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Polymer-bound 6′ sialyl-*N*-acetyllactosamine protects mice infected by influenza virus

A.S. Gambaryan [a], E.Y. Boravleva [a], T.Y. Matrosovich [b], M.N. Matrosovich [a,b], H.-D. Klenk [b], E.V. Moiseeva [c], A.B. Tuzikov [c], A.A. Chinarev [c], G.V. Pazynina [c], N.V. Bovin [c,*]

[a] *M.P. Chumakov Institute of Poliomyelitis and Viral Encephalitides, Russian Academy of Medical Sciences, 142782 Moscow, Russia*
[b] *Institute of Virology, Philipps University, Robert-Koch 17, 35037 Marburg, Germany*
[c] *Shemyakin Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya, 16/10, 117997 Moscow, Russia*

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Abstract

To develop a mouse model for testing receptor attachment inhibitors of human influenza viruses, the human clinical virus isolate in MDCK cells A/NIB/23/89M (H1N1) was adapted to mice by serial passaging through mouse lungs. The adaptation enhanced the viral pathogenicity for mice, but preserved the virus receptor binding phenotype, preferential binding to 2–6-linked sialic acid receptors and low affinity for 2–3-linked receptors. Sequencing of the HA gene of the mouse-adapted virus A/NIB/23/89-MA revealed a loss of the glycosylation sites in positions 94 and 163 of HA1 and substitutions 275Asp → Gly in HA1 and 145Asn → Asp in HA2. The four mouse strains tested differed significantly in their sensitivity to A/NIB/23/89-MA with the sensitivity increasing in the order of BALB/cJCitMoise, C57BL/6LacSto, CBA/CaLacSto and A/SnJCitMoise strains. Testing of protective efficacy of the polyacrylamide conjugate bearing Neu5Acα2-6Galβ1-4GlcNAc trisaccharide under conditions of lethal or sublethal virus infection demonstrated a strong protective effect of this preparation. In particular, aerosol treatment of mice with the polymeric attachment inhibitor on 24–110 h after infection completely prevented mortality in sensitive animals and lessened disease symptoms in more resistant mouse strains.

Keywords: Influenza virus; Sialylglycopolymer; Antivirals; Mice; Anti-adhesion

1. Introduction

Influenza viruses initiate infection by attachment to host cells followed by endocytosis and fusion of the viral and endosomal membranes. Attachment is mediated by the interaction of the viral surface glycoprotein hemagglutinin (HA) with host cell surface receptors containing sialooligosaccharides (Paulson, 1985; Herrler et al., 1995). An extracellular agent that resembles the host receptors may inhibit this binding (for a recent review, see Matrosovich and Klenk, 2003). Airway mucins appear to be natural inhibitors for the influenza virus infection in the respiratory tract of humans. The carbohydrate chains of mucins are rich in sialic acids. Given their extreme heterogeneity and ability to attach to various carbohydrate-binding microorganisms, airway mucins can be regarded as a combinatorial library of carbohydrate epitopes developed during evolution to serve as decoys for pathogenic bacteria and viruses (Lamblin and Roussel, 1993; Scharfman et al., 1995). The literature data concerning the protective effect of mucins are inconsistent. Paulson and colleagues showed that mucins contain primarily Sia2-3Gal fragment (Couceiro et al., 1993). However, more recent studies suggested that human airway mucins also contain Sia2-6Gal-terminated moieties (Lamblin et al., 2001). Inhibitors present in human nasal washings protected mice from several neurotropic influenza A virus strains. The neutralizing activity was associated with heat stable and sialidase-sensitive inhibitors, presumably mucins (Andrewes et al., 1954). It is necessary to elucidate the role of mucins under natural conditions of influenza infection for estimation of the therapeutical perspectives of synthetic inhibitors of influenza virus attachment to the host cell receptor.

* Corresponding author. Tel.: +7 953307138; fax: +7 953305592.
E-mail address: bovin@carbohydrate.ru (N.V. Bovin).

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Attempts to design rationally synthetic inhibitors of influenza virus receptor binding were stimulated in the late 1980s by accumulated biochemical data on virus receptors and natural inhibitors (Paulson, 1985; Wiley and Skehel, 1987) and by the resolution of the structure of hemagglutinin complexed with sialic acid (Weis et al., 1988).

To increase the potency of synthetic inhibitors, several approaches for polyvalent presentation of sialoside ligands have been tested. A series of bivalent sialosides were synthesized that differed by the nature and the length of spacers between sialic acid residues, one of them was 100-fold stronger inhibitor than Neu5Acα-Me (Glick et al., 1991). Symmetrical tetravalent sialosides ('tetrahedrons') with formal distances between sialic acid residues of 110–120 Å showed up to 1200-fold enhancement of inhibitory activity over monovalent ligand and tetrahedrons with shorter antennae (Chinarev et al., 1999). Polyvalent inhibitors in the form of sialylglycopolymers (Matrosovich et al., 1990; Mochalova et al., 1994; Lees et al., 1994; Mammen et al., 1995; Sigal et al., 1996; Bovin, 1998), sialic acid-containing liposomes (Kingery-Wood et al., 1992; Spevak et al., 1993; Guo et al., 2002), star-like dendritic sialosides (Roy et al., 1993; Reuter et al., 1999; Tsvetkov et al., 2002), and self-assembling sialoglycopeptides (Tuzikov et al., 2003; Bovin et al., 2004) were considerably or even dramatically more potent than monovalent compounds.

An important finding in this respect is that all non-egg-adapted human influenza viruses tested so far, irrespective of their type and subtype, share a common high binding affinity for Neu5Acα2-6Galβ1-4GlcNAc (6′SLN) (Gambaryan et al., 1997, 1999). This finding suggested that 6′SLN but not merely Neu5Ac or even Neu5Acα2-6Gal represents the receptor determinant recognized by human influenza A and B viruses on the surface of their target cells. Recently, we investigated potential protective effects of virus attachment inhibitors in mice (Gambaryan et al., 2002a). The animals were infected with the mouse-adapted influenza virus A/Aichi/2/68 (H3N2) and were treated with polyacrylamide (PAA) conjugated with (Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1)$_2$-3,6Manβ1-4GlcNAcβ1-4GlcNAc (YDS) which carried two 6′SLN moieties. Single intranasal inoculations before the infecting or multiple treatments with aerosolized YDS-PAA on days 2–5 post infection increased survival, alleviated disease symptoms and decreased lesions in the mouse lungs. However this study was limited to testing the polymeric inhibitors against virus strain with modified receptor properties due to the egg and mouse adaptation.

To overcome this drawback, we developed a more adequate animal model for testing of antiviral attachment inhibitors. Namely, we adapted clinical human H1N1 virus isolate A/NIB/23/89M to mice without significant changes of its typical human-virus-like receptor specificity. Previously we tested several mouse strains for their susceptibility to influenza virus (Gambaryan et al., 2002b); four most distinctive strains were chosen for this study. The goal of this work was to compare the protective potential of mucins and artificial sialylglycopolymer and estimate the availability of the latter as antiviral.

2. Materials and methods

2.1. Sialylglycopolymers

High molecular weight polyacrylamide conjugates bearing of Neu5Acα2-3Galβ1-4Glcβ (3′SL), Neu5Acα2-6Galβ1-4Glcβ (6′SL) and Neu5Acα2-6Galβ1-4GlcNAcβ (6′SLN) were synthesized by coupling spacered oligosaccharides to poly(*N*-oxisuccinimidyl acrylate) as described previously (Tuzikov et al., 2000; Pazynina et al., 2002; Mochalova et al., 2003; Shilova et al., 2005). The sialylglycopolymers were designated, respectively, 3′SL-PAA, 6′SL-PAA and 6′SLN-PAA. The structure of 6′SLN-PAA conjugate presented on Fig. 1. The average molecular mass of the poly(*N*-2-hydroxyethylacrylamide) carrier was about 1500 kDa as estimated by gel-permeation chromatography. Content of sialosaccharide in polymer was 0.76 nmol/μg of polymer.

2.2. Human mucins

Nasal mucins were taken by plastic spatula from four children and tracheal mucin by expectoration from volunteers, diluted 10 times with PBS and centrifuged 20 min at 5000 rpm. Content of sialic acids in supernatants was estimated as described (Yao et al., 1989). Mucin samples were stored at 4 °C and used for the experiments during the first 1–2 days after preparation.

2.3. Viruses

The mouse-adapted variant of Aichi/2/68 (H3N2) influenza virus strain was obtained from the virus repository of D.I. Ivanovsky Institute of Virology, Moscow, Russia. The non-egg-adapted human influenza viruses A/NIB/26/90M (H3N2) and A/NIB/23/89M (H1N1) (Robertson et al., 1991)) were kindly provided by Dr. J.S. Robertson (National Institute for Biological Standards and Control, Blanche Lane, Potters Bar, Herts EN6 3QG, UK). The original viruses and the mouse-adapted variant A/NIB/23/89-MA were propagated in Madin–Darby canine kidney (MDCK) cells from the American Type Culture Collection in the presence of TPCK-trypsin (2.5 μg/ml, Worthington Diagnostics Freehold, NJ) at 37 °C for 48 h. MDCK cells were

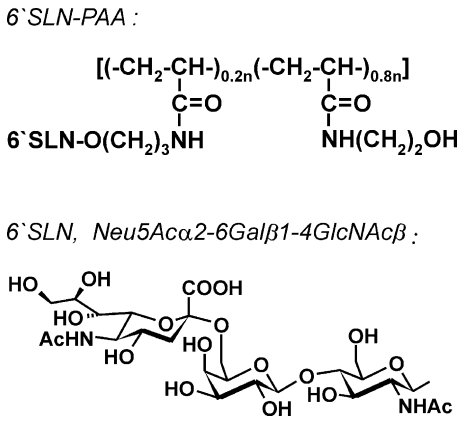

Fig. 1. The structure of 6′SLN-PAA conjugate.

cultured in minimal essential medium (MEM) with 5% bovine fetal serum.

2.4. Preparation of the mouse-adapted (MA)-variant

The MA-variant of the A/NIB/23/89M strain was prepared by 24 subsequent lung-to-lung passages. Outbred 4–6 weeks old albino mice were inoculated intranasally with 50 μl of virus-containing fluids after light anesthesia with ether. For the first passage, undiluted culture fluid was used. Subsequent passages were performed using 10% lung suspension in PBS (pH 7.4) supplemented with antibiotics. Lungs were harvested 48 h after infection.

2.5. Sequencing of the HA gene

Viral RNA was isolated using High Pure RNA isolation kit (Roche, USA). Two overlapping parts of the HA gene (1-727 and 623-1178) were amplified using One Step RT-PCR kit (Qiagen, Germany). The PCR products were purified (QIA Quick PCR purification kit, Qiagen) and sequenced by the dideoxy method using an automatic sequencer (Perkin Elmer, USA).

2.6. Competitive assay of virus binding to soluble receptor analogs

The affinity of the viruses for soluble receptor analogs was assayed by using the solid-phase fetuin binding inhibition assay as previously described (Gambaryan and Matrosovich, 1992). In brief, viruses were adsorbed to the wells of fetuin-coated polystyrene EIA microplates (Costar) at 4 °C overnight. After unbound virus was washed off, 0.05 ml of solution containing a fixed amount of HRP-labeled fetuin and a variable amount of non-labeled inhibitor was added to the plate, which was then incubated for 1 h at 2–4 °C. The solutions were prepared in PBS supplemented with 0.02% BSA, 0.02% Tween 80, and 10 μmol of the sialidase inhibitor, 4-amino-4-deoxy-Neu5Ac2en. The plates were washed, and the amount of labeled fetuin bound was determined by using the standard o-phenylenediamine chromogenic substrate. The dissociation constants (K_{diss}) of the virus complexes with receptor analogs were calculated basing on the concentration of the sialic acid residues in solution, and the results were averaged.

2.7. Determination of viral infectivity

MDCK cells were grown in 96-well microplates for tissue culture (NUNC, Denmark), washed with MEM and four-fold serial dilutions (0.15 ml) of influenza virus samples in MEM were inoculated using four replicate wells per dilution. After incubation of the plates for 14 h at 35 °C in 5% CO_2 atmosphere, the medium was removed; the cells were fixed with 0.02% glutaraldehyde for 30 min and washed with PBS. The solution of HRP-labeled fetuin (0.05 ml per well) in PBS supplemented with 0.01% Tween 20 was added, the plates were incubated for 1 h at 4 °C and washed with PBS-Tween 20. The fetuin-HRP conjugate bound to the surface of virus-infected cells was visualized by incubation with 0.1 ml substrate solution (1 ml 0.5% 3-amino-9-ethylcarbasole in DMSO + 9 ml 0.01% H_2O_2 in 0.05 M sodium acetate buffer, pH 5.7) per well. The numbers of infected cells were counted under the inverted microscope in each of the four replicates wells and the results were averaged. One stained cell corresponded to one infection unit (IU) of the virus.

2.8. Inhibition of viral infectivity in cell culture

Two-fold serial dilutions (0.15 ml) of receptor analog samples in MEM were inoculated onto monolayers (see above), and equal volume of influenza virus containing from 250 to 1000 IU was added per well. Incubation and visualization of infected cells were performed as described above. Concentration of inhibitor that resulted in 90% inhibition of virus infectivity was expressed in μM of sialic acid.

2.9. Mice

Male mice of different strains were of the same age (2 months). BALB/cJCitMoise (B/c) and A/SnJCitMoise (A/Sn) strains were maintained in Mice Breeding Department of Laboratory of Biotechnology, Institute of Bioorganic Chemistry, Moscow. CBA/CaLacSto (CBA) and C57BL/6LacSto (B6) mice were kindly provided by Svetlana S. Kardashova, Laboratory Animals Department, Oncology Center, Moscow.

2.10. Method of infection

Mice were infected by either instilling 50 μl of virus suspension in PBS into each nostril or by using in-house-constructed apparatus for whole body aerosol exposure described previously (Gambaryan et al., 2002a). In the second case, the mice were exposed for 10 min to aerosol containing about 10^5 IU of the virus per liter. The apparatus consisted of a transparent plastic chamber, which was connected to the ultrasound inhalator Musson-1 (Rotor, Altai, Russia) generating virus aerosol (aerosol particle size 3–8 μm). The aerosol entered inside the chamber through the inlet in its upper lid and was exhausted through the outlet in the bottom part of the chamber, which was connected via HEPA filter to peristaltic pump operating at 0.5 l/min.

2.11. Treatment

The infected mice were exposed for 10 min to aerosol atmosphere that was prepared by dispersing 200 μM (by sialic acid) solution of 6′SLN-PAA (Fig. 1) in the same apparatus as used for the infecting. Solution of the sialylglycopolymer in H_2O was poured into special vessel of Musson-1 inhalator and sprayed into 20 l chamber; 2 ml of solution was used for every treatment. Content of drug in aerosol was approximately 0.02 μmol/l. The second infected group was treated with placebo (polymeric carrier without the carbohydrate). Two control non-infected groups were treated with 6′SLN-PAA or placebo similarly. Calculation of the virus and drug deposition in the respiratory tract was performed based on the virus or drug concentration in aerosol, the

mouse tidal breath volume per minute and the exposition time, as described elsewhere (Ovcharenko and Zhirnov, 1994).

2.12. Parameters of the disease

Three parameters were used to record animal sensitivity to virus infection: survival, weight dynamics and lung weight increase after infection. All mice were labeled and followed for 12 days after infection, because normally no mortality was observed in the infected animals afterwards. On day 6 after infection (the day of minimal weight for surviving mice), the difference between the average weights of infected animals was calculated and expressed as percentage of the averaged weight of non-infected mice. The obtained value was designated as WL (weight loss). On the 12th day after infection, the percentage of survived animals (*S*, %) was calculated for all groups. The survived animals were euthanized; lungs were excised and weighed. The difference between average weights of lungs from infected and non-infected mice was calculated and expressed as percentage of non-infected animals' lung weight. This value was defined as LWI (Lung weight increase). Average parameters (WL and LWI) were calculated only if all animals in compared groups survived. All experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (U.S. Department of Health and Human Services, National Institute of Health Publication No. 93-23, revised 1985).

Statistical analysis of survival dynamic was performed using Wilcoxon two-sample test and of WL and LWI was performed using paired Student's *t*-test (http://www.statistics.com/content/javastat.html#Comparisons).

3. Results

To assess the antiviral effect of synthetic sialylglycopolymer, its affinity to viruses and ability to suppress the virus infectivity were compared to those of the airway mucins. The content of sialic residues in preparations of mucins (calculated on sialic acid) was in the range 0.5–2 mmol/l. Dissociation constants of human influenza virus with human tracheal and nasal mucins and sialylglycopolymer 6′SLN-PAA and concentration of 90% neutralization of virus infectivity are given in Table 1. Virus affinity to mucin, expressed in total concentration of sialic acid, is rather high; however, mucins did not appreciably inhibit the viral infection in cell culture. In contrast, the synthetic inhibitor 6′SLN-PAA strongly suppressed the infection of MDCK cells under the same experimental conditions.

There was need in adequate experimental model in order to assay protective effects of 6′SLN-PAA in animals. To this end, we decided to adapt to mice clinical human influenza viruses that were isolated in MDCK cells and were never passaged in chicken embryos. Our attempts to adapt H3N2 and type B viruses failed but we succeeded in adaptation of H1N1 virus A/NIB/23/89M to mice by consecutive 24 lung-to-lung passages in mice. The receptor specificity of the virus at distinct passages is shown in Table 2. The virus retains increased affinity to 6′SLN-PAA after adaptation.

Table 1
Dissociation constants (K_{diss}, μM Sia) of virus complexes with human mucins and sialylglycopolymer, and concentration of 90% neutralization of virus infectivity in MDCK cells ($IC_{90\%}$, μM Sia)

Inhibitors	Viruses			
	A/NIB/23/89M (H1N1)		A/NIB/26/90M (H3N2)	
	K_{diss}	$IC_{90\%}$	K_{diss}	$IC_{90\%}$
Human tracheal mucin	0.1	>10	0.5	>10
Human nasal mucin	0.2	>10	1	>10
6′SLN-PAA	0.01	0.05	0.02	0.5

K_{diss} of the virus complex with soluble receptor analogs was assayed by using the solid-phase fetuin binding inhibition assay; $IC_{90\%}$ was assayed in 96-well plates with MDCK cells monolayer infected by viruses in presence of receptor analog samples (Material and methods section).

Sequencing of the HA of the mouse-adapted virus A/NIB/23/89M (23M-MA) demonstrated that it differs from the clinical material (Robertson et al., 1991) by three replacements in HA1 (96Thr → Lys, 163Asn → Lys and 275Asp → Gly) and one replacement in HA2 (145Asn → Asp) (H3 numbering system). However, one of them, (163Asn → Lys) – was found in parent virus taken for adaptation. Hence, 163Asn → Lys substitution appeared in the course of passaging in MDCK culture.

After 24 passages in mice the productivity of the virus 23M-MA in culture and ability to propagate in mouse lungs had been increased (data not shown). Pathogenicity of this virus was compared to that of the starting virus NIB/23/89M and the mouse adapted virus A/Aichi/1/68 (Aichi-MA) using four strains of mice with different sensitivity. Groups of eight mice were infected by 10^3 IU of each virus and were monitored daily for 12 days.

The results of one representative experiment are given in Table 3. Two to three days after infection with Aichi-MA, the mice decreased consumption of food and sharply lost their weight. The virus caused 100% mortality in the A/Sn, CBA and B6 animals whereas weight of more resistant B/c mice gradually increased after day 6, and all animals survived. Virus NIB/23/89M did not cause visible disease symptoms in any group of mice tested. Weight curves of control and infected animals did not display significant differences; the only consequence of infection was the formation of antibodies to the virus (data not shown). Mouse-adapted virus 23M-MA caused deaths

Table 2
Receptor specificity of A/NIB/23/89M (H1N1) and its mouse-passaged variants (MA)

Virus	K_{diss} of virus-inhibitor complexes (μM Sia)		
	3′SL-PAA	6′SL-PAA	6′SLN-PAA
Parent NIB/23/89M	1	1	0.01
After 3 passages	1	1	0.01
After 16 passages	0.5	0.5	0.01
After 24 passages (23M-MA)	0.5	0.3	≤0.01

Viruses were passaged in cell culture prior to testing.

Table 3
Percentage of survival (*S*, %)[a], weight loss (WL, %)[b] and lung weight increase (LWI, %)[c] during influenza infection in four strains of mice

Virus	B/c			B6			CBA			A/Sn		
	S	WL	LWI	*S*	WL	LWI	*S*	WL	LWI	*S*	WL	LWI
Aichi-MA	100	15 ± 4	70 ± 20	0 (7.25)	24 ± 6	–	0 (8)	23 ± 5	–	0 (6.75)	27 ± 4	–
NIB/23/89M	100	3 ± 5	10 ± 20	100	5 ± 7	15 ± 20	100	6 ± 8	14 ± 7	100	6 ± 7	15 ± 10
23M-MA	100	6 ± 5	60 ± 30	100	15 ± 8	50 ± 30	50 (9.1)	20 ± 6	–	50 (8.5)	23 ± 7	–

Each group of mice contained eight animals infected intranasally with 10^3 IU of virus per mouse. *P*-value for comparison WL between animals infected Aichi-MA or 23M-MA and control group was <0.01, while for NIB/23/89M was nearly 0.5 (paired Student's *t*-test). For B/c mice LWI instead of WL was statistically treated.
[a] Determined on day 12, the mean day of death is shown in brackets (calculated for dead animals only).
[b] Determined on day 6.
[c] Determined on day 12, when all mice survived.

of some infected A/Sn and CBA mice whereas all infected B/c and B6 mice survived. However, the B6 mice clearly responded to infection with their weight curves having distinct minimum on days 6–7 after infection. Weight curves of infected and control B/c animals did not differ significantly but it was possible to detect illness of the infected mice by the increase in the lung weight. Virus 23M-MA, unlike the widely used mouse-adapted strain Aichi/2/68-MA, causes milder disease symptoms in mice, and represents a more adequate model of a typical influenza disease in humans. In subsequent experiments we used mouse strains with different sensitivity to 23M-MA to account for variable severity of influenza in humans.

The B/c, A/Sn and CBA mice were used to test the protective effect of 6′SLN-PAA during infection with 23M-MA virus. Infection and drug administration were performed using the aerosol method. Mice were labeled and divided into groups. Three regimens of treatment with the sialylglycopolymer were tested. In the first regimen, mice were treated with 6′SLN-PAA aerosol only once, 20 min before the infecting (10^3 IU of the virus per mouse). The calculated total dose of the polymer received by each animal was about 5 nmol with respect to sialic acid. According to the second regimen, mice were treated with 6′SLN-PAA aerosol started 24 after infecting (10^3 IU per mouse) by 10 min treatments, eight doses per day during 4 days (every 2 h with 10 h night break). The calculated dose was about 2 μg of the polymer (∼1.5 nmol by sialic acid) per mouse in a single treatment. In the third regimen, the analogical treatments with 6′SLN-PAA aerosol were started after detection of the first disease symptoms, namely, 48 h after infection. In this case, the virus dose was increased to 10^4 IU per mouse, which resulted in a 100% mortality of non-treated animals.

Four groups of mice were used in each of the experiments. Two control non-infected groups were treated by either 6′SLN-PAA or placebo. No significant differences in weight dynamics between these groups were observed in any of the experiments, demonstrating a lack of any significant toxic effects of 6′SLN-PAA. Two other groups of mice were infected and treated with either 6′SLN-PAA or placebo.

Data on survival and weight loss of mice subjected to various treatment regimens are given in Table 4. Treatment of non-infected animals with 6′SLN-PAA did not affect their weight. Treatment of infected animals displayed significant therapeutic effect in infected mice in all cases. Treatment before the infecting dramatically increased survival of mice (*P*-value for comparison *S* and WL between placebo–treated and 6′SLN-PAA-treated infected animals was <0.01 in Wilcoxon two-sample test and in paired Student's *t*-test). Multiple treatments started 24 h after infection was even more efficient and completely prevented mortality. The preparation was effective even when mice were infected with lethal dose of virus and treatment was started as

Table 4
Effect of different treatment regimens with aerosolized 6′SLN-PAA on influenza virus infection in CBA mice

Infection	Treatment	Virus dose, treatment regimen[a]					
		10^3 IU, pretreatment[b]		10^3 IU, 24 h post-treatment[c]		10^4 IU, 48 h post-treatment[c]	
		S	WL	*S*	WL	*S*	WL
No	Placebo	100	0 ± 3	100	0 ± 5	100	0 ± 4
No	6′SLN-PAA	100	2 ± 4	100	−3 ± 5	100	2 ± 3
23M-MA	Placebo	30 (8.5)	19 ± 7	40 (8.1)	17 ± 8	0 (6.6)	25 ± 6
23M-MA	6′SLN-PAA	90	8 ± 4	100	6 ± 4	40 (8.6)	18 ± 4

Non-infected groups contained 5 animals and infected contained 10 animals. Mice were infected by aerosol exposure with indicated virus dose. *P*-value for comparison LW between placebo–treated and 6′SLN-PAA-treated infected animals was <0.001 (paired Student's *t*-test); *P* value for comparison *S* between placebo–treated and 6′SLN-PAA-treated infected animals was <0.01 in Wilcoxon two-sample test.
[a] Definition for *S* and WL – see footnotes for Table 3.
[b] Single treatment with 6′SLN-PAA (5 nmol by sialic acid per mouse) performed 20 min before the infecting.
[c] Treatments with either placebo or 6′SLN-PAA by 10 min exposures every 2 h with 10 h night break. The 6′SLN-PAA dose was about 1.5 nmol by sialic acid per mouse during a single treatment.

Table 5
Effect of aerosolized 6′SLN-PAA on influenza virus infection in three strains of mice[a]

Infection	Treatment	B/c		CBA		A/Sn	
		S	WL	*S*	WL	*S*	WL
No	Placebo	100	0 ± 4	100	0 ± 4	100	0 ± 5
No	6′SLN-PAA	100	1 ± 3	100	−1 ± 4	100	0 ± 3
23M-MA	Placebo	90	8 ± 5	60 (8.5)	15 ± 7	10 (8.2)	20 ± 6
23M-MA	6′SLN-PAA	100	4 ± 4	100	7 ± 4	100	8 ± 4

Non-infected groups contained 5 animals, infected groups contained 10 animals. Mice were infected by aerosol exposure to about 10^3 IU of virus per mouse and treated with either placebo or 6′SLN-PAA on days 2–5 after infection, by 10 min exposures every 2 h with 10 h night break. The 6′SLN-PAA dose was about 1.5 nmol by sialic acid per mouse during a single treatment. *P*-value for comparison WL between placebo–treated and 6′SLN-PAA-treated infected animals was <0.001 (paired Student's *t*-test).

[a] Definition for *S* and WL – see footnotes for Table 3.

late as 48 h after infection: although the mice lost their weight significantly, 40% of the animals survived (*P*-value <0.01 in Wilcoxon two-sample test).

Weight and survival curves for one of the strains (A/Sn) infected by 10^3 IU of virus and treated starting of the treatment from 24 h after infection, are given in Fig. 2. A marginal weight gain can be observed in non-infected group of animals. By contrast, infected and placebo treated animals displayed sharp weight loss on days 3–8, and 9 out of 10 animals in this group eventually died. The weight loss in animals treated with 6′SLN-PAA aerosol starting 24 h after infection was about three times lower than in the placebo-treated group. These animals gained weight gradually starting with days 7–8 post infection, and none of them died.

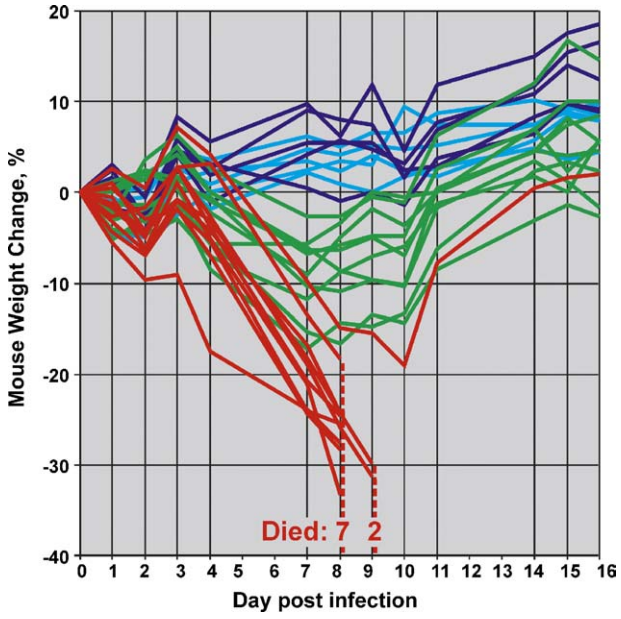

Fig. 2. Weight dynamics and survival of A/Sn mice from non-infected control group treated with placebo (blue, 5 mice); non-infected control group treated with 6′SLN-PAA (cyan, 5 mice); and virus infected mice treated either with placebo (red, 10 mice) or 6′SLN-PAA (green, 10 mice). Mice were treated on days 2–5 after infection (10^3 IU per mouse) by 10 min exposures every 2 h with 10 h night break. The 6′SLN-PAA dose was about 1.5 nmol by sialic acid per mouse during a single treatment. Abrupt drop of curves denotes the death of mouse. *P*-value for WL difference between the two control groups and between the two infected groups was 0.528 and <0.001 accordingly (in paired Student's *t*-test).

Data on survival and weight loss for B/c, CBA and A/Sn mice are given in Table 5. Treatment of non-infected animals with 6′SLN-PAA did not affect their weight. Infection with the 23M-MA virus caused weight loss and death of 10% of the B/c mice, 40% of the CBA mice and up to 90% of the A/Sn mice. Treatment with 6′SLN-PAA lessens the weight loss and completely abolishes mortality in sensitive mouse strains.

4. Discussion

Development of synthetic inhibitors of influenza virus receptor binding was hampered by data on natural inhibitors that are abundant on the surface of target cells. Indeed, in our experiments human airway mucins inhibited virus attachment to receptor analogs even at 1000-fold dilution. This should apparently induce strong competition with cellular receptors. In this view, utilization of synthetic sialylglycopolymers for additional interference with the virus attachment to cells does not seem promising. However, in the course of natural infection the virus is able to efficiently overcome the mucin barrier. Moreover, despite the high binding affinity of mucins for the virus in the in vitro binding assay, they did not appreciably inhibit the viral infection in cell culture (Table 1). In contrast, the synthetic inhibitor 6′SLN-PAA strongly suppressed the infection of MDCK cells under the same experimental conditions. This marked difference between the synthetic inhibitor and the natural mucins inhibitors could be likely explained firstly, by higher stability of 6′SLN-PAA to cleavage by proteases or/and neuraminidase, and secondly, by higher content of receptor moiety (6′SLN) in the synthetic inhibitor as compared to airway mucins. More effective suppression of viral growth by synthetic inhibitor than by mucins stimulated our investigations of the possibility to use the 6′SLN-PAA against influenza virus.

We have demonstrated earlier that a polymer bearing bidentate undecasaccharide, YDS-PAA, protects mice infected with mouse-adapted H3N2 influenza virus A/Aichi/2/68 (Gambaryan et al., 2002a). However, A/Aichi/2/68 is not a typical human virus because of its initial adaptation to chicken embryos followed by mouse adaptation. It is known that adaptation to chicken embryos changes receptor specificity of the virus

(Gambaryan et al., 1997, 1999). Another apparent drawback of this virus strain is that its high pathogenicity for mice does not allow to modeling the typical course of influenza disease in humans.

To avoid these problems, development of more adequate experimental model was attempted. We successfully adapted clinical human isolate in MDCK cells A/NIB/23/89M (H1N1) (Robertson et al., 1991) to mice. Three amino acid replacements discriminate 23M-MA from the parent virus, taken for adaptation, namely, 96Thr → Lys, and 275Asp → Gly in H1 and 145Asn → Asp in H2. 96Asn → Lys replacement resulted in loss of glycosylation site near the receptor-binding site. The loss of glycosylation site at amino acid 94 has been also observed in the case of adaptation H1N1 virus A/USSR/90/77 to mice (Shilov et al., 1984). The replacement 275Asp → Gly could increase the mobility of the HA globular head due to a close proximity of this amino acid to disulfide bridge between 52Cys and 277Cys in the stem and the head of the HA. The 145Asn of HA2 is located near the cleavage site of HA0. It seems likely, therefore, that the mutation 145Asn → Asp in the HA2 of our mouse-adapted virus could affect the efficiency of HA cleavage in a way described previously for the mutation 156Thr → Asn in HA2 of adapted to mice A/HK/1/68 virus (Brown et al., 2001).

We monitored the receptor specificity of the virus at distinct mouse passages. The parent virus displayed the typical receptor-binding phenotype of human H1N1 influenza viruses, namely, a low affinity for 3′SL-PAA and 6′SL-PAA, and much higher affinity for 6′SLN-containing sialylglycopolymer. The receptor properties of the mouse-adapted virus were practically identical to the parent strain (Table 2).

Using this improved animal model, we found that 6′SLN-PAA protects mice from the H1N1 influenza virus. Because 6′SLN is the high-affinity receptor shared by all human influenza A and B viruses (Gambaryan et al., 1997, 1999; Mochalova et al., 2003), 6′SLN-containing polymeric inhibitors could afford protection from all human epidemic strains. Furthermore, it is currently believed that the virus recognition of 6′SLN-terminated receptors is indispensable for the efficient human-to-human spread of the virus (Matrosovich et al., 1999, 2000). If this hypothesis is true, drugs on basis of 6′SLN could be effective for the protection against newly emerging pandemic influenza virus strains.

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