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Linear polysialoside outperforms dendritic analogs for inhibition of influenza virus infection *in vitro* and *in vivo*

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Inhibition of influenza A virus infection by multivalent sialic acid inhibitors preventing viral hemagglutinin binding to host cells of the respiratory tract is a promising strategy. However, optimal geometry and optimal ligand presentation on multivalent scaffolds for efficient inhibition both *in vitro* and *in vivo* application are still unclear. Here, by comparing linear and dendritic polyglycerol sialosides (LPGSA and dPGSA) we identified architectural requirements and optimal ligand densities for an efficient multivalent inhibitor of influenza virus A/X31/1 (H3N2). Due to its large volume, the LPGSA at optimal ligand density sterically shielded the virus significantly better than the dendritic analog. A statistical mechanics model rationalizes the relevance of ligand density, morphology, and the size of multivalent scaffolds for the potential to inhibit virus-cell binding. Optimized LPGSA inhibited virus infection at IC_{50} in the low nanomolar nanoparticle concentration range and also showed potent antiviral activity against two avian influenza strains A/Mallard/439/2004 (H3N2) and A/turkey/Italy/472/1999 (H7N1) post infection. *In vivo* application of inhibitors clearly confirmed the higher inhibition potential of linear multivalent scaffolds to prevent infection. The optimized LPGSA did not show any acute toxicity, and was much more potent than the neuraminidase inhibitor oseltamivir carboxylate *in vivo*. Combined application of the LPGSA and oseltamivir carboxylate revealed a synergistic inhibitory effect and successfully prevented influenza virus infection in mice.

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1. Introduction

Seasonal influenza A infections cause ~500,000 deaths and 3–5 million cases of severe illness annually worldwide [1,2]. The high mutation frequency of viral proteins limits both the use of vaccination [3] and of approved therapeutics against viral proteins that mediate uncoating (e.g. amantadine) or release (e.g. oseltamivir) of the virus [4]. However, new approaches targeting conserved domains of the major viral spike protein hemagglutinin (HA) are still of great interest, since HA is required for early steps of the infection: binding to the sialic acid (SA) residues on the cell surface, and upon endocytosis, fusion with the endosomal membrane [5]. Recent developments of antibodies binding to the stem domain or to the SA binding pocket of HA are promising candidates with a broad spectrum activity [6,7].

Mimicking the host cell surface by multivalent presentation of SA residues on suitable polymeric scaffolds is an alternative to prevent infection by inhibiting the virus binding [8–12]. E.g., linear

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sialylated polyacrylamides (PAA) have been shown to efficiently compete for virus binding to the host cells [8]. However, typically they are cytotoxic [13], and of low biocompatibility [14]. Only a few sialylated multivalent inhibitors of influenza virus have been tested *in vivo*, however, without any promising perspective for application [15,16]. Major concerns are the geometrical requirements for multivalent ligand presentation optimal for *in vivo* application. Dendritic scaffolds can present a rather structurally defined set of ligands compared to its linear more flexible counterparts. In contrast, depending on their properties carbohydrate-conjugated linear polymers may offer a highly hydrated water swollen coiled scaffold with enhanced steric shielding properties in comparison to those of dendritic scaffolds. Likewise, the optimal ligand density varies depending on the architecture type [17]. The importance of optimal ligand density has been addressed recently using spherical 6'-sialyllactose-PAMAM conjugates for efficient influenza virus inhibition [18]. Finally, a low molecular weight of the polymer (<40 kDa) for systemic clearance and a low toxicity are required for *in vivo* use [19].

Given the significance of scaffold geometries for contributions of steric shielding to inhibition of virus binding, a preference for linear or dendritic polymeric scaffolds is not a *priori* given [17]. We have now undertaken a systematic study on a series of linear and dendritic polyglycerols (LPG and dPG) with a similar linker strategy. Due to its low toxicity and good *in vivo* clearance, PGs are a promising scaffold for a multivalent ligand display [20–22]. Setting the hypothetical models by Whitesides and coworkers [23] on a quantitative basis, our recent studies have emphasized that apart from multivalent interaction size dependent steric shielding is indeed a key factor [24]. Hence, both affinity as well as steric shielding determine the efficacy of a multivalent inhibitor to block virus particles. The highly water soluble SA-conjugated linear PGs possess a dynamic scaffold structure due to their flexibility amplifying steric shielding effects, as suggested by theoretical considerations showing that the linear scaffolds inhibit virus binding more efficiently than the dendritic scaffolds. Taken together, we here present the design and synthesis of a non-toxic, low molecular weight multivalent inhibitor for influenza A virus that displays promising antiviral efficacy *in vivo*.

2. Materials and methods

2.1. Synthesis and characterization

All reagents and solvents were purchased from commercial suppliers and used without further purification. Reactions requiring dry or oxygen-free conditions were carried out under argon in Schlenk glassware. ^1H spectra were recorded on Bruker AMX 500 (500 MHz) and Delta Joel Eclipse 700 (700 MHz) spectrometer at 25 °C and calibrated by using the deuterated solvent peak. Infrared (IR) spectra were recorded with a Nicolet AVATAR 320 FT-IR 5 SXC (Thermo Fisher Scientific, Waltham, MA, USA) with a DTGS detector from 4000 to 650 cm^{-1}. A TSQ 7000 (Finnigan Mat) instrument was used for ESI measurements and a JEOL JMS-SX-102A spectrometer was used for the high-resolution mass spectra. Molecular weight distributions of LPG_{10} **1**, dPG_{10} **2**, and the post functionalized polymers were determined by means of GPC coupled to a refractive index detector (RI) for obtaining the complete distribution (Mn, Mp, Mw, dispersity). Measurements were carried out under highly diluted conditions (5 mg/ml) from a GPC consisting of an Agilent 1100 solvent delivery system with pump, manual injector, and an Agilent differential refractometer. Three 30 cm columns (PPS: Polymer Standards Service GmbH, Germany; Suprema 100 Å, 1000 Å, 3000 Å with 5 and 10 μm particle size) were used to separate aqueous polymer samples using water with 0.1 N $NaNO_3$ as the mobile phase at a flow rate of 1 ml/min. The columns were operated at room temperature (rt) with the RI detector at 50 °C. The calibration was performed by using certified standards pullulan (linear) and dextran (branched) from PSS. WinGPC Unity from PSS was used for data acquirement and interpretation. The molecular weight of the dPG_{500} **3** was determined by gel permeation chromatography (GPC) using a DAWN-EOS multiangle laser light scattering (MALLS) (Wyatt technology Inc., Santa Barbara CA) and optilab RI detectors; the details have been described previously [25]. An aqueous 0.1 N $NaNO_3$ solution ($pH = 7.0$) was used as the mobile phase and d*n*/d*c* value for PG used for the molecular weight calculation was 0.12 ml/g [26]. DLS measurements of the various polymers were conducted by using a NanoDLS particle sizer (Brookhaven Instruments Corp.) at 25 °C. Aqueous samples were filtered through 0.2 mm filters prior to analysis. Water of Millipore quality was used in all experiments. An USHIO super high mercury lamp (USH 102d, 100 W) was used for UV assisted thiol-ene click reaction. Naturally occurring sialic acids constitute a family of more than 50 structurally distinct nine-carbon 3-deoxy-ulosonic acids, the most widespread derivative being 5-*N*-acetyl-neuraminic acid (Neu5Ac). We used the abbreviation of sialic acid (SA) for Neu5Ac.

2.2. Virus material

X31 virus (influenza strain A/Aichi/2/68 H3N2, reassorted with internal segments of A/PuertoRico/8/1934 H1N1), A/Mallard/439/2004 (H3N2) or A/turkey/Italy/472/1999 (H7N1) were harvested from allantoic fluid of embryonated chicken eggs. Virus isolates were clarified upon low speed centrifugation (300×*g*, 10 min). For binding (inhibition) experiments clarified allantoic fluid was further concentrated by ultracentrifugation (100,000×*g*, 1 h).

2.3. Cell culture

MDCK-II (Madin-Darby canine kidney epithelial) cells (ATCC) have been maintained in DMEM (supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 units/ml penicillin) at 37 °C and 5% CO_2.

2.4. Hemagglutination inhibition assay (HAI)

Inhibitors were twofold serially diluted in PBS. Then, 4 HAU X31 virus containing approximately $4 \cdot 10^7$ virus particles were added to all the wells. The amount of virus particles per volume was estimated as published by Desselberger et al. [27]. After 30 min incubation under slight agitation at rt, 50 μl of a 1% human erythrocyte solution (German Red Cross, ~$2 \cdot 10^6$ cells/μl) was added, gently mixed, and incubated for 60 min at rt. The inhibitor constant $K_{i(HAI)}$, reflects the lowest inhibitor concentration, which is necessary to achieve full inhibition of virus induced hemagglutination. To check for full hemagglutination inhibition, the microtiter plate was tilted by 60° to cause droplet formation from the red blood cell pellet [28].

2.5. Erythrocyte binding inhibition assay

Experiments were performed as previously described [29]. Briefly, influenza A virus (X31) was labelled with 20 μM octadecyl rhodamine B chloride (R18) in PBS and the free dye was removed upon virus pelleting. 5 μg labelled virus was incubated with twofold serial dilutions of binding inhibitors, and incubated for 30 min at rt (0.5 μg/μl X31, ≈ 16 HAU/μl). The virus-inhibitor complex was mixed with 40 μl of a 1% human erythrocyte solution (~$2 \cdot 10^6$ cells/μl) and incubated for 30 min at rt. Unbound virus

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