CHEMBIOCHEM

Multivalent HSA Conjugates of 3′-Sialyllactose are Potent Inhibitors of Adenoviral Cell Attachment and Infection

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Adenoviruses of serotypes 8, 19 and 37 are the major cause of the severe eye infection EKC (epidemic keratoconjunctivitis). In general, all adenoviruses interact with their cellular receptors through the fibre proteins, which extend from the virus particle. Recently, adenovirus type 37 (Ad37) was found to bind and infect human corneal cells through attachment to carbohydrate structures that carry terminal α-(2–3)-linked sialic acids. Herein we present a synthetic route to a 3′-sialyllactose derivative and corresponding multivalent HSA conjugates with varying orders of valency. The potential of these compounds as inhibitors of EKC-causing adenovirus of serotype Ad37, was studied with both a binding assay and an infectivity assay. The results revealed that these compounds effectively prevent Ad37 from binding to and infecting human corneal epithelial (HCE) cells. Moreover, the inhibition is significantly increased with higher orders of multivalency.

Introduction

Adenoviruses constitute the *Adenoviridae* family of viruses and cause infections worldwide in humans as well as in a variety of animals.[1–4] Three adenovirus (Ad) serotypes of subgenus D, that is, Ad8, Ad19 and Ad37, are the major causes of the severe ocular infection epidemic keratoconjunctivitis (EKC).[5,6] The disease is transferred by contact and it is therefore most common in densely populated areas, predominantly in East Asia[7–9] but also in Northern America, India, Palestine and Europe.[5,10] For example, between half a million and one million individuals fall ill with EKC every year in Japan alone.[11] The initial characteristics of EKC are red eyes, ocular irritation and tearing caused by viral replication in the conjunctiva.[6] Not only the conjunctiva, but also the cornea is infected, and invasion of cells from the immune defence in the cornea leads to subepithelial infiltrates that may last for several years[12] and cause various levels of reduced sight. As a result of the infection, the patient is handicapped for weeks, leading to substantial suffering and economic losses. In some cases, the infection leads to permanent reduction of the sight.[5] At present there are no licensed antiviral agents available for treatment of EKC.

In general, all adenoviruses interact with their cellular receptors through the fibre proteins, which extend from the virus particle. The virus has twelve fibre proteins, each of which consists of a homotrimer.[13] Recently, Ad8, Ad19 and Ad37 were found to bind and infect host cornea cells through attachment to carbohydrate structures. For Ad37, these were shown to be glycoproteins carrying at least one terminal sialic acid residue linked by an α(2–3)-glycosidic bond to a saccharide chain.[14,15] It is important to point out that the ability of these adenoviruses to cause EKC is correlated with their ability to bind to sialic acid residues on the cell surface.[14] Thus, by employing sialic acid as a component in an antiviral drug it might be possible to block virus attachment and thus to treat patients suffering from EKC. Because the virus attaches to the host cell via multiple fibre proteins, it is likely that the ideal inhibitor should be multivalent, for example, a neoglycoprotein prepared by conjugation of several sialylated oligosaccharides to a protein carrier.

Multivalent interactions are frequently used in nature so that weak ligand–receptor interactions become biologically relevant.[16] Such naturally occurring multivalent events have become a popular research area among chemists that study biological recognition. Affinity enhancement afforded by multivalent binding has been demonstrated by several research groups.[17–23] One of the first and most notable applications of multivalent inhibitors is the prevention of the binding of the influenza virus hemaglutinin to host cells.[24–26] One major advantage of a multivalent binding system over a monovalent system is that recognition in multivalent interactions could occur by several different mechanisms. These include the chelate effect, subsite binding, steric stabilisation, statistical rebinding and receptor clustering.[16,27] Naturally occurring, multivalent carbohydrate structures are widespread but their application in probing multivalent cell-surface binding systems is limited since they are often only found in minute amounts, structurally heterogeneous and complex. By generating a synthetic multivalent inhibitor it is possible to vary several important structural characteristics such as i) scaffold structure, ii) or-

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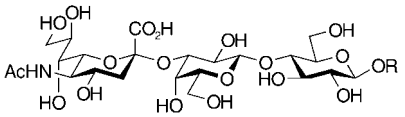
der of valency, iii) size, iv) flexibility and v) the spacing of binding elements.[28]

Our intention with this work was to generate a synthetic inhibitor of EKC-causing adenoviruses of serotypes Ad8, Ad19 and Ad37. Here we present the synthesis of a sialylated lactose derivative **2**, and corresponding multivalent human serum albumin (HSA) conjugates. Evaluation of the compounds as inhibitors of binding and infection of EKC-causing adenovirus to host cells revealed a significant multivalency effect.

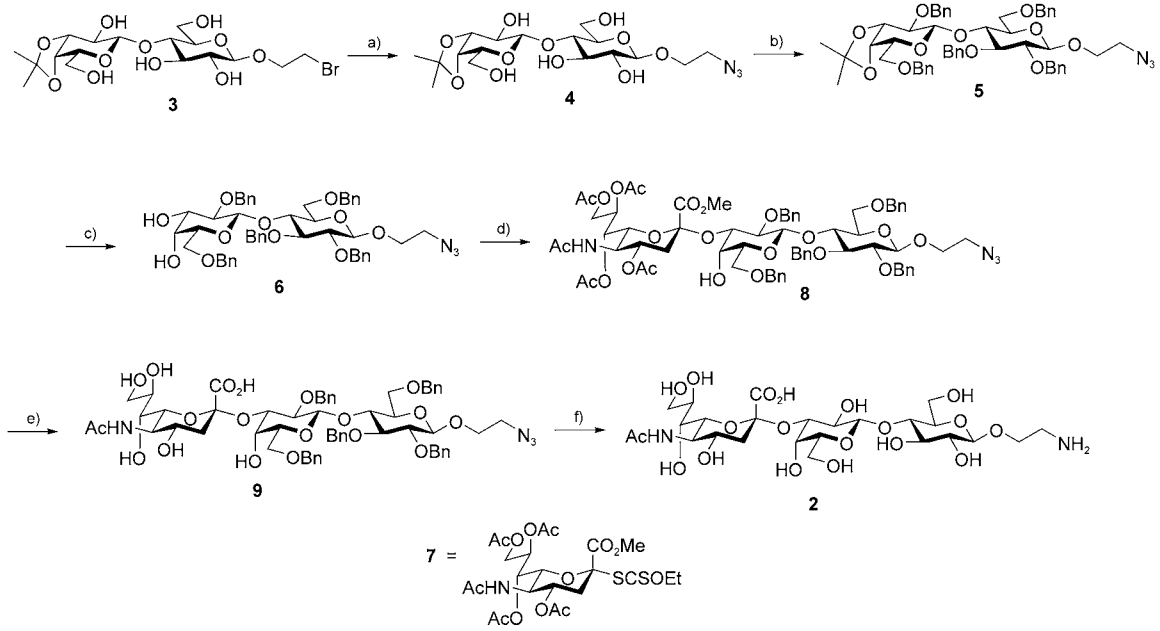


1 R = H
2 R = $CH_2CH_2NH_2$

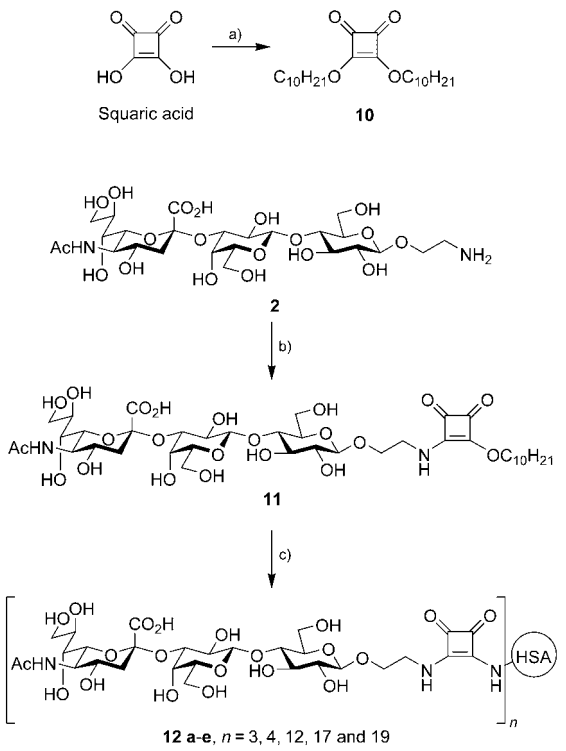
Results and Discussion

Synthesis

The receptor structure for EKC-causing adenoviruses has a terminal sialic acid residue linked by an α(2–3)-glycosidic bond to a saccharide chain.[14,15] Since all α(2–3) sialyltranferases characterised so far only transfer sialic acid to galactose, it is most likely that the neighbouring saccharide is galactose. Studies of the binding of the viral fibre knob protein to glycolipids by using a TLC overlay assay have suggested that the carbohydrate structure consists of a complex saccharide and that 3′-sialyllactose, **1**, is the core (unpublished results in collaboration with Susanne Teneberg). Therefore the 3′-sialyllactose derivative **2**, which can be conjugated to a protein carrier, was selected as target in order to find a synthetic inhibitor of EKC-causing adenoviruses. Lactose was used as the starting material, and synthesis of the 3′,4′-*O*-isopropylidene-protected 2-bromoethyl lactoside **3** (Scheme 1) followed the procedure described by Bengtsson et al.[29] Compound **3** was successfully converted into the 2-azidoethyl lactoside **4** in 97% yield by treatment with sodium azide and crown ether in dimethylformamide (DMF).[30] Benzylation of the unprotected hydroxyl groups with benzyl bromide and sodium hydride in DMF gave **5** in 68% yield. Hydrolysis of the 3′,4′-isopropylidene acetal was achieved by using a mixture of trifluoroacetic acid and water in dichloromethane to give the partially protected lactoside **6** in quantitative yield. Sialylation at the 3′-position of **6** was then performed with xanthate **7**[31] by using methylsulfenyl bromide[32] and silver trifluoromethanesulfonate as promoters[33,34] in a mixture of dichloromethane and acetonitrile at low temperature.[29] After careful purification by column chromatography and preparative HPLC, the protected trisaccharide **8** was obtained in 56% yield. The α-anomeric configuration of the sialic acid residue was established by determination of the coupling constant[35] between C-1′′ and H-3′′$_{ax}$ (J = 6.7 Hz). Since this trisaccharide has an unprotected hydroxyl group in position 4′, it is possible to glycosylate it further to more complex saccharides if preferred. Deacetylation of **8** in methanolic sodium methoxide, followed by hydrolysis of the sialic acid methyl ester, gave the partially protected trisaccharide **9** in 87% yield. Cleavage of the benzyl protective groups, and simultaneous reduction of the azido group were accomplished by catalytic hydrogenation over Pd/C in hydrochloric ethanol[36] to give the unprotected amino ethyl glycoside **2** in 98% yield. Conjugation of **2** to HSA was performed via the squaric decyl ester glycoside[37,38] **11** (Scheme 2) to give the neoglycoprotein



Scheme 1. *Synthesis of 3′-sialyllactose derivative* ***2****. Reagents and conditions: a) NaN_3, [15]crown-5, DMF, 97%; b) BnBr, NaH, DMF, 68%; c) TFA/H_2O (9:1), CH_2Cl_2, quant.; d)* ***7****, AgOTf, MeSBr, −60°C, CH_3CN/CH_2Cl_2 (9:4), 56%; e) 1. NaOMe, MeOH 2. H_2O, 87% over two steps; f) H_2(g) Pd/C, EtOH/HCl, 98%. TFA = trifluoroacetic acid.*



*Scheme 2. Synthesis of multivalent 3′-sialyllactose HSA conjugates, **12a–e**, with varying degrees of multivalency. Reagents and conditions: a) Decan-1-ol, toluene, 78%; b) **10**, DMF, Et_3N, 40%; c) $NaHCO_3$, (pH 9.0), HSA.*

12, following the procedures described by Bergh et al.[30] By varying the number of equivalents of **11**, the degree of incorporation of saccharide to HSA could be varied; this resulted in five neoglycoproteins, **12a**, **12b**, **12c**, **12d** and **12e**, with 3, 4, 12, 17 and 19 incorporated saccharides per HSA, respectively. The average degree of incorporation was determined by MALDI-TOF MS by using the centre of the single-charged neoglycoprotein peak.

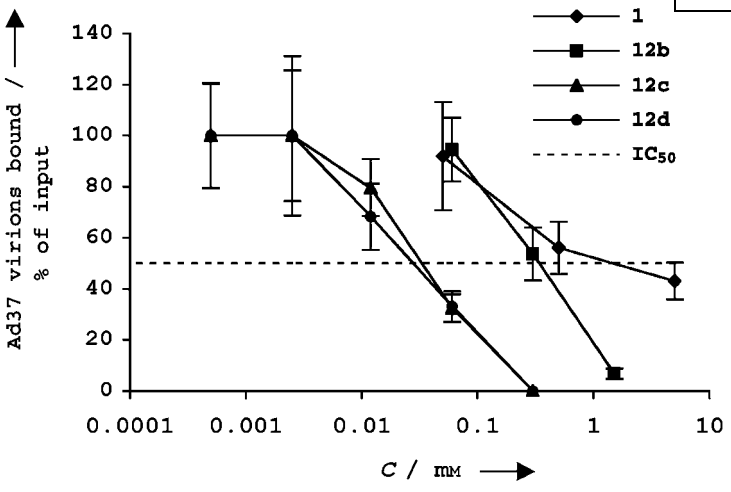


*Figure 1. Dose-dependent inhibition of Ad37 attachment to HCE cells with commercial 3′-sialyllactose and multivalent 3′-sialyllactose HSA conjugates **12b–d** (4-, 12-, and 17-valent). ^{35}S-labelled Ad37 virions were incubated with a dilution series of the different sialyllactose conjugates and then added to HCE cells. Unbound virions were removed by washing, and the cell-associated radioactivity was measured. The results are presented as a mean of two independent experiments. The experiments were repeated three times with reproducible results.*

Biological evaluation

In order to investigate whether the multivalent sialyllactose derivatives could inhibit the attachment of Ad37 virions to human corneal epithelial (HCE) cells and to examine the effect of multivalency, a binding assay based on ^{35}S-labelled virions was used.[39,40] ^{35}S-labelled Ad37 virions were preincubated with commercially available 3′-sialyllactose **1**, or the multivalent derivatives **12b**, **12c** or **12d**, before adding the virions to cells. After incubation, unbound virions were washed away, and cell-associated radioactivity was counted (Table 1). The results show that both monovalent 3′-sialyllactose and the multivalent sialyllactose derivatives were able to inhibit attachment of Ad37 (Figure 1). However, the data clearly show that increased multivalency leads to a very significant gain of inhibitory power as compared to the monovalent case. The best multivalent compound, that is, **12d**, which has the highest degree of multivalency, shows an IC_{50} value that is more than 100-fold better than 3′-sialyllactose (**1**, Figure 1).

Table 1. Inhibition of Ad37 attachment to HCE cells by mono- and multivalent conjugates of sialyllactose.[a]

Compound	C[b] [mM]	% of control[c]	S.D.[d] [%]
1 3′-sialyllactose	5	43	±5
	0.5	56	±8
	0.05	92	±19
12b (4-valent)	1.5	7	±2
	0.3	54	±8
	0.06	95	±4
12c (12-valent)	0.3	0	±0
	0.06	33	±4
	0.012	80	±5
	0.0025	100	±21
	0.0005	100	±10
12d (17-valent)	0.3	0	±0
	0.06	33	±4
	0.012	68	±10
	0.0025	100	±29
	0.0005	100	±15

[a] This experiment was repeated three times with similar results. [b] Concentrations refer to individual saccharide, i.e., for **12d**, 0.3 mM in the table corresponds to 0.3/17 mM conjugate. [c] Mean values of two replicates. [d] Standard deviation.

In order to further examine the effect of multivalency, we investigated whether the conjugates could prevent Ad37 from infecting HCE cells. An infectivity assay that visualises adenovirus-infected cells as fluorescent focus units (FFU) was used. Unlabelled virions were preincubated with 3′-sialyllactose **1** or conjugate **12e** in increasing concentrations (Table 2),

Table 2. *Inhibition of Ad37 infection of HCE cells by mono- and multivalent conjugates of 3′-sialyllactose.*[a]

Compound	C[b] [mM]	% of control[c]	S.D.[d] [%]
1 3′-sialyllactose	0.01	94	±15
	0.1	75	±1
	1	49	±10
12e (19-valent)	0.001	51	±12
	0.01	21	±6
	0.1	4	±0.5
	1	3	±1

[a] This experiment was repeated two times with similar results. [b] Concentrations refer to individual saccharide, i.e., for **12e**, 1 mM in the table corresponds to 1/19 mM conjugate. [c] Mean values of two replicates. [d] Standard deviation.

before the mixtures were incubated with HCE cells. After a synchronised infection, that is, all virions entered the cells at the same time, the cells were fixed and stained with rabbit polyclonal anti-Ad37 serum,[41] followed by FITC (fluorescein thioisocyanate-) labelled swine anti-rabbit IgG antibodies, and examined in an immunofluorescence microscope.[15] It was found that conjugate **12e** effectively prevents Ad37 virions from infecting HCE cells (Figure 2). In addition, the results further demonstrate the improvements in inhibitory power that can be obtained with multivalent structures. Relative to the monovalent structure of 3′-sialyllactose, the 19-valent conjugate **12e** shows an IC_{50} value that is a 1000-fold lower.

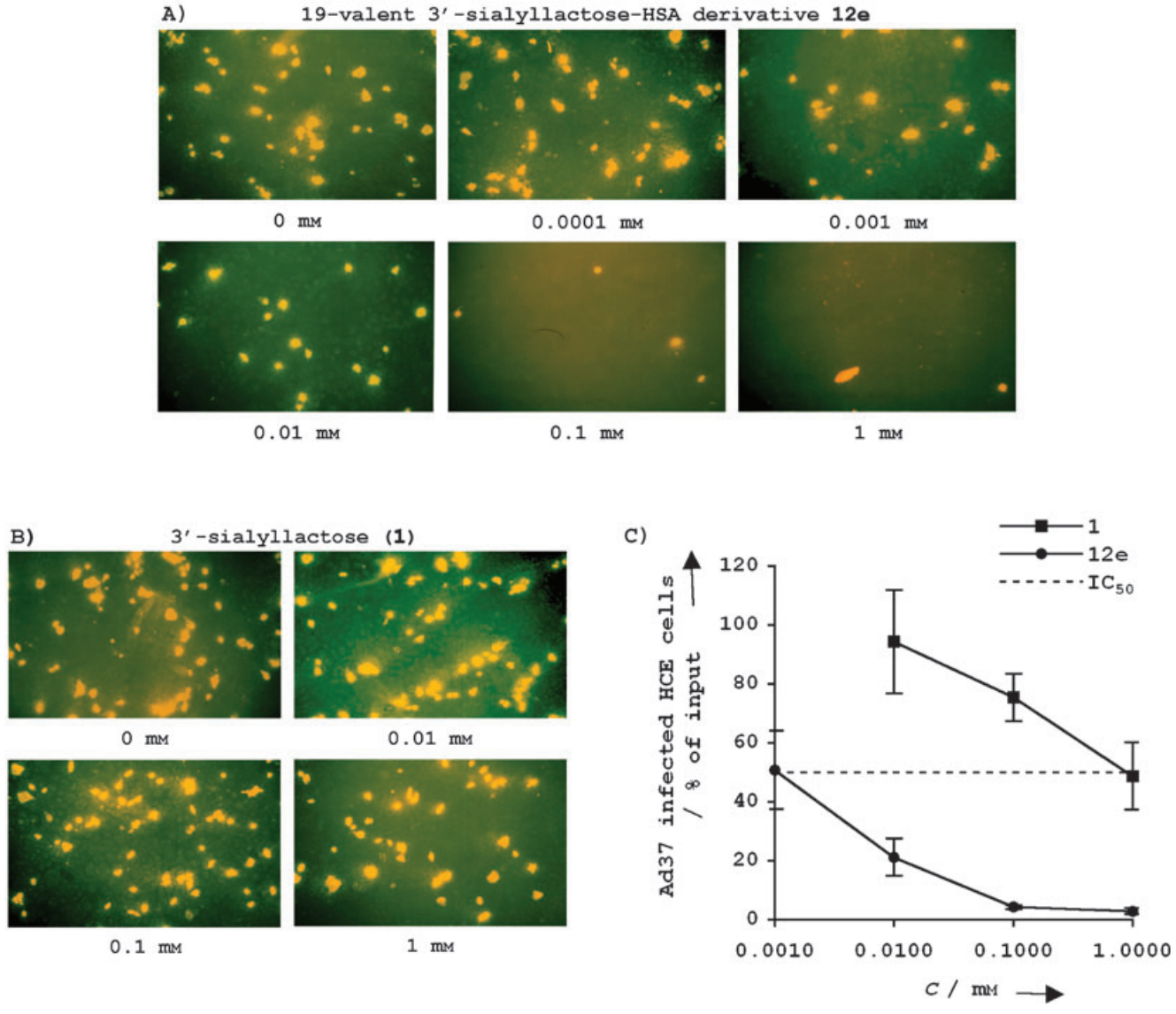


Figure 2. *Dose-dependent inhibition of Ad37 infection of HCE cells with A) multivalent sialyllactose conjugate **12e** and B) commercial 3′-sialyllactose. C) Graphical presentation of the data shown in (A) and (B). Unlabelled virions were preincubated with **12e** or 3′-sialyllactose at different concentrations before the mixtures were transferred to HCE cells. The dilution of virions was adjusted in order to obtain 100 fluorescent focus units (FFU) per view field in the control wells (untreated cells). One yellow dot in A and B corresponds to one FFU, that is, one infected cell. With incubation at +4°C, attachment of virions to cells but not internalisation was permitted, then unbound virions were removed by washing. Incubation at 37°C permitted internalisation of virions in cells. At 44 h postinfection, the cells were rinsed, fixed, stained, and examined in an immunofluorescence microscope. The experiments have been repeated twice with reproducible results.*

Conclusion

In conclusion, this study presents the synthesis and evaluation of a new multivalent synthetic inhibitor of EKC-causing adenovirus of serotype Ad37. At present, there are no licensed antiviral agents available for treatment of EKC. Because as many as 500 000–1 000 000 persons in Japan encounter EKC annually,[11] it is of great interest to find potent inhibitors of the causative agents of this disease, that is, adenoviruses of serotypes Ad8, Ad19 and Ad37. The life cycle of human adenoviruses includes several steps in which initial attachment of the viruses to cellular receptors plays a pivotal role in virus tropism. Thus, structures that mimic the cellular receptors of EKC-causing adenoviruses and effectively bind to the viruses could prevent the viruses from binding and infecting the corneal epithelial cells. Since the viruses attach to the host cells through multiple fibre proteins, it is likely that the ideal structure should be multivalent. Saccharides related to 3′-sialyllactose have been suggested to act as a cellular receptor for Ad37, therefore multivalent synthetic structures that have 3′-sialyllactose on HSA were prepared. The synthesis of 3′-sialyllactose derivative **2** from lactose was performed in 11 steps with an overall yield of 12%. The protection-group strategy enables further glycosylation to larger structures at the 4′-position if needed in future studies. Conjugation of **2** to HSA through the squaric decyl ester glycoside **11** resulted in a series of conjugates with an increasing order of multivalency (**12a**–**e**). The potential of these compounds as inhibitors of EKC-causing adenovirus of serotype Ad37 was studied by both a binding assay and an infectivity assay. The results revealed that our compounds effectively prevent Ad37 from binding to and infecting human corneal epithelial (HCE) cells. Moreover, these results clearly indicate that the inhibition is significantly increased with higher orders of valency. Multivalent conjugates have been shown to be potent antiviral agents in several cases.[16,26] However, the present study is the first one to reveal that synthetic multivalent conjugates can be used to inhibit EKC-causing adenoviruses. Saccharide–HSA conjugates could easily be transferred to the eye through a salve or eye drops, and thereby they would escape the metabolic processes of the body. The result presented herein could therefore be useful in efforts to develop an antiviral drug for treatment of EKC.

Experimental Section

General chemical methods and materials: ^{1}H and ^{13}C NMR spectra of compounds **2**, **4**, **5**, **6**, **9**, and **11** were recorded with a Bruker DRX-400 spectrometer at 400 MHz and 100 MHz, respectively. The ^{1}H NMR spectrum of compound **8** was recorded with a Bruker ARX-500 spectrometer at 500 MHz, and its ^{13}C NMR spectrum was recorded with a Bruker DRX-400 spectrometer at 100 MHz. Chemical shifts are referenced to solutions in $CDCl_3$ (residual $CHCl_3$ (δ_H = 7.26 ppm), $CDCl_3$ (δ_C = 77.0 ppm) as internal standard), CD_3OD (residual CD_2HOD (δ_H = 3.35 ppm), CD_3OD (δ_C = 49.0 ppm) as internal standard) or D_2O (residual HDO (δ_H = 4.81 ppm) as internal standard) at 298 K. Chemical shifts and proton resonance assignments were obtained from COSY, TOCSY, ROESY and ^{1}H,^{13}C HMQC experiments. The α-anomeric configuration of the sialic acid residue in **8** was established by determination of the coupling constant[35] between C-1′′ and H-3′′$_{ax}$ (J = 6.7 Hz). Proton resonances that could not be assigned and aromatic resonances are not reported. The mass spectrum for **6** was recorded on a Water micromass ZQ by using negative electrospray ionisation (ES-). High-resolution fast atom bombardment mass spectra (HRMS) were recorded with a JEOL SX102 A mass spectrometer. Ions for FABMS were produced by a beam of xenon atoms (6 keV) from a matrix of glycerol and thioglycerol. Matrix-assisted laser desorption/ionisation time-of-flight mass spectroscopy (MALDI-TOF MS) was carried out with an Voyager DE-STR instrument (Applied Biosystems, Boston, MA). Preparative HPLC separations were performed on a Beckman System Gold HPLC by using a Kromasil Silica gel column (250× 20 mm, 5 μm, 100 Å) with a flow rate of 12 mL min^{-1}, detection at 254 nm and eluent system: A, CH_2Cl_2; and B, EtOH, or a Kromasil C-8 column (250×20 mm, 5 μm, 100 Å) with a flow rate of 11 mL min^{-1}, detection at 254 nm and eluent system: A, aq. 0.1% CF_3CO_2H; and B, 0.1% CF_3CO_2H in MeCN. Analytical HPLC was performed on a Beckman System Gold HPLC with a Kromasil Silica Gel column (250×4.6 mm, 5 μm, 100 Å) and a flow rate of 2.0 mL min^{-1}, detection at 254 nm and eluent system: A, CH_2Cl_2; and B, EtOH, or a Kromasil C-8 column (250×4.6 mm, 5 μm, 100 Å) with a flow rate of 1.5 mL min^{-1}, detection at 254 nm and eluent system: A, aq. 0.1% CF_3CO_2H; and B, 0.1% CF_3CO_2H in MeCN. Column chromatography was performed on Silica Gel (Matrex, 60 Å, 30–70 μm, Grace amicon) and thin layer chromatography (TLC) was carried out on Silica Gel F_{254} (Merck), detected under UV light and developed with aqueous sulfuric acid (10%). Solutions were concentrated by rotary evaporation. Before concentration, the solutions were dried over Na_2SO_4. CH_2Cl_2 and CH_3CN were dried by distillation over CaH_2. DMF was dried by distillation and MeOH was dried over 3 Å molecular sieves. BnBr was purified by distillation before use. All other chemicals were used as received.

2-Azidoethyl 4-*O*-(3,4-*O*-isopropylidene-β-D-galactopyranosyl)-β-D-glucopyranoside (4): NaN_3 (14 mg, 0.21 mmol) and [15]crown-5 (46 mg, 0.21 mmol) were added to a solution of **3** (84 mg, 0.17 mmol) in DMF (1 mL). The mixture was stirred at 75 °C for 25 h and then concentrated at reduced pressure. The resulting yellow oil was lyophilised before purification by column chromatography (CH_2Cl_2/MeOH/H_2O; 65:35:5) to give compound **4** (74.4 mg, 97%); $[\alpha]_D^{20}$ = +15° (c = 1.0, MeOH); ^{1}H NMR (CD_3OD): δ = 1.37, 1.52 (s, 3H each; Me_2CO_2), 3.31 (dd, $J_{H1'}$ = 7.9, $J_{H3'}$ = 9.0 Hz, 1H; H2′), 4.10 (dd, J_{H2} = 7.3, J_{H4} = 5.5 Hz, 1H; H3), 4.24 (dd, J_{H3} = 5.5, J_{H5} = 2.2 Hz, 1H; H4), 4.39 (d, $J_{H2'}$ = 7.9 Hz, 1H; H1′), 4.41 (d, J_{H2} = 8.2 Hz, 1H; H1); ^{13}C NMR (CD_3OD): δ = 26.5, 28.4, 52.0, 61.9, 62.4, 69.4, 74.4, 74.8, 75.0, 75.3, 76.3, 76.4, 79.5, 81.0, 104.2, 104.3, 111.1; HRMS (FAB) calcd for $C_{17}H_{29}N_3O_{11}$: 474.1700 [M+Na], found: 474.1703.

2-Azidoethyl 2,3,6-tri-*O*-benzyl-4-*O*-(2,6-di-*O*-benzyl-3,4-*O*-isopropylidene-β-D-galactopyranosyl)-β-D-glucopyranoside (5): NaH (533 mg, 22.2 mmol) was added gradually to a solution of **4** (990 mg, 2.19 mmol) in DMF (35 mL) at 0 °C. The mixture was stirred at 0 °C for 40 min before benzyl bromide (2.08 g, 12.21 mmol) was added dropwise, and then the mixture was stirred at room temperature for 20 h. MeOH (10 mL) was added at 0 °C, and then the mixture was diluted with toluene and washed with water. The aqueous phase was extracted with toluene, and the combined organic phases were dried and concentrated at reduced pressure. Column chromatography (heptane/EtOAc 6:1→3:1) of the crude product gave the title compound **5** (1.35 g, 68%). $[\alpha]_D^{20}$ = +14° (c = 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ = 1.35, 1.40 (s, 3H; Me_2CO_2), 4.41 (2d, J = 7.8, J = 7.6 Hz, 2H; H1 and H1′), 4.3–4.95 (10H; 5 CH_2Ph); ^{13}C NMR ($CDCl_3$): δ = 26.4, 28.0, 51.0, 68.1, 68.2, 68.9, 72.0, 73.2,

73.2, 73.4, 73.6, 75.0, 75.1, 75.4, 76.3, 79.4, 80.7, 81.8, 82.8, 101.9, 103.6, 109.8, 138.2, 138.4, 138.5, 138.6, 138.9. HRMS (FAB) calcd for $C_{52}H_{59}N_3O_{11}$: 924.4048 [*M*+Na], found: 924.4034.

2-Azidoethyl 2,3,6-tri-*O*-benzyl-4-*O*-(2,6-di-*O*-benzyl-β-D-galacto-pyranosyl)-β-D-glucopyranoside (6): A mixture of TFA and H_2O (9:1, 25 mL) was added to a cooled (0 °C) solution of **5** (1.35 g, 1.57 mmol) in CH_2Cl_2 (25 mL). The reaction mixture was stirred at 0 °C until the hydrolysis of the 3′, 4′-isopropylidene acetal was completed (80 min). The reaction was followed by TLC (toluene/EtOH 10:1). Acetic acid was added to the mixture before concentration at reduced pressure. Reconcentration repeated from toluene and $CHCl_3$ resulted in the partially protected lactoside **6** (1.15 g, quantitative yield) as a light yellow oil. The crude product was used in the next step; MS(ES-) calcd for $C_{49}H_{55}N_3O_{11}$: 907.4 [*M*+$HCOO^-$+1 H), found: 907.3.

2-Azidoethyl 2,3,6-tri-*O*-benzyl-4-*O*-{2,6-di-*O*-benzyl-3-*O*-[methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosyl)onate]-β-D-galactopyranosyl}-β-D-glucopyranoside (8): The crude compound **6** (500 mg, 0.58 mmol), *O*-ethyl S-[methyl(5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosyl)onate] dithiocarbonate (**7**, 691 mg, 1.16 mmol) and powdered molecular sieves (3 Å, 750 mg) were stirred in a mixture of CH_3CN and CH_2Cl_2 (9:4, 20 mL) for 1 h under nitrogen. Silver trifluoromethanesulfonate (265 mg, 1.03 mmol) was added, and the reaction mixture was cooled to −60 °C. Methylsulfenylbromide in CH_2Cl_2 (2.33 M, 421 μL, 0.98 mmol) was added dropwise over 5 min, and the mixture was then stirred for 8 h. Diisopropylamine (1.6 mL, 11.5 mmol) was added, and stirring was continued for 0.5 h. The mixture was allowed to attain room temperature and was then filtered and concentrated at reduced pressure. Column chromatography of the residue (toluene/EtOH 15:1) followed by preparative HPLC gave the title product **8** as a white foam (437 mg, 56 %). $[\alpha]_D^{20} = +5°$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 1.87$ (s, 3 H; Ac), 1.89 (s, 3 H; Ac), 1.98 (s, 3 H; Ac), 2.02 (s, 3 H; Ac), 2.06 (br s, 1 H; H3″$_{ax}$), 2.09 (s, 3 H; -Ac), 2.52 (dd, $J_{H4''} = 4.6$, $J_{H3''ax} = 12.9$ Hz, 1 H; H3″$_{eq}$), 3.76 (s, 3 H; COO*Me*), 4.39 (d, $J_{H2'} = 7.7$ Hz, 1 H; H1′), 4.57 (d, $J_{H2} = 7.7$ Hz, 1 H; H1), 4.89 (m, 2 H; H4″, CH_2Ph), 5.08 (d, $J_{H5''} = 9.9$ Hz, 1 H; NH″), 5.31 (dd, $J = 2.1$, $J = 8.1$ Hz, 1 H; H7″), 5.39–5.45 (m, 1 H; H8″); ^{13}C NMR ($CDCl_3$): $\delta =$ 20.5, 20.7, 20.8, 21.1, 23.2, 36.5 (C3″), 49.3 (C5″), 51.0, 53.0, 62.3, 67.2, 67.9, 68.1, 68.4, 68.7, 69.1, 72.5, 72.7, 73.0, 73.3, 74.9, 75.0, 75.1, 75.4, 76.4, 76.4, 77.2, 78.4, 81.8, 82.8, 98.3 (C2″), 102.3 (C1), 103.6 (C1′), 138.4, 138.4, 138.6, 139.0, 139.1, 168.4 (C1″, $J_{C1'', H3''ax}$ 6.7 Hz), 169.9, 170.0, 170.3, 170.6, 170.8; HRMS (FAB) calcd for $C_{69}H_{82}N_4O_{23}$: 1357.5268 [*M*+Na], found: 1357.5250.

2-Azidoethyl 2,3,6-tri-*O*-benzyl-4-*O*-{2,6-di-*O*-benzyl-3-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-β-D-galactopyranosyl}-β-D-glucopyranoside (9): Compound **8** (100 mg, 0.075 mmol) was stirred in methanolic sodium methoxide (0.03 M, 17 mL) overnight. Water (200 μL) was added, and, after 3 h, the solution was carefully neutralised with silica gel, filtered and concentrated at reduced pressure. Column chromatography of the residue (toluene/EtOH 2:1→1:1) gave 75 mg of the title product **9** (87 %). $[\alpha]_D^{20} = +9°$ ($c = 0.5$, MeOH); ^{1}H NMR ($CDCl_3$): $\delta = 2.00$–2.10 (m, H3″$_{ax}$, 4 H; COC*H*$_3$), 2.76 (dd, $J_{H4''} = 4.4$, $J_{H3''ax} = 13.0$ Hz, 1 H; H3″$_{eq}$), 3.31 (br t, $J = 8.6$ Hz, 1 H; H2′), 4.11, (dd, $J = 3.3$, $J = 9.7$ Hz, 1 H; H4); ^{13}C NMR ($CDCl_3$): $\delta = 22.6$, 40.0 (C3″), 52.2, 53.7, 64.5, 69.0, 69.3, 69.3, 69.9, 70.3, 70.5, 72.8, 74.2, 74.5, 74.9, 75.5, 76.0, 76.1, 76.2, 76.4, 77.2, 77.6, 79.3, 82.9, 83.8, 100.8 (C2″), 103.9, 104.7, 139.5, 139.9, 140.0, 140.1, 140.4, 172.1, 175.4; HRMS (FAB) calcd for $C_{60}H_{72}N_4O_{19}$: 1175.4689 [*M*+Na], found: 1175.4684.

2-Aminoethyl 4-*O*-{3-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-β-D-galactopyranosyl}-β-D-glucopyranoside (2): HCl (0.5 M, 0.44 mL, 0.22 mmol) and Pd/C (10 %, 80 mg) were added to a solution of **9** (57 mg, 49.5 μmol) in EtOH (40 mL). The mixture was hydrogenated (H_2(g), 1 atm) for 3 h, filtrated through Celite, concentrated at reduced pressure and lyophilised to provide the title compound **2** pure enough for further synthesis (33 mg, 98 %) (in some cases it was necessary to purify the compound with preparative HPLC). $[\alpha]_D^{20} = -1.2°$ ($c = 0.3$, H_2O); ^{1}H NMR (D_2O): $\delta = 1.87$ (br t, 1 H; H3″$_{ax}$), 2.07 (s, 3 H; Ac), 2.79 (m, 1 H; H3″$_{eq}$), 4.57 (m, 2 H; H1, H1′); ^{13}C NMR ($CDCl_3$): $\delta = 22.1$, 39.3, 39.5, 51.7, 60.0, 61.0, 62.9, 65.9, 67.6, 67.9, 68.2, 69.4, 71.4, 72.8, 73.1, 74.3, 74.8, 75.2, 75.5, 78.2, 99.2, 102.0, 102.7, 172.6, 175.1; HRMS (FAB) calcd for $C_{25}H_{43}N_2O_{19}$: 721.2254 [*M*+2 Na−H], found: 721.2245.

2-[(2-Decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethyl 4-*O*-{3-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-β-D-galactopyranosyl}-β-D-glucopyranoside (11): Didecylsquarate **10** (58 mg, 0.15 mmol) and Et_3N (4.6 μL, 33 μmol) were added to a solution of **2** (16.5 mg, 24.3 μmol) in DMF (1.5 mL). The reaction mixture was stirred at room temperature for 21 h before additional didecylsquarate (29 mg, 0.08 μmol) and Et_3N (2.3 μL, 17 μmol) were added. After 24 h, the solution was concentrated at reduced pressure, and the residue was purified by column chromatography (CH_2Cl_2/MeOH/H_2O 70:25:5) to afford the title product **11** (11 mg, 49 %). Analytical data were in agreement with those previously reported by Bergh et al.[30]

Conjugation of 11 to HSA (12 a–e): Compound **11** (**a** 1.9 mg, 2.08 μmol; **b** 1 mg, 1.09 μmol; **c** 2 mg, 2.19 μmol; **d** 4 mg, 4.38 μmol and **e** 6.7 mg, 7.34 μmol) was added to HSA (**a** 24 mg, 0.36 μmol; **b** 12 mg, 0.18 μmol; **c** 8 mg, 0.12 μmol; **d** 8 mg, 0.12 μmol and **e** 13 mg, 0.20 μmol) in $NaHCO_3$ buffer (0.5–1 mL, pH 9.0, 20 g $NaHCO_3$ in 1000 mL H_2O), and the mixture was stirred at room temperature for 24 h. The reaction mixture was then dialysed against water (2×1000 mL) and lyophilised affording the neoglycoprotein **12** as a white powder (**a** 30 mg, **b** 11.4 mg, **c** 9.1 mg, **d** 9.3 mg and **e** 14.7 mg). The average degree of incorporation (**a** 3 glycosides/HSA, **b** 4 glycoside/HSA, **c** 12 glycosides/HSA, **d** 17 glycoside/HSA and **e** 19 glycoside/HAS) was determined by MALDI-TOF MS by using the centre of the single charged neoglycoprotein peak.

Cells and viruses: Human corneal epithelial (HCE) cells were grown as monolayers in steroid hormone epithelial medium (SHEM) as described previously.[42] The correct identity of Ad37 (strain 1477) was ascertained by restriction enzyme patterns according to Adrian et al. 1986.[43] Virions of corresponding strains were then propagated with or without ^{35}S-labelling, as described previously.[39,40] The specific radioactivity of ^{35}S-labelled particles was 1×10^{-5} cpm per virion.

Binding assay: 2×10^9 ^{35}S-labelled virions/well in 96-well microplates were incubated in binding buffer (100 μL; BB: Dulbecco's modified eagle's medium (DMEM, Sigma chem. co.), pH 7.4, containing 1 % bovine serum albumin (Sigma chem. co.)) with or without 3′-sialyllactose, **12 b**, **12 c** or **12 d** in concentrations as indicated in Table 1, at +4 °C for 1 h. These mixtures were then added to 2×10^5 cells prepelleted in another 96-well plate. After resuspension, the mixtures were incubated at +4 °C for 1 h in order to obtain equilibrium between bound and unbound virions. After the cells had been washed, the cell-associated radioactivity was counted by using a Wallac 1409 scintillation counter. Data are presented as % of control, that is, the value obtained in the absence of inhibitor.

Fluorescent focus assay: 2×10^9 unlabelled virions per well in 24-well plates were incubated in BB (0.5 mL), with or without 3′-sialyllactose **1** or **12e** in concentrations as indicated in Table 2, at 4 °C. After 1 h, the mixtures were transferred to new 24-well plates containing 2×10^5 adherent HCE cells per well and incubated at 4 °C, thereby allowing virions to bind, but not infect. One hour later, when equilibrium had been achieved, unbound virions were removed by washing and incubated at 37 °C. In this way, a synchronised infection was obtained: that is, all virions entered the cells at the same time. 44 h after infection, the cells were rinsed in phosphate-buffered saline (PBS), fixed with 99% methanol and incubated with rabbit polyclonal anti-Ad37 antibodies diluted 1:200 in PBS pH 7.4 at RT. One hour later, the cells were washed in PBS and stained with FITC-labelled swine anti-rabbit IgG antibodies (Dakocytomation, Glostrup, Denmark) diluted 1:200 for 1 h at RT. Finally, the cells were washed and examined in an immunofluorescence microscope (Xiovert 25, Carl Zeiss, Germany; 10× magnification). Data are presented as % of control, that is, the value obtained in the absence of inhibitor.

Keywords: antiviral agents · glycoconjugates · glycosylation · multivalency · sialic acids

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