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Synthesis of a library of polycationic lipid core dendrimers and their evaluation in the delivery of an oligonucleotide with hVEGF inhibition

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Abstract—This article follows on from our previous work in the area of non-viral gene delivery using polycationic dendrimers (PCDs). Herein we report on the synthesis and efficacy of a new library of lipid core PCDs in the delivery of the anti-angiogenic oligonucleotide (ODN-1) to retinal pigment epithelial cells. ELISA was used to monitor hVEGF levels in cells transfected with dendriplexes, Cytofectin GSV™ and control (non-transfected). At 48 h, hVEGF titres had returned to that of the untransfected control for Cytofectin GSV™ however, a number of dendriplexes continued to exhibit a marked reduction in hVEGF titres.

1. Introduction

The transfer of DNA/RNA into eukaryotic cells using safer alternatives to viral vectors is an area that continues to receive a great deal of research interest.[1–3] Antisense therapy aims to replace defective DNA by transfer of corrected exogenous genes or portions thereof to target cells, so transferring replication to the rectified DNA sequences.[4] Attempts to deliver naked DNA/oligonucleotides (ODNs) have met with limited success. Electroporation is one such technique that uses short electrical pulses to create pores in the cell membrane so facilitating uptake of naked DNA/ODNs.[5] Major drawbacks with the use of electroporation are that the technique is laborious with large amounts of DNA/ODN required to achieve transfection, coupled with high cell mortality.[6] Viral vectors are to-date the most efficient gene delivery systems, however, concerns are emerging about both the short- and long-term risks they pose. Concerns exist over potentially lethal immune reactions and limitations on the size of DNA that can be introduced into cells using viruses.[7] A major obstacle in the delivery of oligonucleotides using a non-viral approach is exposure to extra- and intracellular barriers, for example, nucleases.[8,9] Several chemically synthesized cationic delivery systems such as cationic liposomes, polymers and dendrimers have been developed to overcome the hurdles associated with gene delivery.[10–12] A dendrimer generally comprises of multiply branched generations of a polycationic functionality to allow for electrostatic complexation with DNA/ODN. In recent years, the delivery of ODNs using dendrimers and studies to determine uptake into cells have been reported primarily using polyethyleneimine-(PEI), polyamidoamine-(PAMAM) and more recently

Abbreviations: Boc, *tert*-Butyloxycarbonyl; Dde, *N*-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl); CH_3CN, acetonitrile; DCC, dicyclohexyl carbodiimide; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; ES-MS, electrospray mass spectroscopy; HBTU, *O*-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate; HF, hydrogen fluoride; HOBt, 1-hydroxybenzotriazole; ITC, isothermal titration microcalorimetry; LAA, lipoamino acid; MBHA, 4-methyl benzhydryl amine; ODN-1, Oligodeoxynucleotide; RP-HPLC, reverse phase-high performance liquid chromatography; RPE, retinal pigment epithelium; TFA, trifluoroacetic acid; THF, tetrahydrofuran; hVEGF, human vascular epithelial growth factor; TMS, trimethyl silane; TIPS, triisopropyl silane.

Keywords: Dendrimer; Oligonucleotide; hVEGF; Antisense therapy.
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polypropylenimine(PPI)starburst dendrimers.[13–16] These systems have delivered promising results and have been elaborated further to increase transfection efficiency by complexation of ODNs with liposomes to form lipoplexes.[17]

To this end PCDs incorporating lipoamino acids (LAAs) show much promise as non-viral gene delivery systems in the transport of oligonucleotides to target sites.[1,2,18] PCDs confer stability against digestive nucleases and aid transport of oligonucleotides across biological membranes.[19,20] We investigated the efficiency of delivering an anti-angiogenic oligonucleotide by complexing with a library of poly-lysine/arginine PCDs.[2,21] ODN-1 (GAGCCGGAGAGGGAGCGC GA; MW = 6282) has a sequence homologous to a portion of the 5′ untranslated region (5′ UTR) of hVEGF—the main factor associated with uncontrolled intraocular revascularization. ODN-1 has been shown to inhibit expression of hVEGF.[22] The overexpression of hVEGF and resulting macular degeneration/diabetic retinopathy is the major cause of blindness in patients aged over 60 years in the developed world.[23,24] Here we describe the synthesis of a library of lipid core poly-lysine/arginine dendrimers using solid-phase Boc synthetic techniques. We envisaged that the amphiphilic nature of the lipid core PCDs would raise solubility issues, however, this was not realized and by introducing a carbohydrate moiety into the synthetic procedure a number of glycodendrimers (**40**–**45**) were afforded.[25] Complexation of the PCDs with ODN-1 was carried out in deionized water using a 6:1 charge ratio (+:−) as determined previously by isothermal microcalorimetric titrations.[26] ODN-1/dendrimer complexes were applied to retinal pigment epithelial cells (RPE51) and media collected at 24 and 48 h with transfection efficiency measured indirectly by hVEGF reduction due to the presence of ODN-1. Results from these findings were compared to those from the Cytofectin GSV™/ODN-1 control complex.[27]

2. Chemical synthesis

2.1. Lipoamino acids

Lipoamino acids of varied chain length (C_8-, C_{10}-, C_{12}-, C_{14}- and C_{18}-enantiomeric mixtures) were prepared following literature methods by reaction of diethyl acetoamidomalonate (**1**) with the appropriate bromoalkane under reflux (Scheme 1).[28] The resulting intermediate was refluxed with 90% v/v HCl acid and neutralized to afford the desired lipoamino acid (**2**). To prepare the lipoamino acid for solid-phase synthesis (SPS), the *N*-terminus was protected using Boc_2O (**3**).

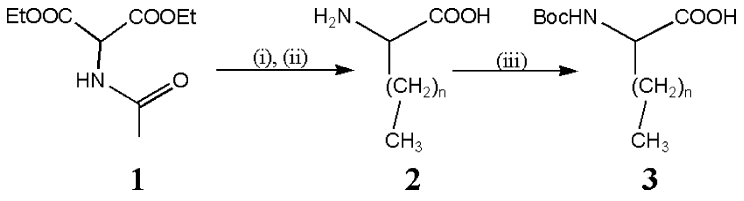
2.2. Galactosyl glutarate

To enable incorporation of a carbohydrate group onto the dendrimer using solid-phase synthesis, *tert*-butyl glutarate was coupled via an amide linkage to D-galactose (Scheme 2).

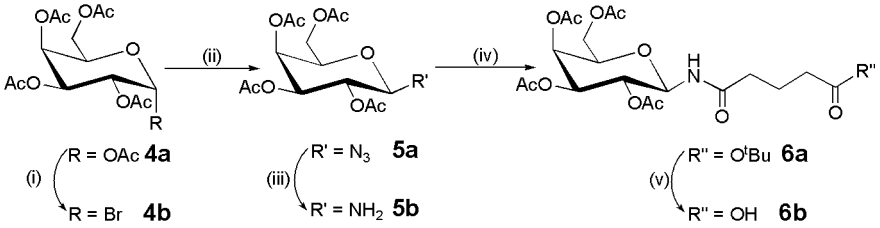
Peracetylated galactose was stirred with 30% HBr/AcOH to afford peracetyl galactosyl bromide (**4b**, α-anomer) followed by reaction with NaN_3 to yield the corresponding azide (**5a**, β-anomer). Reduction of the azide allowed direct condensation with mono-*tert*-butyl glutarate to afford **6a**. Finally, acidolytic cleavage of the ester using TFA yielded the desired building block (**6b**) ready for dendrimer synthesis (see Fig. 1 for general structure).[29]

2.3. Dendrimers and glycodendrimers

Dendrimers were synthesized using stepwise solid-phase Boc-chemistry (see Tables 1 and 2). Glycodendrimers **40**–**45** incorporated building block **6b** coupled via the acid to the ε-NH_2 termini of L-lysine (Fig. 2). To prevent aggregation of the polar heads and lipidic tails within each dendrimer, a spacer (C_6) was introduced. Boc-lipoamino acids were used in C_8, C_{10}, C_{12}, C_{14} and C_{18} chain



Scheme 1. Synthesis of Lipoamino acids.[28] Reagents and conditions: (i) Br–$(CH_2)_n$–CH_3, NaOEt, reflux (ii) H^+ reflux, then NaOH to pH 7 (iii) Boc_2O, $^tBuOH/H_2O$, NaOH (pH 13), then citric acid to pH 7.



Scheme 2. Synthetic strategy towards galactosyl-glutarate.[29] Reagents: (i) 30% HBr/AcOH (ii) NaN_3, *n*-Bu_4NHSO_4, $NaHCO_3$ in DCM (iii) 10% Pd/C in EtOH, H_2 (iv) $HOOC(CH_2)_3COOtBu$, DCC, DIPEA, THF (v) 90% $TFA_{(aq)}$, TIPS/DCM.

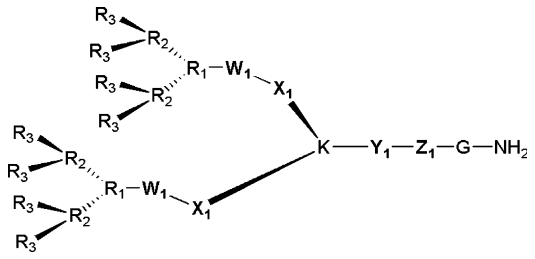


Figure 1. General structure of dendrimers **16**–**35**.

Table 1. C_8, C_{10}, C_{12}, C_{14} and C_{18}, lipoamino acids; C_6, $–OC(CH_2)_5NH–$ (spacer); G, glycine; K, lysine; R, arginine

Dendrimer	R_3	R_2	R_1	W_1	X_1	Y_1	Z_1
16	—	—	K	C_6	C_8	C_8	C_8
17	—	K	K	C_6	C_8	C_8	C_8
18	—	K	K	C_6	$(C_8)_2$	C_8	C_8
19	—	—	K	C_6	C_{10}	C_{10}	C_{10}
20	—	K	K	C_6	C_{10}	C_{10}	C_{10}
21	—	—	K	C_6	$(C_8)_2$	—	—
22	—	K	K	C_6	$(C_8)_2$	—	—
23	—	—	K	C_6	$(C_{10})_2$	—	—
24	—	K	K	C_6	$(C_{10})_2$	—	—
25	K	K	K	C_6	$(C_{10})_2$	—	—
26	—	R	K	C_6	C_{10}	—	—
27	—	R	K	C_6	$(C_{10})_2$	—	—
28	—	R	K	C_6	$(C_{10})_2$	—	C_{10}
29	—	R	K	C_6	$(C_{10})_2$	C_{10}	C_{10}
30	—	—	K	C_6	C_{12}	—	—
31	—	K	K	C_6	C_{12}	—	—
32	—	—	K	C_6	$(C_{12})_2$	—	—
33	—	K	K	C_6	$(C_{12})_2$	—	—
34	—	—	K	C_6	$(C_{12})_2$	—	C_{12}
35	—	K	K	C_6	$(C_{12})_2$	—	C_{12}

Table 2. Abbreviations as in Table 1; GD, glycodendrimer; Gal-Lys, **6b** coupled to ε-NH_2 termini of L-lysine

GD	R_1	U	W	X	Y	Z
40	K	—	C_6	C_{14}	Gal-Lys-	C_{14}
41	K	—	C_6	C_{18}	Gal-Lys-	C_{18}
42	K	C_6	C_{14}	Gal-Lys-	—	—
43	K	C_6	C_{18}	Gal-Lys-	—	—
44	K	—	—	—	Gal-Lys-	$(C_{14})_2$
45	—	—	—	—	Gal-Lys-	$(C_{18})_2$

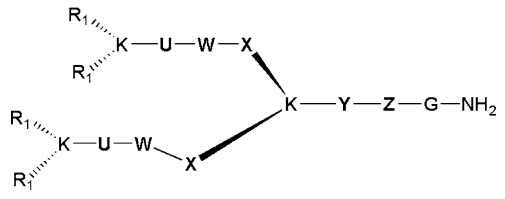


Figure 2. General structure of glycodendrimers **40**–**45**.

lengths. Upon completion of the synthesis, all dendrimers were cleaved from the resin using the high HF protocol, purified using RP-HPLC and characterized by mass spectrometry.

3. Results and discussion

3.1. Microscopic examination

Initial microscopic analysis was performed on RPE cells treated with dendrimers or dendriplexes to assess degradation of cells (Fig. 3).

It is evident that microscopic analysis of RPE cells transfected with Cytofectin GSV™/ODN and dendrimer/ODN complexes appears to be healthy (Figs. 3b and c) and viable for further in vitro studies. An effect of the cationic dendrimers on RPE cell morphology becomes apparent when administered alone, which can be attributed to their surfactant-like properties (Fig. 3d). For a number of dendrimer/ODN-1 complexes a similar morphological change was observed 48 h post-transfection. This change was observed via microscopic analysis (as per Fig. 3d) and the detail of cell morphology change observed for each complex is available in the supporting information.

3.2. ELISA

ELISA was performed on media collected from dendrimer/ODN-1 conjugates **16**–**35** and **40**–**45** in addition to Cytofectin GSV™/ODN-1 (Cyt) and non-transfected control (Cytofectin GSV™ alone, Con) at 24 and 48 h post-transfection (Fig. 4).

All but one (**20**) of the dendrimers resulted in a moderate reduction in hVEGF expression compared to the commercially available transfection agent Cytofectin GSV™ in the initial 24 h period. This clearly demonstrates release of ODN-1 from the dendriplex and delivery to the target site within the nucleus of the cell. Cells transfected using Cytofectin GSV™ exhibited the expected profile of 40–50% reduction in the initial 24 h, followed by no significant reduction in the subsequent 24 h. The profile of **25** was very similar to Cytofectin GSV™ and it may be interesting to explore modifications of **25** to attempt prolonging its efficacy.

Of the library studied, dendrimers **16**, **40** and **41** displayed a sustained reduction of hVEGF concentration between 40% and 50% after 48 h, however, cell morphology resembled that in Figure 3d after this time period. The reduction in hVEGF titre (≈20%) in the second 24 h period of study for dendrimer **16** is encouraging. The structure of **16** consists of three short C_8-LAAs with a net 4+ charge. Previous studies in this area identified dendrimers consisting of two C_{14}-LAAs with an 8+ charge or two C_{18}-LAAs with a 4+ charge as being very efficient at transfecting RPE cells with hVEGF concentrations remaining at between 50% and 60% of control after 48 h.[1] It is interesting that **16** although comprising of much shorter LAAs has a transfection efficiency comparable to those previous dendrimers. Furthermore, the introduction of more LAAs (i.e., **17** and **18**) did not correlate to better activity by way of hVEGF reduction.

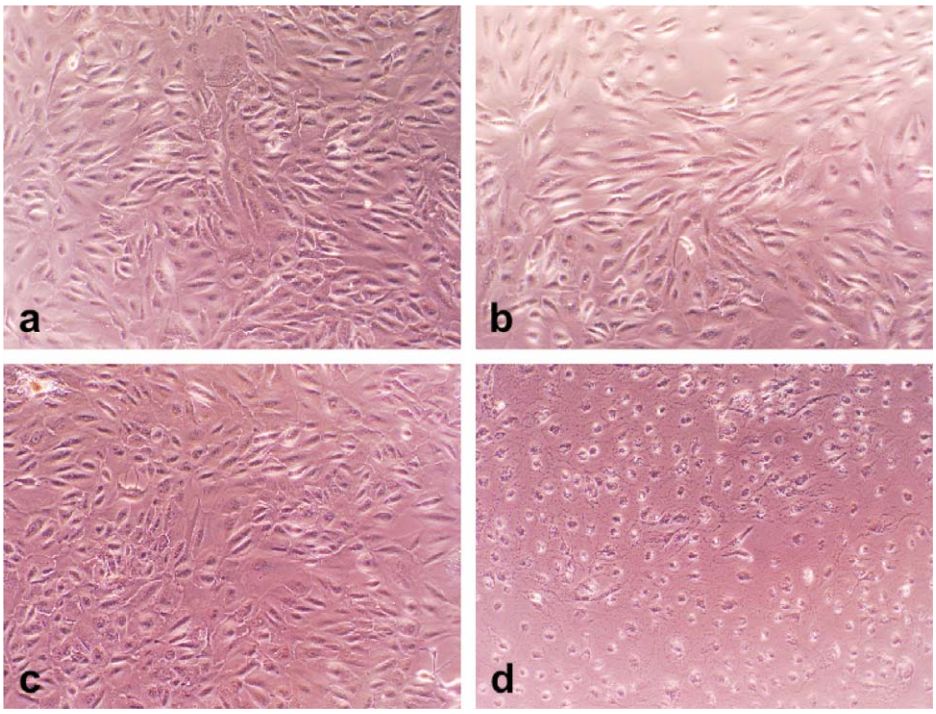


Figure 3. Microscopic analysis of RPE cells 24 h post-transfection with: (a) Control cells (untransfected); (b) Cytofectin GSV™/ODN-1 complex; (c) Dendrimer/ODN-1 complex; (d) Unconjugated dendrimers (1 mg/mL).

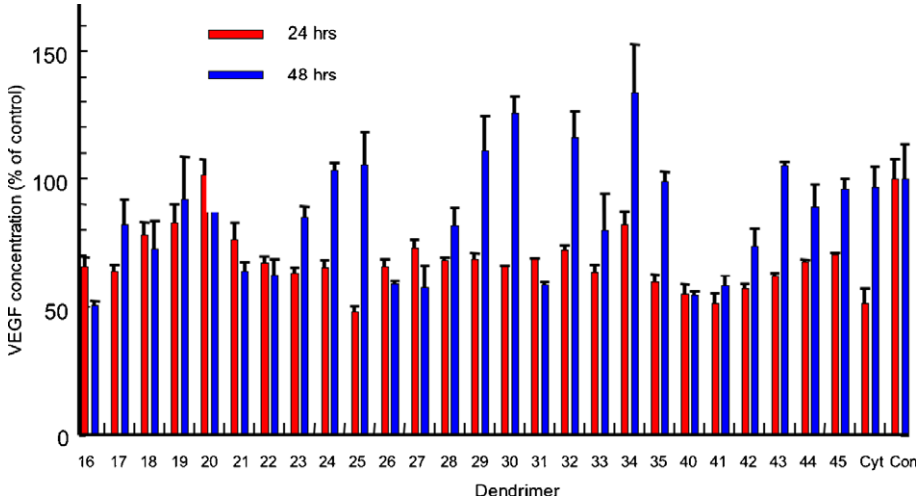


Figure 4. Expression of VEGF (% of untransfected control).

A number of dendrimers were functionalized with galactose—chosen at random as the carbohydrate moiety, primarily to enhance hydrophilicity. The introduction of galactose was also undertaken to explore the possibility of exploiting any active transport mechanisms, however, a larger glycodendrimer library comprising of numerous carbohydrates would be required to fully address this issue. Of the glycodendrimers constructed **40** and **41** displayed similar hVEGF titres at 24 and 48 h (≈60% of control) and each comprises of three C_{14} and C_{18} LAAs, respectively, with both dendrimers carrying a net 8+ charge. Once again, decreasing (**42** and **43**) or increasing (**44** and **45**) the number of LAAs did not further reduce hVEGF titres.

4. Conclusion

Synthesis and evaluation of a library of polycationic dendrimers and glycodendrimers towards transfection of RPE cells has been demonstrated. From the library of dendrimers synthesized there does not seem to be an obvious trend relating dendrimer structure to hVEGF titre, although a number of complexes were moderately efficient in transfecting human cells (RPE51). Dendrimers **16**, **40** and **41** displayed a marked reduction in hVEGF titres after 48 h compared to the commercially available standard Cytofectin GSV™, however, a significant change in cellular morphology was also present 48 h post-transfection for these and a

number of other dendrimers (see supporting information). To enhance the hydrophilicity of dendrimers with relatively long LAAs (C_{14}/C_{18}), a carbohydrate moiety was introduced (**40**–**45**), although in hindsight insolubility in aqueous systems was not an issue. Although glycodendrimers **40** and **41** displayed a marked reduction in hVEGF at 48 h, this was also accompanied by a marked change in cell morphology. Dendrimers **42**–**45** were efficient in transfecting cells at 24 h, however, this returned to control levels after a further 24 h with moderate-no change in cellular morphology. Here we have shown that incorporating three LAAs and galactose as a carbohydrate moiety provides moderate hVEGF reduction in the initial 24 h post-transfection, however, it returns to control levels in the subsequent 24 h. There is evidence of altered cellular morphology from extended exposure of the RPE cells to the dendriplexes and further studies need to be carried out to determine the significance of this. Further work is underway to address the delivery of oligonucleotides to cell lines using glycodendrimers derived from various carbohydrates whilst maintaining healthy viable cells over an extended time course. The effects of complex size, shape and surface charge on transfection efficiency and cell viability in various cell lines are also underway.

5. Experimental

MBHA resin was purchased from Reanal (Hungary) and protected amino acids were obtained from Nova-Biochem (Australia). DMF and TFA of peptide synthesis grade were purchased from Auspep (Parkville, Australia). HPLC grade acetonitrile was purchased from Labscan Asia Co. Ltd (Bangkok, Thailand). The mobile phase was a mixture of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in 90%$_{(aq)}$ CH_3CN) at a flow rate of 1 mL/min.

5.1. General procedure for synthesis of dendrimers 16–35 and 40–45

p-MBHA resin (100–200 mesh, 0.4 mmol/g loading) was swelled in DMF in a fritted glass vessel for 90 min. An activation mixture of HBTU (0.5 M in DMF, 4 equiv), DIPEA (0.442 mL, 4 equiv) and amino acid (4 equiv) was shaken with the resin for 15 min. Negative ninhydrin reaction (5 min) showed nearly quantitative coupling ($\geqslant$99.6%) and the Boc group was removed using 100% TFA (2× 1 min) followed by in situ neutralization.[30,31] Dendrimers were synthesized on a 0.3 mmol scale, and the following amino acid side-chain protection was used: Lys(Boc), Lys(Dde). Between all manipulations the resin was washed exhaustively with DMF. Upon completion of synthesis terminal Boc groups were removed and in the case of glycodendrimers **40**–**45** the resin was also shaken with freshly prepared 1 M NaOMe (8 mL, 30 min), to remove the galactose-acetyl-protecting groups prior to washing with DMF and then methanol. Dde was removed using 2% v/v hydrazine monohydrate in DMF (2× 20 min washes) followed by coupling of building block **6b** (5-oxo-5-(2,3,4,6-tetra-*O*-acetyl-β-D-galactosylamino)pentanoic acid, 3 equiv) using the procedure described above. The resin was dried over KOH under vacuum. The dendrimers were cleaved from the resin using HF (10 mL) and *p*-cresol (1 mL) at 0 °C for 1 h. The cleaved dendrimers were precipitated using diethyl ether, then re-dissolved in 50%$_{(aq)}$ CH_3CN and lyophilized to afford an amorphous powder.

5.2. Purification

Analytical RP-HPLC was performed on a Shimadzu instrument (LC-10AT liquid chromatograph, SCL-10A system controller, SPD-6A UV detector, a SIL-6B auto injector with a SCL-6B system controller and columns C4 (Vydac, 5 μm pore size, id = 4.6, 250 mm), C18 (Zorbax, 3.5 μm pore size, id = 4.6, 150 mm)) to identify the synthesized dendrimers and ascertain the appropriate gradient conditions for preparative HPLC. Preparative HPLC was undertaken with ≈100 mg of crude dendrimer separated on a Waters HPLC system (Model 600 controller, 490E UV detector, F pump and TSK Gel C4/C18 columns with 10μm pore size and 22 mm id) using an acetonitrile/water gradient and characterized by ES-MS (Perkin Elmer API 3000 instrument). The resulting dendrimers were used as diastereomeric mixtures.

5.3. Complexation

Oligodeoxynucleotide, ODN-1 (1.0 mg/mL in deionized water), was added to each dendrimer (1.0 mg/ mL in deionized water), mixed for 15 min, diluted with 200 μL sterile water and lyophilized. Complexes were reconstituted in deionized water (100 μL) prior to transfection. The molar ratio of 6:1 (+:−) dendrimer/ODN-1 was used for complexation as previously determined by ITC experiments.[32,33] Cytofectin GSV™/ODN complexes were prepared according to the manufacturer's instructions (Glen Research, Stirling, VA, USA) using Optimem® media.

5.4. In vitro experiment and ELISA

Cells of human RPE origin, RPE51, were grown to 80% confluency in a 24-well plate and transfected with 1 μM (final concentration) ODN-1 in quadruplet sets using either Cytofectin GSV™ (as per the manufacturer's instructions) or the dendrimer/ODN-1 complexes. The cells were grown under hypoxic conditions (5% CO_2/ 2% O_2) for 48 h with media removed at both 24 and 48 h intervals. One hundred microlitres of media from each sample at 24 and 48 h intervals was directly used in the assay. The samples were then used in a sandwich ELISA as per the manufacturer's instructions (Cytelisa™ Human VEGF kit, CYTIMMUNE Sciences Inc., Maryland, USA). Statistical analysis was performed on the data, which consisted of an ANOVA with post hoc analysis using Tukey/Kramer and a Fishers LSD procedure ($p < 0.05$).

5.5. NMR and MS

NMR measurements were performed on a Bruker Avance 500 instrument using a 5 mm SEI probe at

25 °C. Spectra were processed using Topspin® 1.3 software and TMS was used as the internal standard. Mass spectrometric measurements were performed using a triple-quadrupole PE Sciex API 3000 mass spectrometer with positive ion electrospray (ES). Accurate mass spectrometric measurements (HR-MS) were performed using a Finnigan MAT 900 XL-Trap instrument with a Finnigan API III electrospray source in positive ion electrospray mode.

5.5.1. *tert*-Butyl 5-oxo-5-(2,3,4,6-*tetra*-*O*-acetyl-β-D-galactosyl-1-amino)pentanoate (6a). ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.23 (m, 1H, C_1-Gal), 5.37 (dt, 1H, J = 1.1, 2.9, 14 Hz, C_5-Gal), 5.17 (m, 1H, C_2-Gal), 5.06 (m, 2H, C_3,C_4-Gal), 4.02 (m, 1H, C_6-Gal), 2.19–1.90 (br m, 18H, $4\times CH_3$, $3\times CH_2$ (pent)), 1.38 (s, 9H, $C(CH_3)_3$). ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.75, 172.39, 171.51, 170.59, 170.23, 169.98 (C═O), 80.69 ($C(CH_3)_3$), 78.63(C_1-Gal), 72.46 (C_5-Gal), 71.04 (C_2-Gal), 68.51 (C_4-Gal), 67.35 (C_3-Gal), 61.30 (C_6-Gal), 35.67 ($HNCOCH_2$), 34.56 (CH_2COO^tBu), 28.28 ($C(CH_3)_3$), 20.94, 20.87, 20.80, 20.74, 20.66 ($4\times COCH_3$, $CH_2CH_2CH_2$). ES-MS *m*/*z* 540.43 ($[M+Na]^+$, $C_{23}H_{35}NO_{12}$ requires 517.52).

5.5.2. 5-Oxo-5-(2,3,4,6-*tetra*-*O*-acetyl-β-D-galactosylamino)pentanoic acid (6b). ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.50 (d, 1H, J = 9.3 Hz C_1-Gal), 5.41 (dt, 1H, J = .1, 2.9, 14 Hz, C_5-Gal), 5.23 (t, 1H, C_2-Gal), 5.09 (m, 2H, C_3,C_4-Gal), 4.11–4.00 (br m, 3H, 2× C_6-Gal, NH), 2.40–2.20 (br m, 4H, 2× CH_2 (pent)), 2.11, 2.03, 2.00, 1.96 (s, 12H, 4× CH_3), 1.91 (m, 2H, CH_2 (pent)). ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.37, 172.66, 171.43, 170.49, 170.04, 169.80 (C═O), 78.37 (C_1-Gal), 72.30 (C_5-Gal), 70.83 (C_2-Gal), 68.36 (C_4-Gal), 67.17 (C_3-Gal), 61.11 (C_6-Gal), 35.11 ($HNCOCH_2$), 32.64 (CH_2COOH), 20.72, 20.66, 20.57, 20.52 (CH_3), 19.96 ($CH_2CH_2CH_2$). HR-MS *m*/*z* calcd for $C_{19}H_{27}NO_{12}Na$ requires 484.1431. Found 484.1431.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.03.029.

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