAB-dendr–$(NH_2)_8$ furnished the construction of the final carbamate bond. After deprotection of peracetate and neutralization, the reaction residues were dialyzed against water in a membrane with molecular weight cutoff 1000. Finally, lyophilization of the dialyzed compound afforded the desired product **Di-Gal** in ca. 70% yield for the last two steps.

Biological assay

Our synthetic galactosyl compounds **Mono-Gal**, **Di-Gal**, and **Tri-Gal** were tested for their transfection ability in



Scheme 2. Reactions and conditions: (a) CBr_4PPh_3 (1.25 equiv), rt, 30 min; (b) NaN_3 (5 equiv), CH_3CN–H_2O (v/v = 9:1), reflux, 12 h; (c) PPh_3 (2 equiv), THF–H_2O (v/v = 9:1), rt, 12 h; then reflux, 30 min, 69% for three steps; (d) compound **11** (1.5 equiv), THF–Et_3N, reflux, 3 h, 73%; (e) Bu_4NF (1.25 equiv), THF, rt, 12 h, 90%; (f) CDI (1.25 equiv), DMAP (0.2 equiv), dry CH_2Cl_2, rt, 45 min, 89%; (g) DAB-dendr–$(NH_2)_8$ (4 equiv), dry THF, reflux, rt, 1.5 h, 75%; (h) 0.04 M NaOMe–MeOH, rt, 1 h; then Dowex 50WX4-100 ca. 78%.



Scheme 3. Reactions and conditions: (a) $N(CH_2CH_2NH_2)_3$ (3.25 equiv), DMAP (1 equiv), dry MeCN, rt, 3 h, 88%; (b) compound **12** (2.2 equiv), THF–Et_3N, reflux, 3 h, 69%; (c) Bu_4NF (1.4 equiv), THF, rt, 12 h, 82%; (d) CDI (1.7 equiv), DMAP (0.3 equiv), dry CH_2Cl_2, rt, 1 h, 87%; (e) DAB-dendr–$(NH_2)_8$ (4 equiv), dry THF, reflux, 1.5 h; (f) 0.04 M NaOMe–MeOH, rt, 1 h; then Dowex 50WX4-100 ca. 70% for the last two steps.

BL-6 and HepG2 cells. Data in Table 1 showed that the transfection activity of these compounds was independent of galactose residues in BL-6 cells. Under our experimental conditions, a similar transfection activity was seen for all the three compounds. However, a different transfection activity pattern was obtained in HepG2 cells. Data in Table 2 clearly show that with an increase of the number of galactose residues, the transfection activity was increased. Namely, for compound **Di-Gal** and **Tri-Gal**, the transfection activity was about twenty times higher than that of compound **Mono-Gal**.

To confirm that the high transfection activity seen in HepG2 cells is indeed mediated by galactose residues and asialoglycoprotein receptors, we carried out an inhibition assay using asialofetuin, a neuraminidase-treated serum protein from Sigma. Asialofetuin has a molecular weight of 48,000 and contains ca. 12.4 galactosyl residues per protein molecule.[21] For inhibition assay, HepG2 cells were pre-incubated with asialofetuin before the transfection with galactosyl compound/DNA complexes. The transfection activity of the compound **Di-Gal** and **Tri-Gal** decreased in the presence of 100 equivalent of galactosyl residues of asialofetuin (Fig. 1). Asialofetuin does not seem to interfere with the transfection activity of compound **Mono-Gal** (data not shown).

Table 1. Transfection activity of galactosyl compounds on BL-6 cells[a]

Amounts of galactosyl compounds used (nmol)	Level of luciferase gene expression (pg/mg protein)		
	Mono-Gal	**Di-Gal**	**Tri-Gal**
5	2.4×10^4	4.9×10^4	7.4×10^4
7.5	2.5×10^4	9.5×10^4	9.5×10^4
10	2.4×10^4	1.2×10^5	7.4×10^4

[a]BL-6 cells (5×10^4 cells per well) were seeded in a 48-well plate 24 h before the addition of DNA/galactosyl compound complexes. Each well received freshly prepared complexes containing 1 μg of pCMV-Luc plasmid DNA and different amounts of galactosyl compounds. Transfection proceeded for 5 h in serum free medium. Gene expression was analyzed 48 h after transfection. Data represent mean ($n=3$).

Table 2. Transfection activity of galactosyl compounds on HepG2 cells[a]

Amounts of galactosyl compounds used (nmol)	Level of luciferase gene expression (pg/mg protein)		
	Mono-Gal	**Di-Gal**	**Tri-Gal**
5	1.2×10^2	3.7×10^4	6.5×10^4
7.5	4.9×10^2	2.0×10^4	5.5×10^4
10	9.3×10^2	2.1×10^5	5.0×10^4

[a]HepG2 cells (5×10^4 cells per well) were seeded in a 48-well plate 24 h before the addition of DNA/galactosyl compound complexes. Each well received freshly prepared complexes containing 1 μg of pCMV-Luc plasmid DNA and different amounts of galactosyl compounds. Transfection proceeded for 5 h in serum free medium. Gene expression was analyzed 48 h after transfection. Data represent mean ($n=3$).

Results and conclusions

For an efficient gene delivery, the carrier compounds need to protect the DNA against nuclease-based degradation and deliver gene to target cells. To achieve this goal, here we present our strategy for synthesis of bifunctional compounds with one end capable of binding to DNA and the other capable of binding to the target cells. We synthesized a series of compounds with increasing number of primary amines to achieve an effectively binding with DNA. At a later stage, we varied the galactose residues on the vector to reach a multivalent binding effect for the asialoglycoprotein receptor on hepatocytes. The two functional domains were conjugated through a carbamate bond on a hydrocarbon chain, which is expected to provide a sheath around the DNA in complexes.

Results from transfection experiments showed that compounds **Mon-Gal**, **Di-Gal**, and **Tri-Gal** have a similar transfection activity in BL-6 cells, a cell line of murine melanoma that does not have asialoglycoprotein receptors, suggesting that these compounds transfect BL-6 cells in a nonspecific manner. For HepG2 cells, however, the effect of structural variation of these compounds was reflected. For example, in contrast to a good transfection activity of compound **Mono-Gal** in BL-6 cells, the level of luciferase gene expression in HepG2 cells was approximately two orders of magnitude lower, indicating that HepG2 cells are not as sensitive as BL-6 cells to compound **Mono-Gal** based transfection. As expected, HepG2 cells transfected with compounds **Di-Gal** or **Tri-Gal** exhibited a much higher level of luciferase gene expression. As the difference between **Mono-Gal, Di-Gal** and **Tri-Gal** is the number

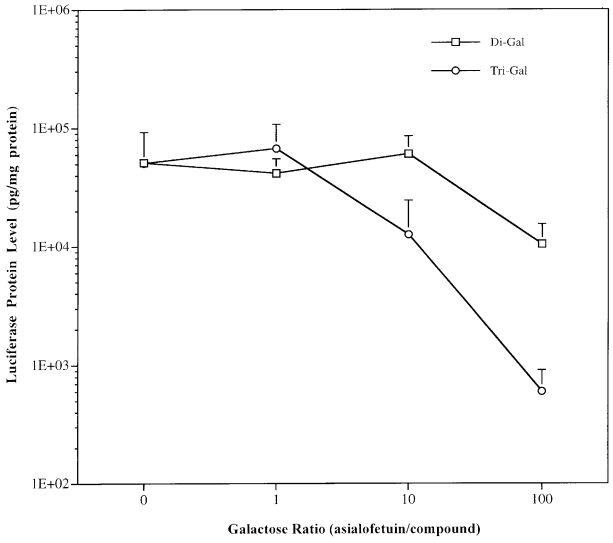


Figure 1. Effect of asialofetuin on the transfection activity of DNA/Di-Gal and DNA/Tri-Gal complexes in HepG2 cells. HepG2 cells (5×10^4 cells per well) were seeded in a 48-well plate 24 h before the addition of desired amounts of asialofetuin protein. Cells were incubated with asialofetuin for 5 h and then received freshly prepared complexes containing 1 μg of pCMV-Luc plasmid and 5 nmol of galactosyl compounds. Transfection proceeded for 5 h in serum free medium. Gene expression was analyzed 48 h after transfection. Data represent means ± SD ($n=3$).

of galactosyl residue in each molecule, a higher level of luciferase gene expression in HepG2 cells transfected by compounds **Di-Gal** and **Tri-Gal** would suggest that the multivalent galactosyl residue enhanced their transfection activity. The effective inhibition of **Di-Gal** and **Tri-Gal** transfection activity by asialofetuin, a natural substrate for asialoglycoprotein receptors, support the notion that transfection of HepG2 cells by compounds **Di-Gal** and **Tri-Gal** involves asialoglycoprotein receptors of HepG2 cells.

In summary, we have developed an efficient route toward the synthesis of bifunctional cationic compounds with DAB-dendr–$(NH_2)_8$ as the DNA binding domain and galactose ligand for binding to the asialoglycoprotein receptor. The transfection results suggest that the galactosyl compounds **Di-Gal** and **Tri-Gal** could effectively deliver DNA into HepG2 cells. Asialofetuin could effectively block the transfection process of DNA/**Tri-Gal** complexes, indicating that asialoglycoprotein receptor-mediated endocytosis is likely involved in the transfection. We hope that the strategy illustrated here is useful for the development of receptor based gene delivery.

Experimental

^{1}H NMR spectra were recorded on a Bruker AMX 300 spectrometer. Column chromatography was performed using silica gel 70–270 mesh. Thin-layer chromatography (TLC) was performed with F254 plates. Compounds on chromatography plates were visualized by spraying 5% ethanolic sulfuric acid, 4% phosphomolybdic acid in ethanol, or 3% ninhydrin in ethanol by charring on hot plates. Unless otherwise stated, all materials were obtained from commercial suppliers and were used without further purification. DAB-dentr–$(NH_2)_4$ and DAB-dendr–$(NH_2)_8$ were purchased from Aldrich. Tetrahydrofuran was freshly distilled from sodium benzophenone ketyl under argon prior to use. Dichloride methylene and acetonitrile was refluxed in CaH_2 before use. For preparation of 0.04 M NaOMe Methanolic solutions, dry methanol was freshly distilled from Mg–iodine under argon and immediately treated with desired amount of sodium metal under anhydrous conditions.

Synthesis of galactosyl compound Mono-Gal

Compound 11. To a solution of 50 mL of dry CH_2Cl_2 containing 3.16 g (10 mmol) of compound **10** was added 3.30 g (12.5 mmol) of Ph_3P and 4.25 g (12.5 mmol) of CBr_4 at 0 °C. The reaction mixture was stirred at room temperature for 30 min. After removal of CH_2Cl_2 under vacuum, the reaction residues were diluted with 150 mL of hexanes and stirred at room temperature for 30 min. After filtration, the filtrate was condensed under vacuum. To the resulted residues were added 3.25 g (50 mmol) of NaN_3, 45 mL of MeCN, and 5 mL of H_2O. The reaction mixture was refluxed under nitrogen for 12 h. After condensation under vacuum, the residues were diluted with 150 mL of ethyl acetate and washed with 2×50 mL of saturated NaCl aqueous solution. The organic layer was dried over Na_2SO_4, filtered, and concentrated. The final residues were purified by column chromatography (ethyl acetate/hexanes, 1/10) to afford 3 g (8.8 mmol) of azide compound in a total yield of 88% for the two steps. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.60 (t, J = 6.6 Hz, 2H, CH_2OTBDMS), 3.26 (t, J = 6.8 Hz, 2H, CH_2N_3), 1.60–1.20 (m, 20H, 10×CH_2), 0.90 (s, 9H, *t*-Bu), 0.05 (s, 6H, 2×Me). To a solution of 3 g (8.8 mmol) of above azide compound in 50 mL of THF/H_2O (v/v = 9:1) was added 2.62 g (10 mmol) of Ph_3P. The reaction mixture was stirred at room temperature for 12 h, then refluxed for 0.5 h. The reaction mixture was diluted with 150 mL of chloroform and washed with 2×50 mL of saturated NaCl aqueous solution. The organic layer was dried over Na_2SO_4, filtered, and concentrated. The residues were further purified by column chromatography (ethyl acetate/hexanes, 1:1, then chloroform/methanol/triethylamine, 10:1:1) to afford 2.2 g (7.0 mmol) of primary amine **11** in a yield of 79%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.69 (t, J = 6.6 Hz, 2H, CH_2OTBDMS), 2.69 (t, J = 6.9 Hz, 2H, CH_2NH_2), 1.6–1.2 (m, 22H, 10CH_2 and NH_2), 0.90 (s, 9H, *t*-Bu), 0.05 (s, 6H, 2×Me).

Compound 13. A mixture of 650 mg (1.2 mmol) of imidazole carboxylic acid ester **12**, 570 mg (1.8 mmol) of primary amine **11**, and 0.2 mL of triethylamine was dissolved in 10 mL of dry THF. The resulted reaction mixture was refluxed under nitrogen for 3 h, and then diluted with 100 mL of ethyl acetate. The organic layer was washed with 2×25 mL of 5% of KOH aqueous solution to remove the byproduct imidazole, and then washed with 50 mL of saturated NaCl aqueous solution. The organic layer was dried over Na_2SO_4, filtered, and concentrated. The residues were purified by column chromatography (ethyl acetate/hexanes, 1:1) to afford 701 mg (0.88 mmol) of compound **13** in a yield of 73%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.39 (d, J = 3.1 Hz, 1H, H-4), 5.18 (dd, J = 10.5, 8.0 Hz, 1H, H-2), 5.02 (dd, J = 10.5, 3.4 Hz, 1H, H-3), 4.70 (brs, 1H, NHCO), 4.46 (d, J = 7.8 Hz, 1H, H-1), 4.17 (dd, J = 11.2, 6.8 Hz, 1H, H-6a), 4.12 (dd, J = 11.3, 6.9 Hz, 1H, H-6b), 4.04 (t, J = 6.1 Hz, 2H, CH_2OCO), 3.89 (m, 2H, H-5 and alkyl chain H-1′), 3.58 (t, J = 6.5 Hz, 2H, CH_2OTBDMS), 3.47 (m, 1H, alkyl chain H-1′), 3.15 (m, 2H, CH_2NHCO_2), 2.15 (s, 3H, Ac), 2.05 (s, 6H, 2×Ac), 1.99 (s, 3H, Ac), 1.60–145 (m, 8H, 4×CH_2), 1.27 (m, 20H, 10×CH_2), 0.89 (s, 9H, *t*-Bu), 0.04 (s, 6H, 2×Me).

Compound 14. To a solution of 701 mg (0.88 mmol) of compound **13** in 7 mL of THF was added 1.1 mL of tetrabutylammonium fluoride solution (1.0 M in THF) at 0 °C. The resulted mixture was stirred at room temperature overnight, then diluted with 50 mL of ethyl acetate. The organic layer was washed with 25 mL of saturated NaCl aqueous solution, dried over Na_2SO_4, filtered, and concentrated. The residues were purified by column chromatography (ethyl acetate/hexanes, 3:1) to afford 540 mg (0.79 mmol) of primary alcohol in a yield of 90%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.39 (d, J = 3.3

Hz, 1H, H-4), 5.18 (dd, $J=10.5$, 8.0 Hz, 1H, H-2), 5.01 (dd, $J=10.5$, 3.4 Hz, 1H, H-3), 4.70 (brs, 1H, NHCO), 4.45 (d, $J=7.9$ Hz, 1H, H-1), 4.17 (dd, $J=11.2$, 6.5 Hz, 1H, H-6a), 4.12 (dd, $J=11.3$, 6.9 Hz, 1H, H-6b), 4.04 (t, $J=6.1$ Hz, 2H, CH_2OCO), 3.89 (m, 2H, H-5 and alkyl chain H-1), 3.64 (t, $J=6.5$ Hz, 2H, CH_2OH), 3.48 (m, 1H, alkyl chain H-1′), 3.15 (t, $J=6.3$ Hz, 2H, CH_2NHCO_2), 2.15 (s, 3H, Ac), 2.05 (s, 6H, 2×Ac), 1.99 (s, 3H, Ac), 1.70–1.20 (m, 28H, 14×CH_2). To a solution of 540 mg (0.79 mmol) of above primary alcohol in 5 mL of dry CH_2Cl_2 was added 162 mg (1.0 mmol) of CDI and 20 mg (0.16 mmol) of DMAP. The reaction mixture was stirred at room temperature under nitrogen for 45 min, then added 10 mg of SiO_2 to decompose the excess amounts of CDI. After filtration, the filtrate was diluted with 50 mL of ethyl acetate and washed with 2×15 mL of 5% KOH aqueous solution and 25 mL of saturated NaCl aqueous solution. The organic layer was dried over Na_2SO_4, filtered, and concentrated. The residues were purified by column chromatography (ethyl acetate/hexanes, 3:1) to afford 540 mg (0.70 mmol) of compound **14** in a yield of 89%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.13 (s, 1H, imidazole ring), 7.47 (s, 1H, imidazole ring), 7.06 (s, 1H, imidazole ring), 5.38 (d, $J=3.3$ Hz, 1H, H-4), 5.17 (dd, $J=10.5$, 8.0 Hz, 1H, H-2), 5.01 (dd, $J=10.5$, 3.4Hz, 1H, H-3), 4.65 (brs, 1H, NHCO), 4.44 (d, $J=7.9$ Hz, 1H, H-1), 4.40 (t, $J=6.7$ Hz, 2H, CH_2OCO), 4.17 (dd, $J=11.2$, 6.5 Hz, 1H, H-6a), 4.12 (dd, $J=11.3$, 6.9 Hz, 1H, H-6b), 4.02 (t, $J=6.1$ Hz, 2H, CH_2OCONH), 3.90 (m, 2H, H-5 and alkyl chain H-1′), 3.48 (m, 1H, alkyl chain H-1′), 3.14 (m, 2H, CH_2NHCO$_2$), 2.14 (s, 3H, Ac), 2.04 (s, 6H, 2×Ac), 1.98 (s, 3H, Ac), 1.78 (m, 2H, CH_2), 1.70–1.20 (m, 26H, 13×CH_2).

Compound Mono-Gal. A mixture of 70 mg (0.091 mmol) of compound **14** and 282 mg (0.36 mmol) of DAB-dendr–$(NH_2)_8$ in 5 mL of dry THF was refluxed for 1.5 h under nitrogen. The reaction mixture was diluted in 50 mL of chloroform and poured into separation funnel. The organic layer was washed with 15 mL of 5% KOH aqueous solution and stand over night for phase separation. The organic layer was dried over Na_2SO_4, filtered, and concentrated to give 100 mg (0.068 mmol) of residue in a yield of ca. 75%. ^{1}H (300 MHz, $CDCl_3$) δ 5.91 (bs, 2H, 2×NHCO), 5.39 (d, $J=3.2$ Hz, 1H, H-4), 5.17 (dd, $J=10.5$, 8 Hz, 1H, H-2), 5.02 (dd, $J=10.4$, 3.3 Hz, 2H, H-3), 4.45 (d, $J=8.0$ Hz, 1H, H-1), 4.19 (dd, $J=11.2$, 6.6 Hz, 1H, H-6a), 4.10 (dd, $J=11.2$, 6.9 Hz, 1H, H-6b), 4.02 (m, 4H, 2×CH_2OCONH), 3.90 (m, 2H, H-5 and alkyl chain H-1′), 3.45 (m, 1H, alkyl chain H-1′), 3.20 (m, 4H, 2×CH_2NHCO), 2.70 (t, $J=6.7$ Hz, 14H, 7×CH_2NH$_2$), 2.50–2.40 (m, 36H, 6×N$(CH_2)_3$), 2.14 (s, 3H, Ac), 2.05 (s, 6H, 2×Ac), 1.99 (s, 3H, Ac), 1.70–1.40 (m, 32H, 16CH_2), 1.40–1.20 (m, 38H, 10×CH_2 from alkyl chain, 2×CH_2 and 7×NH_2 from dendrimer) ppm. The above compound was dissolved in 10 mL of 0.04 M NaOMe methanol solution. The resulted mixture was stirred at room temperature for 1 h then neutralized with Dowex 50WX4-100 resins. After filtration and removal of methanol under vacuum, the residue was dissolved in distilled water and subjected to dialysis against water in a membrane with the molecular weight cut-off 500 (Spectrum Laboratories, Rancho Dominguez, CA, USA). Lyophilization of the dialyzed compound afforded 70 mg (0.053 mmol) of the desired product **Mono-Gal** in ca. 78% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 4.17 (d, $J=6.9$ Hz, 2H, H-1), 3.98 (m, 4H, 2×CH_2OCO), 3.90–3.80 (m, 2H, alkyl chain H-1′ and H-4), 3.70 (m, 2H, H-6a and H-6b), 3.54–3.45 (m, 4H, H-2, H-3, H-5 and alkyl chain H-1′), 3.15 (m, 4H, 2×CH_2NHCO), 2.75 (m. 14H, 7×CH_2NH$_2$), 2.45 (m, 36H, 6×N$(CH_2)_3$), 170–1.20 (m, 56H, 14×CH_2 from alkyl chain and 14×CH_2 from dendrimer).

Synthesis of galactosy compound Di-Gal

Compound 16. A mixture of 1.26 g (3.07 mmol) of compound **15**, 1.46 g (10 mmol) of tris(2-aminoethyl)amine, 366 mg (3 mmol) of DMAP in 20 mL of dry acetonitrile was stirred under nitrogen at room temperature for 3 h. The mixture was diluted with 150 mL of chloroform and successively washed with 2×50 mL of 5% KOH aqueous solution, 2×50 mL of saturated NaCl aqueous solution, dried over Na_2SO_4, filtered, and concentrated. DMAP was removed by column chromatography. The desired product on SiO_2 was released by treatment with methanol/triethylamine (v/v = 1:1) to afford 1.29 g of compound **16** in a yield of 88%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.71 (brs, 1H, NHCO), 4.03 (t, $J=6.6$ Hz, 2H, CH_2OCO), 3.60 (t, $J=6.6$ Hz, 2H, $CH_2OTBDMS$), 3.23 (m, 2H, CH_2NHCO), 2.76 (t, $J=6$ Hz, 4H, 2×CH_2NH$_2$), 2.57 (t, $J=6$ Hz, 2H, CH_2N), 2.53 (t, $J=6$ Hz, 4H, N$(CH_2)_2$), 1.65–1.40 (m, 8H, 2×NH_2 and 2CH_2), 1.26 (m, 16H, 8×CH_2).

Compound 17. To a mixture of 474 mg (1 mmol) of compound **16** and 1.2 g (2.2 mmol) of imidazole carboxylic acid ester **12** in 10 mL of dry THF was added 200 mg (2 mmol) of triethyl amine. The resultant mixture was refluxed for 3 h then diluted with 100 mL of chloroform and successively washed with 2×50 mL of 5% KOH aqueous solution, 2×50 mL of saturated NaCl aqueous solution, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography (ethyl acetate/hexane from 2:1 to 1:0) to afford 1 g (0.69 mmol) of compound **17** in a yield of 69%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.45 (b, 3H, 3NHCO), 5.36 (d, $J=3.2$ Hz, 2H, H-4), 5.15 (dd, $J=10.2$, 7.9 Hz, 2H, H-2), 4.99 (dd, $J=10.4$, 3.2 Hz, 2H, H-3), 4.43 (d, $J=8$ Hz, 2H, H-1), 4.11 (dd, $J=11.2$, 6.7 Hz, 2H, H-6a), 4.09 (dd, $J=11.2$, 6.7 Hz, 2H, H-6b), 4.00 (t, $J=6$ Hz, 6H, 3×CH_2OCO), 3.87 (m, 4H, H-5 and alkyl chain H-1′), 3.56 (t, $J=6.6$ Hz, 2H, $CH_2OTBDMS$), 3.44 (m, 2H, alkyl chain H-1′), 3.17 (m, 6H, 3×CH_2NHCO), 2.53 (t, $J=5.5$ Hz, 6H, N$(CH_2)_3$), 2.12 (s, 6H, 2×Ac), 2.02 (s, 12H, 4×Ac), 1.98 (s, 6H, 2×Ac), 1.65–1.40 (m, 12H, 6×CH_2), 1.40–1.20 (m, 24H, 12×CH_2), 0.86 (s, 9H, *t*-Bu), 0.01 (s, 6H, 2×Me).

Compound 18. To a solution of 1g (0.69 mmol) of compound **17** in 5 mL THF was added 1 mL (1 mmol) of 1.0 M tetrabutylammonium fluoride solution (THF). The reaction mixture was stirred at room temperature overnight, then diluted with 50 mL of ethyl acetate and

washed with 25 mL of saturated NaCl aqueous solution, dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography (chloroform/methanol, 16:1) to afford 750 mg (0.57 mmol) of primary alcohol in a yield of 82%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.39 (d, J = 3.3 Hz, 2H, H-4), 5.29 (b, 3H, 3×NHCO), 5.20 (dd, J = 10.5, 7.9 Hz, 2H, H-2), 5.02 (dd, J = 10.5, 3.3 Hz, 2H, H-3), 4.45 (d, J = 7.8 Hz, 2H, H-1), 4.20 (dd, J = 11.5, 6.6 Hz, 2H, H-6a), 4.09 (dd, J = 11.3, 6.9 Hz, 2H, H-6b), 4.04 (t, J = 6.6 Hz, 6H, 3×CH_2OCO), 3.90 (m, 4H, H-5 and alkyl chain H-1′), 3.64 (t, J = 6.6 Hz, 2H, CH_2OH), 3.46 (m, 2H, alkyl chain H-1′), 3.21 (m, 6H, 3CH_2NHCO), 2.58 (t, J = 5.6 Hz, 6H, N(CH_2)$_3$), 2.15 (s, 6H, 2×Ac), 2.05 (s, 12H, 4×Ac), 1.99 (s, 6H, 2×Ac), 1.60–1.50 (m, 12H, 6×CH_2), 1.40–1.20 (m, 24H, 12×CH_2). To a mixture of 400 mg (0.30 mmol) of above primary alcohol and 80 mg (0.5 mmol) of CDI in 5 mL of dry methylene dichloride was added 12 mg (0.1 mmol) of DMAP. The reaction mixture was stirred under nitrogen at room temperature for 1 h, then ca. 15 mg of silica gel was added to decompose the excess amount of 1,1′-carbonyldiimidazole. After filtration, the filtrate was diluted with 50 mL of chloroform and successively washed with 2×25 mL of 5% KOH in aqueous solution and 25 mL of NaCl saturated aqueous solution, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography (chloroform/methanol/triethylamine, 20:1:1) to afford 370 mg (0.26 mmol) of compound **18** in a yield of 87%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.10 (s, 1H, imidazole ring), 7.39 (s, 1H, imidazole ring), 7.03 (s, 1H, imidazole ring), 5.40 (b, 3H, 3×NHCO), 5.35 (d, J = 3.3 Hz, 2H, H-4), 5.15 (dd, J = 10.4, 7.9 Hz, 2H, H-2), 4.98 (dd, J = 10.5, 3.4 Hz, 2H, H-3), 4.43 (d, J = 7.9 Hz, 2H, H-1), 4.37 (t, J = 6.6 Hz, 2H, CH_2OCO), 4.20 (dd, J = 11.5, 6.9 Hz, 2H, H-6a), 4.09 (dd, J = 11.3, 6.9 Hz, 2H, H-6b), 4.00 (t, J = 6.6 Hz, 6H, 3×CH_2OCO), 3.88 (m, 4H, H-5 and alkyl chain H-1′), 3.43 (m, 2H, alkyl chain H-1′), 3.17 (m, 6H, 3×CH_2NHCO), 2.54 (m, 6H, N(CH_2)$_3$), 2.11 (s, 6H, 2×Ac), 2.01 (s, 12H, 4×Ac), 1.95 (s, 6H, 2×Ac), 1.70–1.40 (m, 12H, 6×CH_2), 1.40–1.20 (m, 24H, 12×CH_2).

Compound Di-Gal. A mixture of 370 mg (0.26 mmol) of compound **18** and 804 mg (1.04 mmol) of DAB-dendr–(NH_2)$_8$ in 5 mL of dry THF was refluxed under nitrogen for 1.5 h. The reaction mixture was diluted in 50 mL of chloroform and poured into separation funnel. The organic layer was washed with 20 mL of 5% KOH aqueous solution to remove the byproduct imidazole and the excess amounts of DAB-dendr–(NH_2)$_8$, and left over night for a better phase separation. The organic layer was dried over Na_2SO_4, filtered, and concentrated. The residue was dried under vacuum for overnight. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.50–5.30 (br, 4H, 4NHCO), 5.38 (d, J = 3.2 Hz, 2H, H-4), 5.19 (dd, J = 10.4, 8 Hz, 2H, H-2), 5.01 (dd, J = 10.4, 3.3 Hz, 2H, H-3), 4.45 (d, J = 7.9 Hz, 2H, H-1), 4.19 (dd, J = 11.2, 6.6 Hz, 2H, H-6a), 4.10 (dd, J = 11.2, 6.9 Hz, 2H, H-6b), 4.03 (t, J = 6.3 Hz, 8H, 4×CH_2OCO), 3.90 (m, 4H, H-5 and alkyl chain H-1′), 3.45 (m, 2H, alkyl chain H-1′), 3.19 (m, 8H, 4×CH_2NHCO), 2.71–2.50 (t, J = 6.6 Hz, 14H, 7×C$H_2$$NH_2$), 2.50–2.40 (m, 42H, 7×N(CH_2)$_3$), 2.14 (s, 6H, 2×Ac), 2.05 (s, 12H, 4×Ac), 1.99 (s, 6H, 2×Ac), 1.70–1.50 (m, 36H, 18×CH_2), 1.43 (m, 14H, 7×NH_2), 1.40–1.20 (m, 28H, 14×CH_2). The above reaction residue was dissolved in 15 mL of 0.04 M freshly prepared NaOMe methanol solutions. The reaction mixture was stirred at room temperature for 1 h then neutralized with Dowex 50WX4-100 resins. After filtration and removal of methanol under vacuum, the residues were dissolved in distilled water and subjected to dialysis (molecular weight cut-off 1000) for 24 h. Lyophilization of the dialyzed compound afforded 325 mg (0.18 mmol) of the desired product **Di-Gal** in ca. 70% yield for the last two steps. ^{1}H NMR (300 MHz, CD_3OD) δ 4.20 (d, J = 6.3 Hz, 2H, H-1), 4.02 (t, J = 5.6 Hz, 8H, 4×CH_2OCO), 3.91–3.84 (m, 4H, alkyl chain H-1′ and H-4), 3.73 (m, 4H, H-6a and H-6b), 3.55–3.47 (m, 8H, H-2, H-3, H-5 and alkyl chain H-1′), 3.15 (t, J = 6 Hz, 8H, 4×CH_2NHCO), 2.85 (m. 14H, 7×C$H_2$$NH_2$), 2.59 (m, 42H, 7×N($CH_2$)$_3$), 170–1.50 (m, 24H, 12×$CH_2$ from dendrimer), 1.40 (m, 16H, 2×CH_2 from dendrimer and 6×CH_2 from alkyl chain), 1.30 (24H, 12×CH_2 from alkyl chain).

Preparation of DNA/galactosyl compound complexes

Galactosyl compound was disolved in PBS (pH 7.4) at concentration of 5 mM. The hydration was proceeded for 30 min at room temperature and the suspension was then agitated for 2–3 min by vortexing. pCMV-Luc plasmid DNA containing firefly luciferase gene driven by a cytomegalovirus immediate early promoter (CMV) was diluted in serum free CHO-S-SFM medium to give a DNA concentration at 1 μg/125 μL. Galactosyl compound suspension (5 mM) was diluted with Hank's balanced salt solution (HBSS) to generate three different concentrations: 5 nmol/125 μL, 7.5 nmol/125 μL, and 10 nmol/125 μL. DNA complexes in appropriate ratios were prepared by mixing equal volume of diluted DNA solution and galactosyl compound suspension. The mixture (250 μL) was incubated for 5–10 min at room temperature before being added to cells.

Transfection

Murine melanoma BL-6 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS). Human HepG2 cells were cultured in DMEM medium with 10% FBS. For a standard transfection, cells (5×10^4 cells per well) were plated in a 48-well plate and allowed to grow for 24 h before the transfection. Two hundred fifty microliters of transfection reagents (DNA/galactosyl compound complexes) containing one μg of plasmid DNA and desired amounts of galactosyl compound were added to each well. Cells with transfection reagents were incubated for 5 h followed by addition of 27.5 μL of FBS to each well. The transfection solution was replaced with fresh medium containing 10% FBS 24 h post-exposure to the DNA complexes.

Gene expression analysis

For gene expression analysis, cells were collected after an additional incubation for 24 h and a cell lysate was

prepared by PBS washing and addition of 100 μL of lysis buffer (0.1 M Tris–HCl, 0.1% Triton X-100, 2 mM EDTA, pH 7.8) per well. Cell lysates were collected, centrifuged in a microcentrifuge (12,000 rpm, 5 min, 4 °C). Protein concentration of the supernatant was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). The level of luciferase gene expression was evaluated using 10 μL of supernatant and a luciferase assay kit (Promega, Madison, WI, USA) in a luminometer (Autolumat LB953, EG & G, Berthhold, Germany) with 10 s set for measurement. Luciferase activity was normalized to the amount of luciferase per mg of extracted proteins using a standard curve in which luciferase protein (pg) was equal to 7.89×10^{-5} RLU + 0.093 ($R^2 = 0.999$).[22]

Inhibition assay with asialofetuin

Asialofetuin protein (Sigma) was dissolved in distilled water at concentration of 2 μg/μL according to the instruction of the manufacturer. The hydration was proceeded for 30 min at room temperature. HepG2 cells (5×10^4 cells per well) were plated in a 48-well plate and allowed to grow for 24 h before the transfection. Desired amounts of asialofetuin were added to each well. Cells with asialofetuin were incubated for 5 h followed by addition of 250 μL of transfection reagents (DNA/galactosyl compound) containing 1 μg of plasmid DNA and 5 nmol of galactosyl compound. Cells with transfection reagents were incubated for 5 h and added with 27.5 μL of FBS to each well. The transfection solution was replaced with fresh medium containing 10% FBS 24 h post exposure to transfection reagents. After an additional incubation for 24 h, cells were washed with PBS and lysed with lysis buffer (100 μL/well) for 15 min at room temperature. Cell lysates were collected and centrifuged in a microcentrifuge for 5 min. Ten microliters of the supernatant were used for luciferase assay. Protein concentration of the supernatant was determined by protein assay using Bio-Rad protein assay reagents.

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