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Multivalent sialic acid conjugates inhibit adenovirus type 37 from binding to and infecting human corneal epithelial cells

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Abstract

Adenovirus type 37 is one of the main causative agents of epidemic keratoconjunctivitis. In a series of publications, we have reported that this virus uses sialic acid as a cellular receptor. Here we demonstrate *in vitro* that on a molar basis, multivalent sialic acid conjugated to human serum albumin prevents adenovirus type 37 from binding to and infecting human corneal epithelial cells 1000-fold more efficiently than monosaccharidic sialic acid. We also demonstrate that the extraordinary inhibitory effect of multivalent sialic acid is due to the ability of this compound to aggregate virions. We conclude that multivalent sialic acid may be a potential new antiviral drug, for use in the treatment of epidemic keratoconjunctivitis caused by the adenoviruses that use sialic acid as cellular receptor.

Keywords: Adenovirus; EKC; Sialic acid; Multivalent

1. Introduction

The adenovirus family has been classified into six species (A–F) (Benkö et al., 2000). Species D is the largest species, with 31 different serotypes. Three species D serotypes, adenovirus type 8 (Ad8), Ad19, and Ad37 cause severe ocular disease designated EKC (epidemic keratoconjunctivitis (Ford et al., 1987)). EKC-causing adenoviruses are usually spread by direct contact (Azar et al., 1996; Buffington et al., 1993; Jernigan et al., 1993), and possibly as a result of this, they are more common in densely populated areas of the world. Between 500,000 and one million individuals fall ill with EKC every year in Japan alone (Aoki and Tagawa, 2002). Besides keratitis and conjunctivitis, EKC is characterized by pain, edema, lacrimation, photophobia, hemorrhages and formation of pseudomembranes (Ford et al., 1987). At present there are no drugs available against EKC, but some (i.e. NMSO3) are under development (Kaneko et al., 2001).

Whereas most other adenovirus serotypes appear to use CAR (coxsackie adenovirus receptor) or MCP (membrane cofactor protein; CD46) as cellular receptors (Bergelson et al., 1997; Gaggar et al., 2003; Marttila et al., 2005; Roelvink et al., 1998; Segerman et al., 2003; Tomko et al., 1997), the EKC-causing serotypes use sialic acid (SA) saccharides as cellular receptors. For Ad37 it has been demonstrated that the sialic acid receptor is preferentially linked via α2,3 glycosidic bonds to galactose saccharides (α2,3 SA) (Arnberg et al., 2000a,b, 2002a,b; Burmeister et al., 2004; Cashman et al., 2004). Since the sialic acid-interacting domain (the knob domain) of the fiber protein of Ad37 is highly homologous to the knobs of two other EKC-causing adenoviruses (Arnberg et al., 1997), it is to be expected that Ad8 and Ad19a would also use α2,3 SA as a cellular receptor. The sialylated receptor is either a glycoprotein or a ganglioside that containing a complex glycan motif (unpublished results in collaboration with Dr. Susann Teneberg, Göteborg University, Göteborg, Sweden). We demonstrated recently that a precursor (sialyllactose [SLA]) to this motif inhibited Ad37 from infecting HCE (human corneal epithelial) cells at concentrations around 1 mM, whereas multivalent SLA linked to human serum albumin (SLA-HSA) efficiently inhibited Ad37 from binding to and infecting HCE cells at 100- to 1000-fold lower concentrations (Johansson et al., 2005). Based on these findings, we hypothesized that the mechanism by which multivalent sialic acid had a neutralizing effect on Ad37 virions was due to aggregation of virions.

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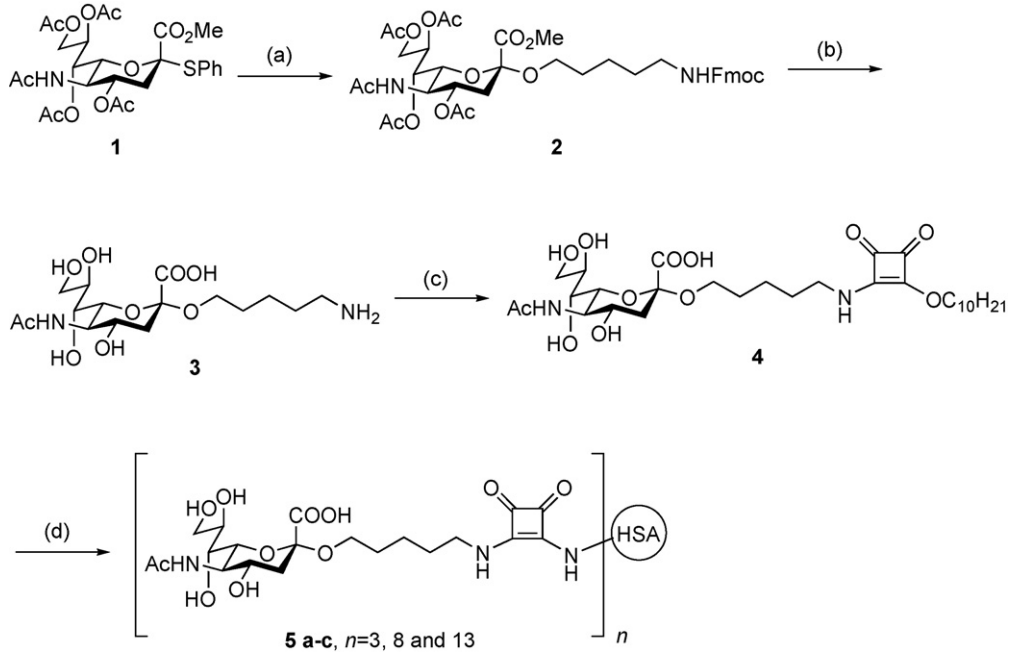
In addition, we have recently crystallized the sialic acid-interacting knob domain of the Ad37 fiber protein in complex with SLA (Burmeister et al., 2004), and found that neither the galactose nor the glucose saccharides contribute to the interaction with the knob of Ad37. Instead, the interaction is mediated entirely by the sialic acid saccharide. Thus, we hypothesized that multivalent sialic acid would inhibit Ad37 from binding to and infecting HCE cells to an extent similar to multivalent SLA. The major advantage of sialic acid conjugates compared to SLA conjugates is that less complicated synthesis is required. Here, we present a short and simple synthetic route to a sialic acid derivative **3** and corresponding multivalent HSA conjugates. Evaluation of these compounds as inhibitors of binding and infection of EKC-causing adenoviruses to host cells revealed a significant multivalency effect similar to that previously obtained for the SLA conjugates. We also present a novel method to quantify the aggregation of Ad37 virions by multivalent sialic acid.

2. Materials and methods

2.1. General chemical methods and materials

^{1}H and ^{13}C NMR spectra of compounds **2** and **4** (see Scheme 1) were recorded with a Bruker DRX-400 spectrometer (BrukerBiospin GmbH, Rheinstetten, Germany) at 400 MHz and 100 MHz, respectively. Chemical shifts are referenced to solutions in CD_3OD [residual CD_2HOD (δ_H 3.35 ppm), CD_3OD (δ_C 49.0 ppm) as internal standard] at 298 K. Chemical shifts and proton resonance assignments were obtained from COSY and ^{1}H–^{13}C-HMQC experiments. The α-anomeric configuration of the sialic acid residue in **2** was established by determination of the coupling constant according to Hori et al. (1988) between C-1 and H-3_{ax} ($J = 6.6$ Hz). Proton resonances that could not be assigned and aromatic resonances are not reported. The mass spectrum for **3** was recorded on a Water micromass ZQ (Waters, Milford, MA, USA) using positive electrospray ionization (ES+). High-resolution fast atom bombardment mass spectra (HRMS) were recorded with a JEOL SX102 A mass spectrometer (JEOL USA Inc., Peabody, MA, USA). Ions for FABMS were produced by a beam of xenon atoms (6 keV) from a matrix of glycerol and thioglycerol. Matrix-assisted laser desorption/ionization-time of flight mass spectroscopy (MALDI-TOF MS) was carried out with a Voyager DE-STR instrument (Applied Biosystems, Boston, MA, USA). Optical rotations were measured with a Perkin-Elmer 343 polarimeter (Perkin-Elmer, Wellesley, MA, USA) at 20 °C. Preparative high-performance liquid chromatography (HPLC) separations were performed on a Beckman System Gold HPLC (Beckman Coulter Inc., Fullerton, CA, USA), using a Kromasil C-8 column (250 mm × 20 mm, 5 μm, 100 Å) (Highchrom Ltd., Berkshire, UK) with a flow rate of 11 mL/min, detection at 214 nm and with the following 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, using a Kromasil C-8 column (250 mm × 4.6 mm, 5 μm, 100 Å; Highchrom Ltd.) with a flow rate of 1.5 mL/min, detection at 214 nm and eluent system as above. Column chromatography was performed on Silica Gel (Matrex Millipore Corp., Bedford, MA, USA), 60 Å, 30-70 μm, Grace Amicon and thin layer chromatography (TLC) was carried out on Silica Gel F_{254} (Merck, Darmstadt, Germany), detected under UV light and developed with aqueous sulphuric acid (10%). Solutions were concentrated using rotary evaporation. CH_2Cl_2 and CH_3CN were dried by distillation over CaH_2. Dimethylformamide (DMF) was dried by distillation and



Scheme 1. Synthesis of multivalent HAS conjugates of sialic acid. Reagents and conditions: (a) Fmoc-aminopentanol, AgOTf, 1_M IBr in CH_2Cl_2, −72 °C, CH_3CN:CH_2Cl_2 (3:2), pure α 26%; (b) NaOMe, MeOH 2. LiOh; (c) didecylsquarate, DMF, Et_3N, 62% from **2**; (d) $NaHCO_3$ (pH 9.0). HSA = human serum albumin, DMF = dimethylformamide.

MeOH was dried over 3 Å molecular sieves. All other chemicals were used as received.

2.2. 5-Fmoc-aminopenthyl methyl(5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonylopyranosyl)onate (2)

1 (Marra and Sinay, 1989; Waglund and Claesson, 1992) (216 mg, 0.37 mmol), Fmoc-aminopentanol (482 mg, 1.48 mmol) and powdered molecular sieves (3 Å, 750 mg) were stirred in a mixture of CH_3CN and CH_2Cl_2 (3:2, 12.5 mL) for 1 h under nitrogen. Silver trifluoromethanesulfonate (190 mg, 0.74 mmol) was added and the reaction mixture was cooled to −72 °C. Iodinemonobromide in CH_2Cl_2 (1 M, 492 μL, 0.49 mmol) was added drop-wise during 5 min and the mixture was then stirred for 3 h. Di-isopropylethylamine (550 μL, 2.2 mmol) was added and stirring was continued for 30 min. The mixture was allowed to attain room temperature and was then filtered and concentrated at reduced pressure. Repeated column chromatography of the residue (Toluene:Acetone; 3:1) followed by preparative HPLC gave pure α isomer of the title product **2** as a white powder (83 mg, 26%). Purification of the discarded fractions with α/β mixtures, using a Daicel-chiralcel OD column (250 mm × 20 mm, 10 μm, Chiral Technologies, Illkirch, France) resulted in additional 56 mg pure α; $[\alpha]_D^{20}$ −8.5° (*c* 1.0, MeOH). ^{1}H NMR (CD_3OD): δ = 1.3–1.6 (m, 6H, –$OCH_2CH_2CH_2CH_2CH_2NH$–), 1.83 (t, 1H, J_{H3eq} = 12.6 Hz, $H3_{ax}$), 1.87 (s, 3H, Ac), 2.01 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.13 (s, 3H, Ac), 2.17 (s, 3H, Ac), 2.64 (dd, 1H, J_{H4} = 4.5 Hz, J_{H3ax} = 12.6 Hz, $H3_{eq}$), 3.15 (t, 2H, $J_{OCH_2CH_2}$ = 6.9 Hz, –OCH_2CH_2–), 3.25–3.32 (m, 1H, –CH_2CH_2NH–), 3.75–3.82 (m, 1H, –CH_2CH_2NH–), 3.84 (s, 3H, –$COOCH_3$), 3.99 (t, 1H, J_{H6} = 10.6 Hz, H5), 4.09 (dd, 1H, J_{H8} = 2.2 Hz, J_g = 12.4 Hz, H9), 4.21 (dd, 1H, J_{H7} = 1.7 Hz, J_{H5} = 10.6 Hz, H6), 4.26 (t, 1H, J_{OCH_2CH} = 6.76 Hz, –OCH_2CH–), 4.35 (dd, 1H, J_{H8} = 5.5 Hz, J_g = 12.4 Hz, H9), 4.40 (d, 2H, J_{OCH_2CH} = 6.76 Hz, –OCH_2CH–), 4.78–4.86 (m, 1H, H4), 5.36, 5.4–5.5 (m, 1H, H8); ^{13}C NMR ($CDCl_3$): δ = 20.65, 20.70, 20.86, 21.23, 22.67, 24.12, 30.30, 30.35, 39.19 (C3), 41.69 (–OCH_2CH_2), 48.61 (–OCH_2CH–), 50.16 (C5), 53.15 (–$COOCH_3$), 63.57 (C9), 65.72 (–CH_2CH_2NH–), 67.52 (–OCH_2CH–), 68.73 (C7), 69.61 (C8), 70.78 (C4), 73.13 (C6), 100.07 (C2), 120.92, 126.17, 128.14, 128.76, 142.62, 145.39, 158.86, 169.79 (C1, $J_{C1, H3ax}$ = 6.6 Hz), 171.58, 171.78, 171.78, 172.43,173.51. HRMS (FAB) calculated for $C_{40}H_{50}N_2O_{15}$ (*M* + Na) 821.3109, found 821.3117.

2.3. 5-Aminopenthyl 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonylopyranosylonic acid (3)

Compound **2** (40.5 mg, 0.051 mmol) was stirred in methanolic sodium methoxide (0.03 M, 5 mL) for 1 h. The solution was neutralized with silica gel, filtered, concentrated and dissolved in MeOH before LiOH (64 μL, 1 M) was added. The solution was stirred at room temperature (RT) over night and then carefully neutralized with silica gel, filtered and concentrated at reduced pressure. The residue was dissolved in water and filtered through a supelco C18 column (Sigma–Aldrich, St. Louis, MO, USA). Lyophilisation gave 23 mg of the crude product **3**. The crude product was used in the next step; MS(ES+) calculated for $C_{16}H_{30}N_2O_9$ (*M* + 1H$^+$) 394.2, found 394.9.

2.4. 5-[(2-Decyloxy-3,4-dioxocyclobut-1enyl)amino]penthyl 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonylopyranosylonic acid) (4)

To a solution of crude **3** (23 mg) in DMF (3 mL) were added didecylsquarate as previously described (Blixt and Norberg, 1999; Tietze et al., 1991) (64.5 mg, 0.16 mmol) and Et_3N (9.8 μL, 71 μmol). The reaction mixture was stirred at RT for 5 h before the solution was concentrated at reduced pressure and the residue was purified by column chromatography (CH_2Cl_2:MeOH:H_2O; 70:25:5), affording 20 mg (62%, two steps) of the title product 4; $[\alpha]_D^{20}$ −1.3° (*c* 1.0, MeOH). ^{1}H NMR (CD_3OD): δ = 0.93 (t, 3H, $J_{CH_2CH_3}$ = 6.9 Hz, –CH_2CH_3), 1.8–1,9 (m, 2H, –C(sp2)OCH_2CH_2), 2.04 (s, 3H, Ac), 2.83 (dd, 1H, J_{H4} = 3.6 Hz, J_{H3ax} = 12.1 Hz, $H3_{eq}$), 4.7–4.8 (m, 2H, –C(sp2)OCH_2CH_2); HRMS (FAB) calculated for $C_{30}H_{50}N_2O_{15}$ (*M* + Na) 653.3261, found 653.3270.

2.5. Conjugation of 4 to HSA (5a–c)

Compound **4** (**a** 1.1 mg, 1.7 μmol; **b** 1.7 mg, 2.6 μmol; **c** 9 mg, 14 μmol) was added to HSA (Sigma–Aldrich) (**a** 19 mg, 0.29 μmol; **b** 14.5 mg, 0.22 μmol; **c** 25 mg, 0.38 μmol) in 0.5–1 mL $NaHCO_3$ buffer (note: 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 dialyzed against water (2 × 1000 mL) and lyophilised affording the neoglycoproteins as a white powder (**a** 14 mg; **b** 13 mg; **c** 25 mg). The average degree of incorporation (**a** 3 glycosides/HSA; **b** 8 glycosides/HSA; **c** 13 glycosides/HSA [SA-HSA]) was determined by MALDI-TOF MS (Applied Biosystems) using the center of the single charged neoglycoprotein peak.

2.6. Cells, viruses and antibodies

2.6.1. Cells

HCE (human corneal epithelial) and A549 cells were grown as described (Araki-Sasaki et al., 1995; Arnberg et al., 2000a).

2.6.2. Viruses

Ad37 (strain 1477) virions were produced as follows: 0.5 mL of Ad37 inoculation material (prepared from infected A549 cells) was added to A549 cells (175 cm^2 flasks) and incubated for 2 h at 37 °C. Non-internalized viruses were removed by washing, and the cells were further incubated at 37 °C in Dulbecco's modified Eagle's Medium (DMEM; Sigma–Aldrich) supplemented with 1% fetal calf serum (FCS; Sigma–Aldrich). About 72 h later, the cells were pelleted, resuspended in Tris–HCl, pH 7.4, and freeze-thawed three times. After another round of centrifugation, the supernatant was loaded onto a discontinuous CsCl gradient (densities: 1.27 g/mL, 1.32 g/mL, and 1.37 g/mL, in

20 mM Tris–HCl, pH 8.0; Sigma–Aldrich) and centrifuged at 25,000 rpm (SV40Ti rotor, Beckman L5-65B ultracentrifuge; Beckman Coulter Inc.) for 2.5 h at +4 °C. The virion band was harvested and desalted on a NAP column (Amersham Biosciences AB, Uppsala, Sweden) in sterile DMEM buffer supplemented with 10% glycerol (Sigma–Aldrich). Aliquoted virions were then stored at −80 °C until further use. ^{35}S-labeled Ad37 virions were produced as above with the following exceptions: 24 h post infection the cells were starved for 2 h in methionine-cysteine-free DMEM (Sigma–Aldrich). Thereafter isotope (1.4 mCi/flask; NEG-772 Easytag express protein labeling mix; Perkin-Elmer) was added. L-Cysteine (final concentration 2 mM; Sigma–Aldrich;) was added 26 h and 50 h post-infection and L-methionine (final concentration 1 mM; Sigma–Aldrich) was added 31 h and 50 h post-infection. The specific radioactivity of labeled virions was determined to be 4×10^{-6} cpm per virion. The identity of Ad37 was determined by digesting viral DNA with restriction enzymes and compared to established patterns for Ad37 prototype strain (Wadell et al., 1981).

2.6.3. Antibodies

Rabbit polyclonal anti-Ad37 serum was prepared as described previously (Wadell et al., 1999).

2.7. Binding assay

2×10^9 ^{35}S-labeled Ad37 virions per well was incubated in 100 μL binding buffer (BB: DMEM containing 1% bovine serum albumin [Roche AB, Stockholm, Sweden], penicillin/streptomycin [Gibco, Carlsbad, CA, USA] and hepes [EuroClone, Milano, Italy]), pH 7.5, at +4 °C, together with different concentrations of 3-, 8- and 13-valent SA-HSA or mono-, di-, or trisialic acid (Taiyo Kagaku Co., Ltd., Yokkaichi, Japan) in 96-well plates. After 1 h the mixtures were added to V-shaped 96-well plates containing pre-pelleted HCE cells (2×10^5 cells/well). After resuspension of cells, the mixtures were incubated for an additional hour at +4 °C. After two washes in BB, the cell-associated radioactivity was measured in a Wallac 1409 liquid scintillation counter (Perkin-Elmer).

2.8. Infectivity assay

Non-labeled Ad37 virions (2×10^9 per well in 500 μL BB) were incubated at +4 °C in 24-well plates together with different concentrations of 3-, 8- and 13-valent SA-HSA or SA. After 1 h, the mixtures were added to HCE cells grown as monolayers on glass slides in 24-well plates (2×10^5 cells/well) and incubated on ice. One hour later, the wells were washed three times with 1% SHEM (supplemented hormone epithelial medium (Araki-Sasaki et al., 1995)) in order to remove unbound virions, and incubated for 44 h at 37 °C to allow infection. The glass slides were rinsed with phosphate-buffered saline (PBS pH 7.4), fixed with methanol (99%) and incubated with a rabbit polyclonal anti-Ad37 serum (diluted 1:200 in PBS) at RT. One hour later, the slides were washed three times in PBS and incubated with swine anti-rabbit FITC-labeled antibody in RT (diluted 1:200 in PBS). The slides were mounted with fluorescent mounting medium (DakoCytomation, Glostrup, Denmark) and examined in an immunofluorescence microscope (Axioskop2, Carl Zeiss, Germany; 10× magnification).

2.9. Aggregation assay

^{35}S-labeled Ad37 virions (5×10^8 per well) were incubated with or without 13-valent SA-HSA (0.05 mM with respect to sialic acid) in BB at +4 °C. One hour later, the samples were centrifuged at different speeds (1000 rpm, 4000 rpm, 7000 rpm, 10,000 rpm, 13,000 rpm) using a Beckman Coulter Microfuge 22R Centrifuge (Beckman Coulter Inc., Fullerton, CA, USA). The radioactivity in the supernatant (top 90 μL) and in the pellet (lower 10 μL) was measured using a liquid scintillation counter as described above.

3. Results

3.1. Monovalent sialic acid or sialic acid-containing oligosaccharides inhibit binding of Ad37 to HCE cells with similar efficiency

Based on recent crystallography data, demonstrating that the only saccharide in the SLA trisaccharide that interacts directly with the Ad37 knob was sialic acid (Burmeister et al., 2004), we hypothesized that other sialic acid containing saccharides would inhibit Ad37 virions from binding to HCE cells to the same extent as 3′SLA trisaccharides. Mono-, di-, tri-sialic acid saccharides as well as SLA were incubated first with isotope-labeled Ad37 virions and then with HCE cells in suspension. Cell-associated virions were then measured as described in Section 2. All monovalent saccharides inhibited Ad37 binding to HCE cells approximately to the same extent. 3′SLA, sialic acid and disialic acid inhibited 50% of Ad37 binding (IC_{50}) at 2–5 mM (Fig. 1), whereas the IC_{50} of trisialic acid was approximately 1 mM.

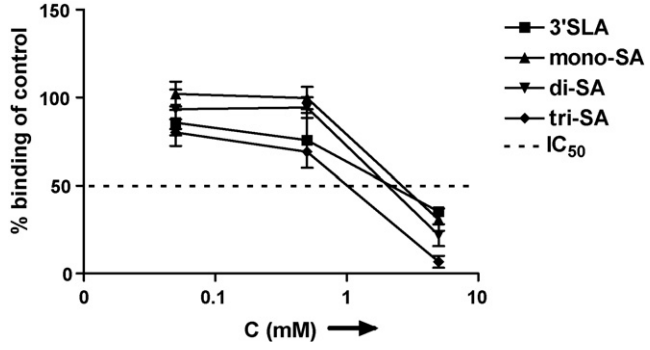


Fig. 1. Monovalent sialic acid saccharides inhibit Ad37 from binding to HCE cells. Purified ^{35}S-labeled Ad37 virions were pre-incubated with saccharides, further incubated with HCE cells, and analyzed in a scintillation counter for cell-associated radioactivity as described in Section 2. The data represents an average of at least three independent experiments.

3.2. Synthesis of multivalent HSA conjugates of sialic acid

Since non-conjugated sialic acid was as potent as non-conjugated 3′SLA at inhibiting binding of Ad37 to HCE cells, we expected that multivalent HSA conjugates of sialic acid would be as potent as those of 3′SLA. Multivalent conjugates of sialic acid were obtained in a short and simple synthetic route compared to the more complex 11 step synthesis of the multivalent 3′SLA conjugates (Johansson et al., 2005). The sialic acid derivative **3** with an aminopentyl spacer, which can be conjugated to a protein carrier, was selected as target in order to find a new simpler synthetic inhibitor of EKC-causing adenoviruses. Sialic acid was used as starting material and synthesis of the sialic acid thiophenyl donor (**1**, Scheme 1) was performed by published procedures (Marra and Sinay, 1989; Waglund and Claesson, 1992). Commercially available Fmoc-aminopentanol was sialylated with **1**, by using the new efficient high yield promoter system, iodine monobromide and silver trifluoromethanesulfonate in a mixture of dichloromethane and acetonitrile at low temperature, as described by others (Meijer and Ellervik, 2002). This gave the 5-Fmoc-aminopentyl sialoside **2** in 90% yield as an α:β/7:1 mixture. Extensive purification by repeated column chromatography and preparative HPLC, gave pure α product in 26% yield. By using a chiral HPLC preparative column it was possible to separate the two isomers completely and thereby increase the yield significantly. The α-anomeric configuration of the sialic acid residue was established by determination of the coupling constant (Hori et al., 1988) between C-1 and H-3_{ax} ($J = 6.6$ Hz). The *O*-acetates and the Fmoc group of **2** were simultaneously removed in methanolic sodium methoxide, followed by hydrolysis of the sialic acid methyl ester by LiOH, resulting in the fully deprotected sialic acid aminopentyl derivative **3**. The crude product was filtered through a supelco C18 column and used without further purification. Conjugation of **3** to HSA was performed via the squaric decyl ester sialoside **4** (Blixt and Norberg, 1999; Tietze et al., 1991) to give the neoglycoprotein **5**, following the procedures described by others (Bergh et al., 2001). By varying the equivalents of **4**, the degree of incorporation of saccharide to HSA could be varied resulting in three neoglycoproteins, **5a**, **5b** and **5c** with 3, 8 and 13 incorporated saccharides per HSA, respectively. The average degree of incorporation was determined by MALDI-TOF MS using the center of the single-charged neoglycoprotein peak.

3.3. Multivalent sialic acid is 1000-fold more efficient than monovalent sialic acid in inhibiting binding of Ad37 to HCE cells

To test whether multivalent sialic acid could inhibit Ad37 from binding to HCE cells to the same extent as multivalent 3′SLA (Johansson et al., 2005), we used mono-, 3-, 8- and 13-valent sialic acid for inhibition of Ad37 binding to HCE cells in the binding assay described above and in Section 2. Mono-, 3-, 8- or 13-valent sialic acid inhibited 50% of the binding at 1 mM, 20 μM, 1 μM and <1 μM, respectively (all with respect to sialic acid saccharide; Fig. 2). Thus, each sialic acid saccharide in the 8- and 13-valent SA-HSA complex was more than 1000-fold more efficient in inhibiting Ad37 from binding to HCE cells as compared to monovalent sialic acid. For mono- and 13-valent sialic acid, the IC_{50} values were in the same range as describe previously for mono- and 12-valent 3′SLA (Johansson et al., 2005).

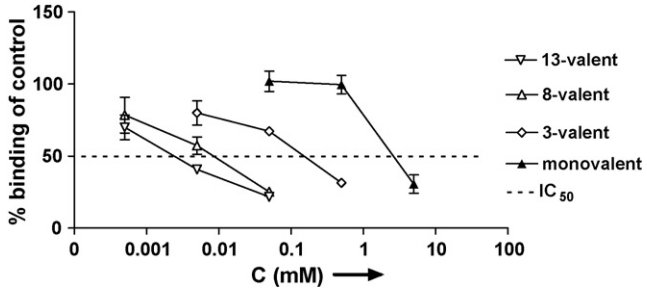


Fig. 2. Multivalent sialic acid saccharides inhibit Ad37 from binding to HCE cells. Purified ^{35}S-labeled Ad37 virions were pre-incubated with mono- or multivalent sialic acid, further incubated with HCE cells, and analyzed in a scintillation counter for cell-associated radioactivity as described in Section 2. The data represents an average of at least three independent experiments.

3.4. Multivalent sialic acid is 1000-fold more efficient than monovalent sialic acid in inhibiting Ad37 infection in HCE cells

To test the inhibitory effect of mono- and multivalent sialic acid at both binding and infection level, we incubated purified (non-labeled) Ad37 virions with mono-, 8- or 13-valent sialic acid and allowed them to infect HCE cells as described in Section 2. As expected, sialic acid inhibited Ad37 from infecting HCE cells in a valency-dependent manner: monovalent sialic acid at concentrations up to 5 mM inhibited infectivity, but not as much as 50%. 8-valent, and 13-valent sialic acid on the other hand inhibited 50% of the infectivity at approximately 3 μM (Fig. 3). Thus, 8- and 13-valent sialic acid reduced Ad37 infection more than 1000-fold as compared to monovalent sialic acid.

3.5. Multivalent sialic acid neutralizes Ad37 by aggregating viral particles

In order not only to confirm, but also to quantify the mechanism of aggregation, we set up a novel experiment, where ^{35}S-labeled virions were incubated with or without 13-valent sialic acid, centrifuged at increasing centrifugal force (*g*) followed by separation of the radioactivity in supernatant (top 90 μL) from the radioactivity "pellet" (lower 10 μL). Scintillation analysis of the radioactivity in the two fractions revealed that increasing (*g*) pelleted ^{35}S-labeled Ad37 virions, but that the virions that had been incubated with 13-valent sialic acid were pelleted more efficiently (Fig. 4). The majority of "free" virions (incubated without sialic acid) in the supernatant even at the highest (*g*), whereas "bound" virions (incubated with 13-valent sialic acid) were pelleted at lower (*g*).

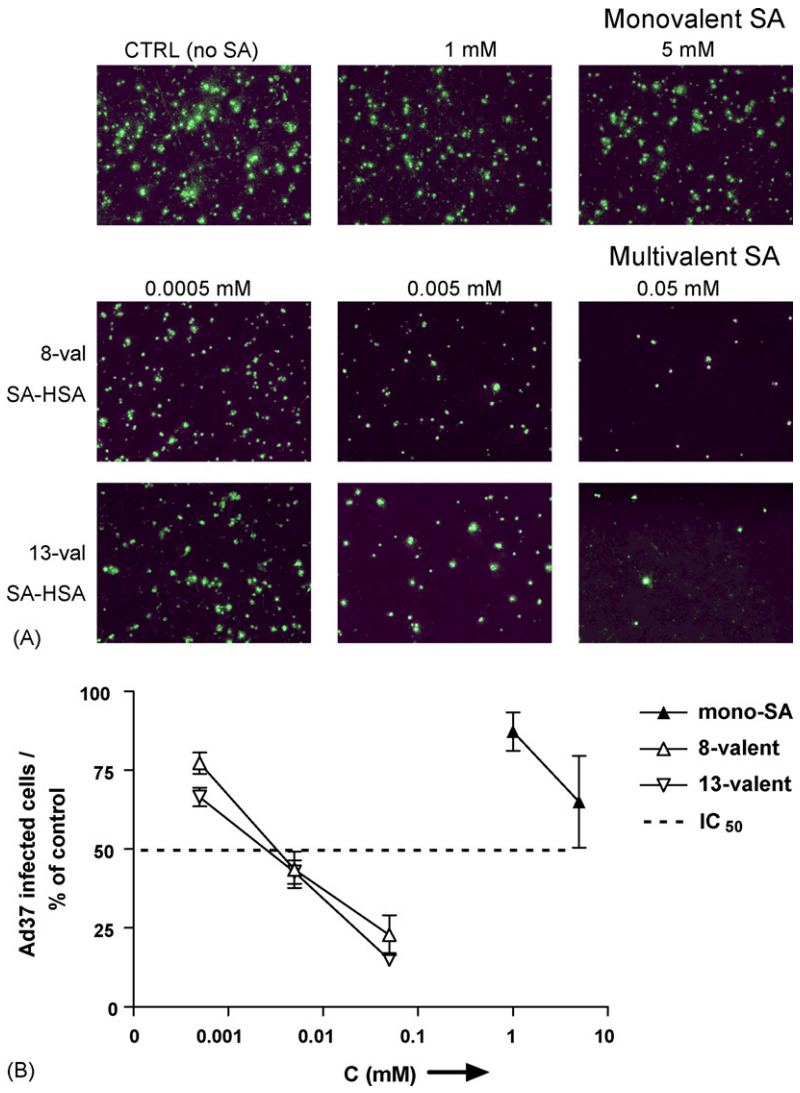


Fig. 3. Multivalent sialic acid protects HCE cells from infection by Ad37. (A) Non-labeled Ad37 virions were pre-incubated with mono- or multivalent sialic acid and further incubated with HCE cells first on ice for 1 h and thereafter at 37 °C for 44 h. The cells were thereafter fixed, stained and analyzed in fluorescence microscope as described in Section 2. (B) Quantification of (A). Each yellow-green dot corresponds on one infected cells. The data represents an average of at least three independent experiments.

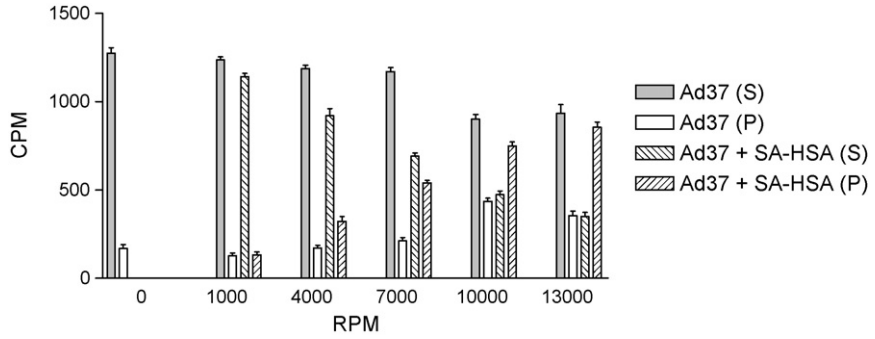


Fig. 4. Multivalent sialic acid aggregates Ad37 virions. ^{35}S-labeled Ad37 virions were incubated with or without 13-valent sialic acid and thereafter centrifuged as indicated. The supernatant (S; top 90 μL) was separated from the pellet (P; lower 10 μL) and analyzed using scintillation counter (see Section 2 for details). The data represents an average of at least three independent experiments.

4. Discussion

The human eye can be infected by multiple pathogens, including many viruses. We have recently described that many ocular viruses, including (i) EKC-causing adenoviruses, (ii) AHC (acute hemorrhagic conjunctivitis)-causing picornaviruses and (iii) avian influenza viruses use, or may use α2,3-linked sialic acid as cellular receptors on human ocular cells (Olofsson et al., 2005). Based on this, we have hypothesized that it would be possible that the ocular infections caused by these viruses could be treated with so called binding inhibitors, consisting of sialic acid saccharides that are able to inhibit viruses from binding to cell surface sialic acid by occupying the sialic acid-binding sites on the viral ligands. We have also hypothesized that this could be more efficient if the binding inhibitor is multivalent with respect to sialic acid, since this structure would aggregate virions, and consequently neutralize these without occupying every single viral ligand (Fig. 5). This hypothesis has been confirmed using mono- and multivalent 3′SLA, where multivalent SLA inhibited binding and infectivity of Ad37 much more efficient as compared to monovalent SLA (Johansson et al., 2005). However, recent crystallography data has indicated that less complex sialoconjugates would be equally efficient as SLA (Burmeister et al., 2004). Therefore we set out to investigate whether mono- and multivalent sialic acid could mimic the effects obtained previously by mono- and multivalent 3′SLA.

First, we demonstrated that monovalent sialic acid and 3′SLA were equally efficient in inhibiting Ad37 from binding to HCE cells by comparing several sialic acid containing saccharides. Trisialic acid was slightly more efficient than the other monovalent saccharides, which may be explained by a slightly higher affinity between the positively charged knob domain of Ad37 (isoelectric point = 9.1; Arnberg et al., 1997) and trisialic acid, which is even more acidic than the other saccharides (pK_a of sialic acid = 2.6).

In order to investigate whether multivalent sialic acid could inhibit Ad37 from binding to and infecting HCE cells to the same extent as multivalent 3′SLA-HSA, we conjugated sialic acid to HSA (SA-HSA) using a mechanism similar to the one used previously for conjugation of 3′SLA-HSA, and measured the inhibitory effect of these compounds on Ad37 binding to and infection of HCE cells. The synthesis of the multivalent sialic acid derivative **5** was performed in four steps with an overall yield of 16%. This low yield was mainly due to problems of separating the α- and β-isomers of compound **2**. However, it is possible to increase the α yield significantly if the separation is performed with preparative chiral HPLC. 13-Valent SA-HSA prevented 50% of virus binding to the same extent as 12-valent 3′SLA-HSA, and the level of inhibition clearly correlated with the level of valency, indicating that multivalent sialic acid cause aggregation of virions.

The sialic acid-mediated reduction in viral binding was also confirmed by infectivity experiments. Multivalent (8- and 13-valent) sialic acid reduced 50% of Ad37 binding at concentrations that were 1000 times lower than monovalent sialic acid. We also found that multivalent SA-HSA was effective in inhibiting Ad37 from infecting HCE cells, at levels similar to those previously obtained using 3′SLA-HSA. The ability of multivalent sialic acid to neutralize viruses by aggregation has never been described, or quantified before. Here we set up a novel assay to quantify aggregation of Ad37 virions by means of multivalent sialic acid. This assay will be useful for initial screening of novel,

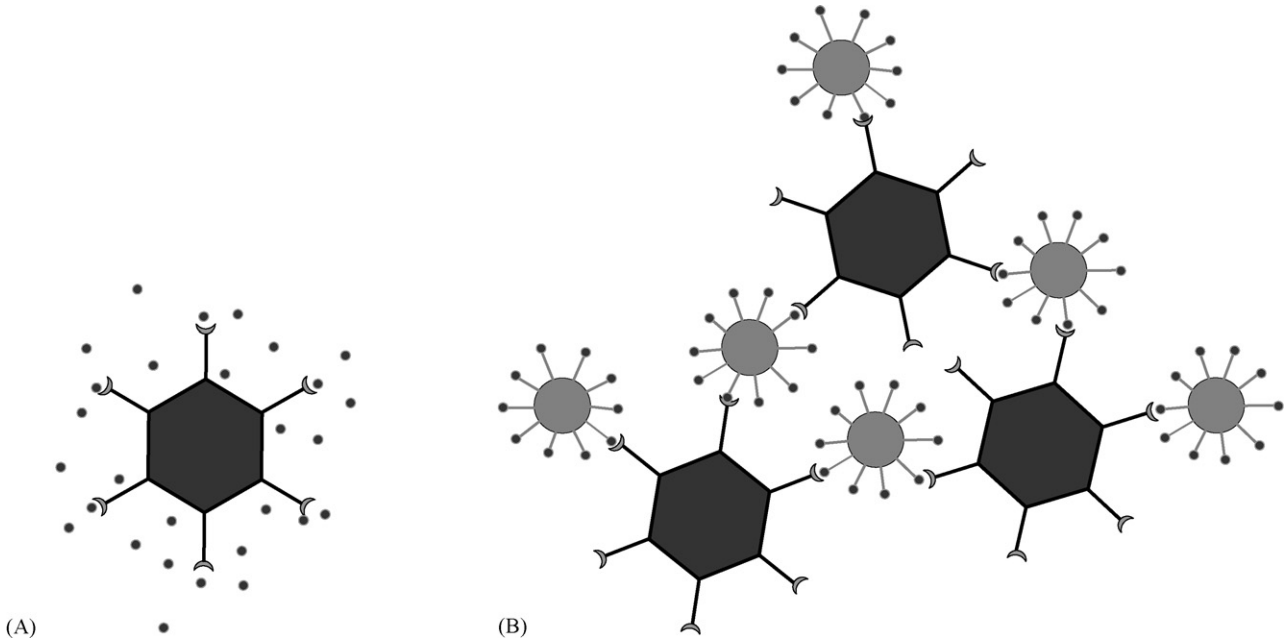


Fig. 5. Mechanism for increased neutralization of virions in the presence of multivalent sialic acid. (A) In the presence of monovalent sialic acid, all viral ligands (fiber knobs) needs to be occupied by sialic acid in order to prevent each virion from binding to and infecting target cells. (B) In the presence of multivalent sialic acid virions are aggregated. Each aggregate (containing an unknown number of virions) may in worst case infect one cell, which would be favorable instead of having non-aggregated virions capable of infecting numerous cells.

multivalent binding inhibitor candidates that target adenoviruses or other viruses.

Our results clearly demonstrate that multivalent sialic acid has a similar inhibitory efficacy as multivalent SLA. For *in vitro* experiments, as well as for *in vivo* usage, there are several advantages of using sialic acid conjugates instead of SLA conjugates. The structures of sialic acid derivatives are much less complex and thereby easier to synthesize. The previous SLA conjugate was prepared in 11 steps as compared to the synthesis of the present sialic acid conjugate that required only four steps. This difference is advantageous considering terms of time and costs of producing an antiviral drug. In addition, a smaller and less complex structure is generally easier to modify than a large structure. This smaller sialic acid conjugate could be systemically varied at different positions in order to increase the affinity to the sialic acid binding site of the viral fiber knob domain. SLA on the other hand is more complex and additional modifications would be difficult to perform due to synthetic problems. By using multivalent sialic acid conjugates in future research towards an antiviral drug against EKC, the possibilities of finding an easy accessible and effective drug seems promising.

Multivalent sialic acid has been demonstrated previously to be very effective against both virus and bacteria in inhibiting microbe binding to/infection of target cells *in vitro* (Reuter et al., 1999; Simon et al., 1997). However, many of the antimicrobial drugs of the binding inhibitor class are delivered to organs that are large and/or are difficult to reach. Here we report about a prototype construct drug that targets the eye, an organ that is small and accessible for topical administration. For maximum effect, the drug should most likely be used at an early stage of infection or as prophylaxis, in order to achieve maximum effect. Besides NMSO3, which seems to be effective against multiple adenovirus serotypes, there are no other candidate antiadenovirus drugs (as to the best of our knowledge). The effects of NMSO3 on adenovirus replication in ocular cells has not yet been investigated but it should be of interest to compare the effects of multivalent sialic acid and NMSO3 using such cells. The economic impact of adenovirus epidemics is significant worldwide (Gordon, 2000) and the need for an antiviral drug that targets EKC-causing adenoviruses is widely expressed (Gordon, 2000; Kinchington et al., 2005; Viswalingam, 1993). Here we present a novel anti-adenovirus drug candidate with ability to efficiently inhibit EKC-causing adenovirus from binding to and infection of HCE cells, which represents the cells that are infected by this group of viruses *in vivo*. We also confirm that neutralization of EKC-causing adenoviruses by means of aggregation is extremely efficient *in vitro*. Finally, we present a novel method for initial screening of future antiviral drugs that rely on the ability to neutralize viruses by aggregation.

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