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Short communication

Polymeric inhibitor of influenza virus attachment protects mice from experimental influenza infection

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Abstract

Synthetic sialic acid-containing macromolecules inhibit influenza virus attachment to target cells and suppress the virus-mediated hemagglutination and neutralize virus infectivity in cell culture. To test the protective effects of attachment inhibitors in vivo, mice were infected with mouse-adapted influenza virus A/Aichi/2/68 (H3N2) and treated with synthetic polyacrylamide-based sialylglycopolymer PAA-YDS bearing moieties of (Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1)$_2$-3,6Manβ1-4GlcNAcβ1-4GlcNAc. Single intranasal inoculations with PAA-YDS 30 min before or 10 min after infection increased the survival of mice ($P < 0.01$). Multiple treatments with aerosolized PAA-YDS on days 2–5 post infection also increased survival ($P < 0.01$), alleviated disease symptoms, and decreased lesions in the mouse lungs. These data suggest that synthetic polyvalent inhibitors of virus attachment can be used for prevention and treatment of influenza.

Keywords: Influenza; attachment inhibitor; sialylglycopolymer; antiviral therapy; mice

Influenza virus attachment to target cells is mediated by specific interactions of the viral hemagglutinin with terminal sialyloligosaccharides of cell-surface glycoproteins and/or glycolipids (reviewed by Herrler et al., 1995; Paulson, 1985; Skehel and Wiley, 2000). It is believed that optimally designed competitive inhibitors of the attachment could be used as anti-influenza drugs. Because monovalent sialosides are rather inefficient inhibitors of the highly polyvalent cooperative interaction of the influenza virus particle with cell surface receptors (see Kiessling and Pohl, 1996; Matrosovich, 1989 for a discussion), efforts have been focused on the development of polyvalent attachment inhibitors in the form of sialylglycopolymers, sialic acid-containing liposomes, and star-like dendritic sialosides (reviewed by

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Kiessling and Pohl, 1996; see also Tuzikov et al., 1997; Chinarev et al., 1999; Choi et al., 1997; Reuter et al., 1999; and references therein). The best preparations designed in these studies were at least 3 orders of magnitude more active as inhibitors of influenza virus receptor binding in in vitro assays than the corresponding monovalent ligands. Besides inhibiting influenza virus-mediated hemagglutination, synthetic sialylglycopolymers and dendritic sialosides neutralized influenza virus infection in cell culture tests (Mochalova et al., 1994; Itoh et al., 1995; Reuter et al., 1999). However, protective effects of such compounds in vivo have not been demonstrated so far. In this study, we addressed this question by testing the anti-influenza activity of a synthetic polymeric attachment inhibitor in the mouse model.

We have shown previously (Gambaryan et al., 1997) that all human influenza A and B viruses share a common high binding affinity for 6′-sialyl(*N*-acetyllactosamine) (Neu5Acα2-6Galβ1-4GlcNAc; 6′SLN). This finding suggested that 6′SLN represents the minimal receptor determinant recognized by human influenza viruses on the surface of their target cells, and therefore this sialyloligosaccharide is likely the best choice for incorporation as an active ligand into a synthetic polymeric inhibitor. Based on this notion, polyacrylamide-based glycopolymer PAA-YDS bearing pendant moieties of a commercially available 6′SLN-containing biantennary oligosaccharide [$(Neu5Ac\alpha2\text{-}6Gal\beta1\text{-}4GlcNAc\beta1\text{-}2Man\alpha1)_2$-3,6 Manβ1-4GlcNAcβ1-4GlcNAc, YDS] was synthesized (Fig. 1). This glycopolymer demonstrated strong binding to a variety of human influenza viruses and a significant antiviral activity in the virus-neutralization assay in cell culture (Tuzikov et al., 2000). We, therefore, selected PAA-YDS for the in vivo experiments on anti-influenza activity of attachment inhibitors in mice.

Outbred albino mice (weight in the range from 14 to 18 g) were purchased from 'Lesnoye' farm, Moscow, Russia. The mouse-adapted variant of A/Aichi/2/68 (H3N2) influenza virus strain was obtained from the virus repository of D.I. Ivanovsky Institute of Virology, Moscow, Russia. The virus was grown in 9-day-old embryonated eggs and stored in aliquots at −80 °C. Two routes of virus challenge and drug treatment were used, intranasal inoculation under ether anesthesia and inhalational treatment by using an in-house made apparatus for whole body aerosol exposure. The apparatus consisted of a transparent plastic chamber (20 l) which was connected to the ultrasound inhalator 'Musson-1' ('Rotor' mechanical plant, Altai, Russia) generating either virus or drug aerosol with a particle diameter ranging from 3 to 8 μm.

The aerosol entered the chamber through the inlet in its upper lid and was exhausted through the outlet in the bottom part of the chamber connected via HEPA filter to a peristaltic pump operating at 0.5 l/min. The median mouse lethal doses (MLD_{50}) of the virus stock under conditions of intranasal and aerosol virus challenge were estimated in preliminary experiments from the number of deaths at 10 days post infection in the groups of four mice infected with serial 10-fold virus dilutions.

In the initial treatment experiment (Fig. 2A), the drug (PAA-YDS) and the saccharide-free PAA carrier (control) were administered intranasally in equivalent weight amounts (7 μg/mouse) using a 25 μl inoculum. Thirty minutes later, mice of the treatment and control groups were infected intranasally with one MLD_{50} of the virus (25 μl suspension in PBS). The mortality was recorded for 14 days after the infection. The treatment with the drug statistically significantly increased the number of mice that survived the infection as compared to mice that were treated

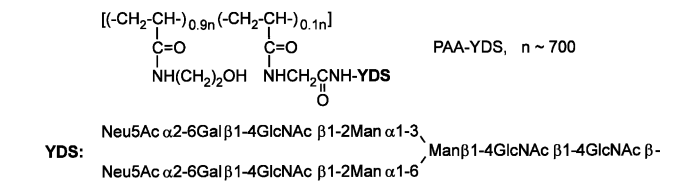

Fig. 1. Structural formula of PAA-YDS. The sialylglycopolymer was synthesized by the coupling of YDS-$NHCOCH_2NH_2$ with poly(4-nitrophenyl acrylate) as described previously (Bovin et al., 1993) and contained one moiety of YDS per 10 monomeric units of the polymeric carrier. The average degree of polymerization (*n*) of the poly(*N*-2-hydroxyethylacrylamide) carrier was estimated by gel-permeation chromatography.

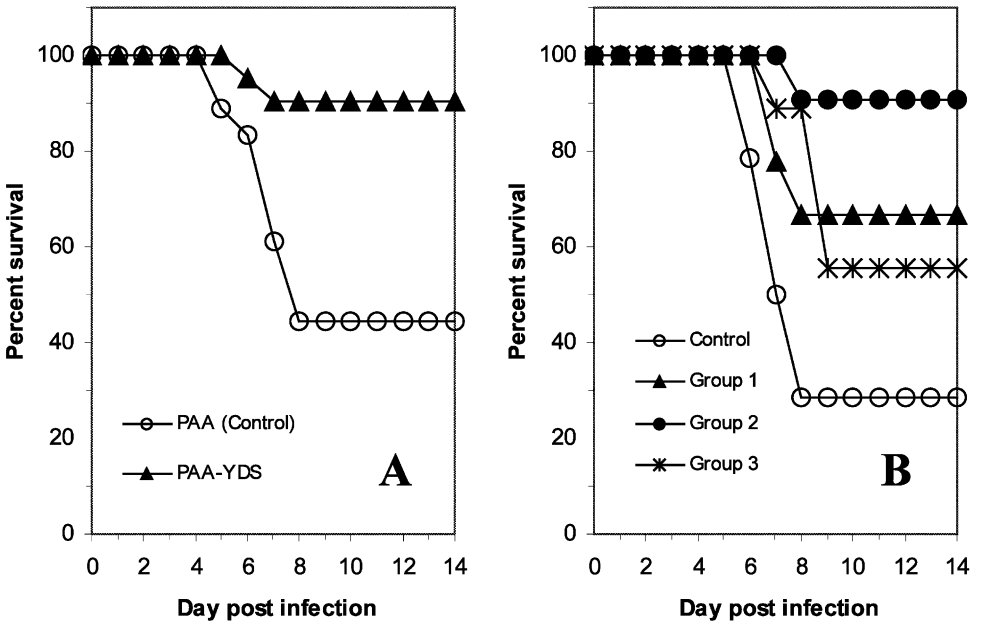

Fig. 2. Effects of treatments with PAA-YDS on survival in influenza virus infected mice. (A) Two groups of 20 mice were inoculated with either PAA-YDS (7 μg/mouse; 4 nmole with respect to sialic acid) (triangles) or with carbohydrate-free PAA (7 μg/mouse) (open circles) 30 min before intranasal infection with 1 MLD_{50} of the virus. (B) Experimental and control groups of mice were simultaneously infected with 5 MLD_{50} of influenza virus aerosol and either treated with PAA-YDS or non-treated (control group, open circles). Intranasal (group 1, triangles and group 2, closed circles) and aerosol (group 3, crosses) treatments with the drug were performed as described in the text. The differences between drug-treated groups and the control group are statictically significant (groups 1 and 2, $P < 0.01$, Fisher's exact test; group 3, $P < 0.01$, Wilcoxon two-sample test). The differences between groups 1, 2 and 3 are not statistically significant.

with sialic acid-free polymer ($P < 0.01$; Fisher's exact test).

In the next experiment (Fig. 2B), three experimental groups of mice and one control group were simultaneously infected with 5 MLD_{50} of the virus via the inhalational route. The control group (14 mice) received no treatment. Each mice within the first two groups received a single dose of 21 μg PAA-YDS (12 nmole sialic acid) per mouse in 50 μl PBS intranasally, either 30 min before (group 1; 9 animals), or 10 min after the infection (group 2; 11 mice). The mice of the third group (9 animals) were treated by PAA-YDS aerosol (five 10 min incubations per day) for 4 consecutive days starting on the first day after infection. For aerosol generation, 0.05 mM solution of the drug in PBS was used. Calculation of the drug deposition in the respiratory tract was performed as described by Ovcharenko and Zhirnov (1994) and suggested that each mouse in the third group received about 3 μg (1.7 nmole sialic acid) per day. Each type of treatment increased survival of the animals relative to the control ($P < 0.01$). Importantly, a protective effect was observed even when the treatment was delayed by 1 day after the infection (group 3).

To further evaluate the therapeutic potential of delayed drug administration, we tested the effect of PAA-YDS on general clinical symptoms of the disease under conditions of sublethal influenza infection. Thirty two mice were infected with 0.2 MLD_{50} of the virus aerosol followed by separation of the animals into two groups of equal size. Mice of the control group received no treatment; the second group was treated with aerosolized PAA-YDS on days 2–5 after infection exactly as described in the previous experiment. Scoring of the general condition of the surviving animals on

Table 1
Physical condition of drug-treated and control mice infected with 0.2 MLD_{50} of influenza virus

Group	Dead/total	Number of animals with disease symptoms[a]		
		No visual symptoms of the disease	Listlessness, debilitation, rough hair coat	Highly depressed, hunched posture, emaciation
Drug-treated	2/16	9	3	2
Control	3/16	4	4	5

Thirty-two mice were infected with the virus aerosol and separated into control and treatment groups. The experimental group was treated with PAA-YDS aerosol, as described in the text, on days 2–5 post infection; the control group was not treated.

[a] Symptoms were scored on day 10 post infection.

the day 10 after infection indicated alleviation of the disease in the treatment group versus the control group (Table 1). On day 14, the lungs of survived animals were removed and scanned on a flat-bed scanner (Fig. 3). The lungs of most control mice contained extensive inflammatory lesions. By contrast, no extensive lesions were observed in the lungs of all but two PAA-YDS-treated mice. Antibody response to influenza virus was observed in both treated and control animals (data not shown). Thus, delayed treatment with the drug did not stop the infection, but significantly reduced its severity.

Previous studies indicated that synthetic sialic acid-containing inhibitors of the influenza viruses can neutralize the virus infectivity in cell culture tests (Itoh et al., 1995; Mochalova et al., 1994; Tuzikov et al., 2000). Our data demonstrate for the first time protective effects of such compounds against influenza virus infection in mice. The data show, in particular, that the Sia–Gal linkage in the synthetic sialosides is sufficiently resistant towards the action of the viral neuraminidase. We expect that combined application of sialosides with neuraminidase inhibitors could additionally protect sialosides from the viral enzyme and increase their anti-influenza activity. Another synthetic attachment inhibitor bearing a trisaccharide 6′SLN coupled to PAA carrier demonstrated protective activity in mice that was comparable to protective effects of PAA-YDS (data not shown). These results argue in favor of further development of polyvalent sialosides for prevention and treatment of influenza.

Although derivatives of polyacrylamide are non-toxic (Bradley, 1999) and non-immunogenic (Dintzis et al., 1989), we do not consider PAA-YDS or other PAA-based sialylglycopolymers as candidate anti-influenza drugs due to the lack of safety data on PAA degradation products in vivo and to a general limited biocompatibility of carbo-chain PAA carrier. This problem could be addressed, for example, by utilization of biodegradable polymeric carriers or by design of multimeric rather than polymeric sialioside inhibitors.

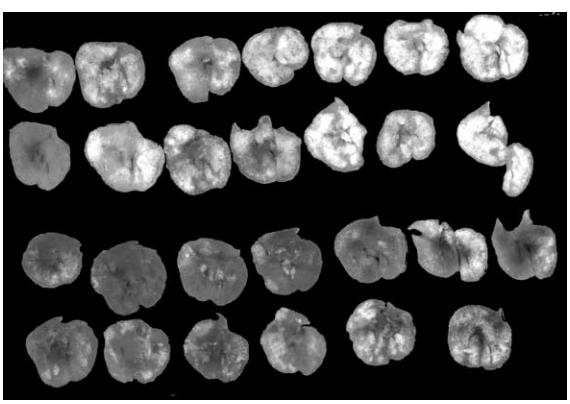

Fig. 3. Lungs of influenza virus-infected mice on day 14 post infection. Two bottom rows: control (infected but non-treated) mice. Two top rows: mice treated with PAA-YDS aerosol on days 2–5 after infection as described in the text.

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References

Bovin, N.V., Korchagina, E.Y., Zemlyanukhina, T.V., Byramova, N.E., Galanina, O.E., Zemlyakov, A.E., Ivanov, A.E., Zubov, V.P., Mochalova, L.V., 1993. Synthesis of polymeric neoglycoconjugates based on *N*-substituted polyacrylamides. Glycoconj. J. 10, 142–151.

Bradley, D., 1999. Plastics in cancer therapy. Drug Discov. Today 4, 540–541.

Chinarev, A.A., Tuzikov, A.B., Gambaryan, A.S., Matrosovich, M.N., Umberty, A., Bovin, N.V., 1999. In: Inoue, Y., Lee, Y.C., Troy, F.A. II (Eds.), Tetravalent Blockers for Influenza Virus Hemagglutinin. Gakushin Publishing Co, Osaka, Japan, pp. 135–143.

Choi, S.-K., Mammen, M., Whitesides, G.M., 1997. Generation and in situ evaluation of libraries of poly(acrylic acid) presenting sialosides as side chains as polyvalent inhibitors of influenza-mediated hemagglutination. J. Am. Chem. Soc. 119, 4103–4111.

Dintzis, R.Z., Okajima, M., Middleton, M.H., Greene, G., Dintzis, H.M., 1989. The immunogenicity of soluble haptenated polymers is determined by molecular mass and hapten valence. J. Immunol. 143, 1239–1244.

Gambaryan, A.S., Tuzikov, A.B., Piskarev, V.E., Yamnikova, S.S., Lvov, D.K., Robertson, J.S., Bovin, N.V., Matrosovich, M.N., 1997. Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6'-sialyl(*N*-acetyllactosamine). Virology 232, 345–350.

Herrler, G., Hausmann, J., Klenk, H.-D., 1995. Sialic acid as receptor determinant of ortho- and para-myxoviruses. In: Rosenberg, A. (Ed.), Biology of the Sialic Acids. Plenum, New York, pp. 315–336.

Itoh, M., Hetterich, P., Isecke, R., Brossmer, R., Klenk, H.-D., 1995. Suppression of influenza virus infection by an N-thioacetylneuraminic acid acrylamide copolymer resistant to neuraminidase. Virology 212, 340–347.

Kiessling, L.L., Pohl, N.L., 1996. Strength in numbers: non-natural polyvalent carbohydrate derivatives. Chem. Biol. 3, 71–77.

Matrosovich, M.N., 1989. Towards the development of antimicrobial drugs acting by inhibition of pathogen attachment to host cells: a need for polyvalency. FEBS Lett. 252, 1–4.

Mochalova, L.V., Tuzikov, A.B., Marinina, V.P., Gambaryan, A.S., Byramova, N.E., Bovin, N.V., Matrosovich, M.N., 1994. Synthetic polymeric inhibitors of influenza virus receptor-binding activity suppress virus replication. Antiviral Res. 23, 179–190.

Ovcharenko, A.V., Zhirnov, O.P., 1994. Aprotinin aerosol treatment of influenza and para-myxovirus bronchopneumonia of mice. Antiviral Res. 23, 107–118.

Paulson, J.C., 1985. Interactions of animal viruses with cell surface receptors. In: Conn, M. (Ed.), The Receptors, vol. 2. Academic Press, Orlando, FL, pp. 131–219.

Reuter, J.D., Myc, A., Hayes, M.M., Gan, Z., Roy, R., Qin, D., Yin, R., Piehler, L.T., Esfand, R., Tomalia, D.A., Baker, J.R. Jr., 1999. Inhibition of viral adhesion and infection by sialic-acid-conjugated dendritic polymers. Bioconjug. Chem. 10, 271–278.

Skehel, J.J., Wiley, D.C., 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu. Rev. Biochem. 69, 531–569.

Tuzikov, A., Gambaryan, A., Juneja, L., Bovin, N., 2000. Conversion of complex sialooligosaccharides into polymeric conjugates and their anti-influenza virus inhibitory potency. J. Carbohydr. Chem. 19, 1191–1200.

Tuzikov, A.B., Byramova, N.E., Bovin, N.V., Gambaryan, A.S., Matrosovich, M.N., 1997. Monovalent and polymeric 5*N*-thioacetamido sialosides as tightly-bound receptor analogs of influenza viruses. Antiviral Res. 33, 129–134.