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Synthetic polymeric inhibitors of influenza virus receptor-binding activity suppress virus replication

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Summary

A new approach to anti-influenza chemotherapy is based on the development of synthetic inhibitors of virus attachment to host cells. These inhibitors are prepared by anchoring the minimum receptor determinant of influenza virus, sialic acid, to polymeric or liposomal carriers. In this study, a series of poly(acrylic acid-co-acrylamides) and dextrans bearing pendant glycylamidobenzylsialoside groups were synthesized and evaluated for their binding to a panel of influenza A and B virus strains and for their ability to inhibit virus infectivity in cell culture. Significant type-, subtype-, and strain-specific variation in virus susceptibility to the synthetic inhibitors was observed. Among the viruses tested, H3 subtype strains evolved in humans since 1975 were the most sensitive, while the earlier H3 viruses and the type B strains were resistant. The virus-inhibitory potency of the polymeric sialosides correlated with their bindings to the virus, and was dependent on the virus affinity for the ligand, the density of the ligand, and the nature and molecular mass of the polymeric carrier. In embryonated eggs, the antiviral effect of poly(acryloylglycylamidobenzylsialoside-co-acrylic acid) was comparable to that of equine α_2-macroglobulin.

Influenza virus; Virus attachment; Sialic acid; Polymeric sialoside; Equine α_2-macroglobulin

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[2] *Dedication*: This paper is dedicated to the memory of our dear teacher, Professor Mikhail Petrovich Chumakov.

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Introduction

The idea of competitive inhibition of virus attachment to host cells by suitably designed artificial analogues of cellular receptors may permit a rational development of new antiviral agents (for discussion see Karlsson et al., 1986; Rossman, 1988; Matrosovich, 1989; Lentz, 1990).

Influenza A and B virus attachment is mediated by the binding of the viral hemagglutinin (HA) to terminal sialyloligosaccharides of cell surface glycoproteins or glycolipids. While virus-mediated hemagglutination and sometimes infection are inhibited competitively by natural sialylglycoproteins (reviewed in Krizanova and Ratnova, 1969; Gottschalk et al., 1972; Paulson, 1985), monovalent natural and synthetic sialosides are rather inefficient inhibitors of the multipoint cooperative interaction of the virus particle with cell surface receptors (Zakstelskaya et al., 1972; Pritchett et al., 1987; Sauter et al., 1989). To overcome this problem, bivalent sialosides, sialoside polymers, and sialoside liposomes have been prepared and investigated (Roy and Laferriere, 1988; Matrosovich et al., 1990; Glick and Knowles, 1991; Spaltenstein and Whitesides, 1991; Sabesan et al., 1992; Kingery-Wood et al., 1992; Spevak et al., 1993). Substantial increase in the hemagglutination-inhibiting potency of polyvalent sialosides as compared to monovalent ligands was demonstrated. However, in most studies the virus-inhibiting properties of the compounds were not tested.

Previously, we have described potent synthetic inhibitors of influenza virus-mediated hemagglutination prepared by anchoring glycylamidobenzyl α-glycoside of *N*-acetylneuraminic acid (Sia) to polyacrylic acid carrier (Matrosovich et al., 1990). In this study, these inhibitors and also new series of polymeric sialosides were investigated for their ability to bind to influenza viruses and inhibit virus infectivity.

Materials and Methods

Viruses and cells

Lyophilized seed stocks of influenza viruses were obtained from the sources indicated in Table 1. One or two passages from the seed stocks in 9-day-old embryonated eggs were made at low dilutions, and the allantoic fluids were harvested and stored frozen at −70°C. The anti-influenza virus serum utilized for the virus detection in tissue culture was obtained by hyperimmunization of rabbits. Influenza subunit vaccine ‘Grippovac’ (Institute of Poliomyelitis, Moscow) was used. This vaccine included hemagglutinins and neuraminidases of A/Philippines/2/82 (H3N2), A/Chile/1/83 (H1N1), and B/USSR/100/83 virus strains. Madin-Darby canine kidney (MDCK) cells were maintained in Eagle’s minimum essential medium (MEM) supplemented with 5% fetal calf serum, penicillin and streptomycin. The same medium without serum was used for the maintenance of infected cultures.

TABLE 1

In vitro anti-influenza virus activity of polymeric sialoside $P_{12.1}$, and equine α_2-macroglobulin (EM)

Virus[a]	Affinity of binding to the virus, K_{aff}, μM Sia[b]			Inhibition of virus infectivity for MDCK cells, ED_{50},μM Sia[c]		
	$P_{12.1}$	Neu5-Ac2Bn[d]	EM	$P_{12.1}$	EM	$P_{0.1}$
H3 subtype						
Aichi/1/68 (X-31)	>50	400	0.1	NI (8)	0.4	NI (500)
England/42/72	>50	200	0.02	NI (8)	0.2	
Port Chalmers/1/73	>50	200	0.02	NI (8)	0.4	NI (500)
Victoria/3/75	20	100	0.02	2	0.1	NI (500)
Texas/1/77	0.3	50	0.006	0.5	0.05	
England/321/77	0.05	16	0.004	<0.1	0.05	
Philippines/2/82	0.05	20	0.006	0.2	0.03	
USSR/3/85	0.03	10	0.004	0.2	0.05	NI (500)
USSR/2/85*	0.4	780	0.2	1	0.8	NI (50)
Riga/9977/86	0.2	60	0.01	0.4	0.05	
Sichuan/2/87	0.4	160	0.25	1	0.8	NI (500)
Shanghai/11/87	0.2	10	0.01	0.5	0.4	NI (500)
H1 subtype						
USSR/90/77	5	400	0.6	2	0.8	NI (50)
Chile/1/83*	10	250	0.6	4	NI (0.8)	NI (500)
Singapore/6/86	2	200	0.6	4	NI (0.8)	NI (50)
Type B						
Hong Kong/8/73*	>50	1000	0.4	NI (8)	1.6	NI (50)
Singapore/222/79*	10	320	0.4	NI (8)	NI (3.2)	
USSR/100/83	10	250	0.4	NI (8)	NI (3.2)	
Ann Arbor/1/86	10	500	0.4	NI (8)	NI (3.2)	

[a] Viruses were obtained from the State Collection of viruses of the D.I. Ivanovsky Institute of Virology, Moscow and from the L.A. Tarasevich State Institute for Standardization and Control of Medical Biologicals, Moscow (*). X-31 and USSR/3/85 were kindly provided by Dr. S.A. Wharton (National Institute for Medical Research, London) and Dr. M.A. Yakhno (D.I. Ivanovsky Institute of Virology, Moscow), respectively.

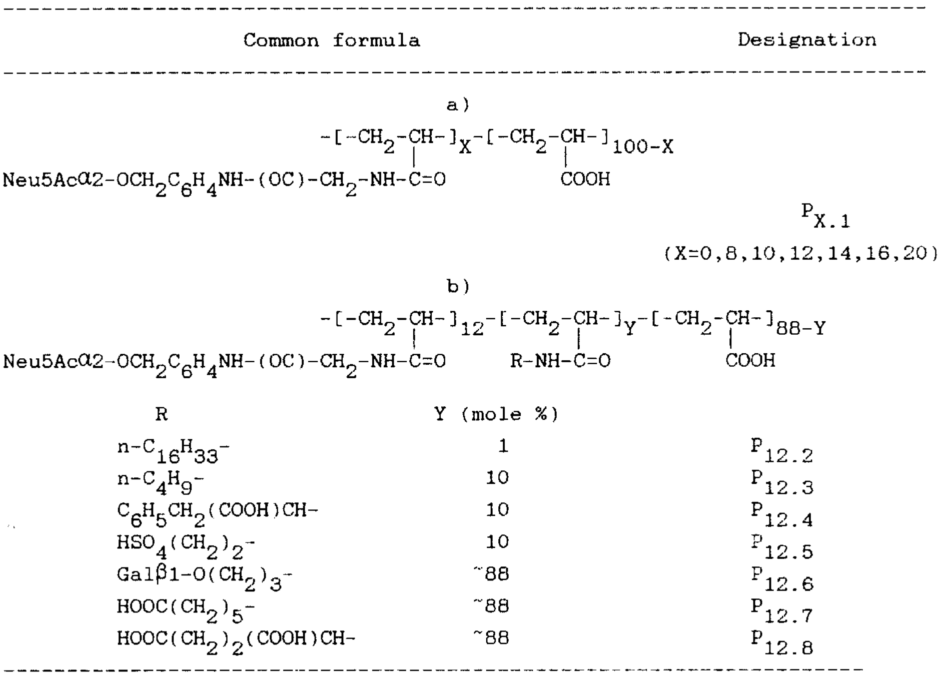
[b] Measured by inhibition of fetuin-HRP binding in a solid phase assay.

[c] Assayed by reduction of virus infectivity for MDCK cells. NI – no statistically significant reduction of infected cell numbers was observed at the highest concentration of the compound. Concentrations are indicated in parentheses in μM Sia for $P_{12.1}$ and EM, and in μg/ml for $P_{0.1}$. The weight concentrations of 1 μM Sia solutions of EM and $P_{12.1}$ were 14 μ/ml and 1.3 μg/ml, respectively.

[d] The data on binding affinity of monomeric α-benzyl sialoside Neu5Ac2Bn was obtained in a separate study on the influenza virus recognition of monovalent sialosides (Matrosovich et al., 1993).

Preparation of inhibitors

(A) Conjugates of polyacrylic acid and poly(acrylic acid-co-acrylamides). Poly(4-nitrophenylacrylate) was synthesized by polymerization of the monomer in benzene essentially as described (Bovin et al., 1993). If not indicated otherwise, the same batch of the polymer was used for the synthesis of inhibitors throughout this study. The synthesis of the monomeric glycylamidobenzyl α-glycoside of Neu5Ac (Sia-GAB) and the procedure used



Common formula	Designation
a) $-[-CH_2-\underset{\text{Neu5Ac}\alpha 2-OCH_2C_6H_4NH-(OC)-CH_2-NH-C=O}{\underset{\vert}{CH}}-]_X-[-CH_2-\underset{COOH}{\underset{\vert}{CH}}-]_{100-X}$	$P_{X.1}$ (X=0,8,10,12,14,16,20)
b) $-[-CH_2-\underset{\text{Neu5Ac}\alpha 2-OCH_2C_6H_4NH-(OC)-CH_2-NH-C=O}{\underset{\vert}{CH}}-]_{12}-[-CH_2-\underset{R-NH-C=O}{\underset{\vert}{CH}}-]_Y-[-CH_2-\underset{COOH}{\underset{\vert}{CH}}-]_{88-Y}$	

R	Y (mole %)	
$n-C_{16}H_{33}-$	1	$P_{12.2}$
$n-C_4H_9-$	10	$P_{12.3}$
$C_6H_5CH_2(COOH)CH-$	10	$P_{12.4}$
$HSO_4(CH_2)_2-$	10	$P_{12.5}$
$Gal\beta 1-O(CH_2)_3-$	~88	$P_{12.6}$
$HOOC(CH_2)_5-$	~88	$P_{12.7}$
$HOOC(CH_2)_2(COOH)CH-$	~88	$P_{12.8}$

Fig. 1. Structural formula of the synthetic sialoside poly(acrylic acid-co-acrylamides).

for the synthesis of copolymers of acryloyl-glycylamidobenzyl sialoside with acrylic acid ($P_{X.1}$ series, Fig. 1a) were described previously (Byramova et al., 1991). The Sia content in polymeric sialosides was assessed spectrophotometrically by using monomeric Sia-GAB as a standard (λ_{max} 248 nm, E 17000 $cm^{-1} \times M^{-1}$).

To prepare polymeric sialosides with modified carrier ($P_{12.n}$ series, Fig. 1b), 4.2 μmol Sia-GAB in 200 μl of DMF were mixed with 70 μl of 10% solution of poly(4-nitrophenylacrylate) in DMF (35 μmol in respect to monomeric unit). Twenty μl of triethylamine were added and the mixture was kept at 20°C for 48–60 h until monovalent sialoside depletion was achieved as judged by TLC. The resulting copolymer was further modified by addition of one of the aminoligands (*n*-hexadecylamine, *n*-butylamine, L-phenylalanine, 2-aminoethylsulfuric acid, 3-aminopropyl-β-D-galactoside, ε-aminohexanoic acid, or L-glutaminic acid) in dimethylformamide (DMF) or DMF-water for 24 h at 20°C. Unreacted nitrophenyl groups were hydrolyzed by addition of 2 ml of 0.1 N aqueous NaOH for 48–72 h at 20°C. Liberated 4-nitrophenol was removed by gel-filtration on a Sephadex LH-20 column (1 × 25 cm, MeCN-H_2O 1:1), and the preparations were lyophilized.

To prepare fractions of $P_{12.1}$ with different molecular masses, a sample of $P_{12.1}$ was synthesized using a separate batch of poly(4-nitrophenyl acrylate).

This sample was fractionated on a Sephacryl S-300 Superfine column equilibrated with 0.2 M NaCl. Five fractions were obtained. The mean effective molecular masses of the fractions were determined using an analytical Sephacryl S-300 Superfine column calibrated with globular proteins.

(B) Conjugate of dextrans. Polymeric sialosides of this type were prepared by periodate oxidation of dextrans T70 and T500 (Pharmacia Fine Chemicals, Sweden, mean molecular masses 70 and 500 kDa, respectively) followed by reductive amination of aldehyde groups of the polyaldehyde dextrans with Sia-GAB in the presence of cyanoborohydride. Briefly, to 0.4 ml solution of 20 mg dextran in 0.025 M Na-acetate buffer (pH 5.75) 0.037 ml of 0.5 M solution of sodium periodate was added, and the mixture was incubated for 1.5 h at the room temperature. After dialysis against water for 16 h at 4°C, 12.5 μM Sia-GAB was added (0.1 mol per mol of glucose unit of dextran), followed 0.5 h later by the addition of 20-fold excess of sodium cyanoborohydride and by incubation of the mixture for 24 h at 4°C. The mixture was finally treated with sodium borohydride solution in 0.5 M Na-borate buffer (pH 8.75) for 3 h at 0°C to reduce unreacted aldehyde groups, adjusted to pH 6 by addition of acetic acid, dialyzed against 0.5 M solution of NaCl, water, and lyophilized. Polymeric sialosides $D70_4$ and $D500_4$ were thus obtained, bearing 4 mol of Sia-GAB moieties per 100 glucose units of the carrier, as estimated spectrophotometrically using the monomeric sialoside as a standard. $D70_{17}$ conjugate (17 mol % of Sia-GAB) was prepared from T70 dextran by the same method apart from using 2-fold higher concentrations of both sodium periodate and Sia-GAB during the synthesis.

(C) Partially purified equine α_2-macroglobulin (EM) was prepared from normal horse serum. The serum was treated with ammonium sulfate at 30% saturation, and the precipitate was discarded. The supernatant was adjusted with ammonium sulfate to 60% saturation, the precipitate was dissolved in PBS and chromatographed on a Sephacryl S-300 column. Fractions were assayed for the total protein amount and for the anti-influenza virus activity in the binding inhibition assay (see below). According to SDS gel-electrophoretic analysis, the peak fraction chosen for subsequent studies contained about 50% EM. To facilitate a comparison with synthetic inhibitors, concentrations of EM were expressed as a measure of sialic acid content, assuming 55 mol Sia per mol EM as reported by Pritchett and Paulson (1989).

Binding inhibition assay

The affinity of the influenza viruses for the sialosides was assayed as described previously (Gambaryan and Matrosovich, 1992).

In brief, viruses in allantoic fluid diluted with PBS to the hemagglutination titer 1:10–1:40 were adsorbed to the wells of fetuin precoated microplates for 2 h at 4°C. The mixture of an inhibitor with the fetuin-horseradish peroxidase (HRP) conjugate in PBS supplemented with 0.01% Tween-20 (PBST) were

TABLE 2

Potency of synthetic polymeric sialosides as inhibitors of A/Philippines/2/82 (H3N2) receptor-binding activity (K_{aff})[a] and infectivity (ED_{50})[b]

	Compound															
	$P_{8.1}$	$P_{10.1}$	$P_{12.1}$	$P_{14.1}$	$P_{16.1}$	$P_{20.1}$	$P_{12.2}$	$P_{12.3}$	$P_{12.4}$	$P_{12.5}$	$P_{12.6}$	$P_{12.7}$	$P_{12.8}$	$D70_4$	$D70_{17}$	$D500_4$
K_{aff}, μM Sia	0.05	0.05	0.05	0.1	0.15	0.2	0.5	0.2	0.2	0.2	0.3	0.2	0.2	1.5	0.7	0.16
ED_{50}, μM Sia	0.2	0.2	0.2	0.8	1	2	1.5	0.4	0.2	0.4	3	1	4	10	1	0.5

[a] Assayed by inhibition of fetuin-HRP conjugate-binding.
[b] Assayed in MDCK cells.

incubated in the wells of the virus-precoated plate for 1 h at 4–6°C, the plate was washed by PBST and the amount of labelled fetuin bound was quantified by a standard assay of peroxidase activity using chromogenic substrate *o*-phenylenediamine.

The affinity constants of the virus-inhibitor complexes (K_{aff}), formally equivalent to the dissociation constants of the complexes, were calculated essentially as described before (Gambaryan and Matrosovich, 1992). To improve the reliability of the results, replicate assays were performed on different days, and the data were averaged. Standard deviations of the mean values of $\log_2 K_{aff}$ thus obtained did not exceed 1. For compounds not inhibitory at the highest concentration used, only lower limits of K_{aff}s could be estimated, as indicated in Tables 1–3.

Antiviral and cytotoxicity assays

The susceptibility of influenza virus strains to natural and synthetic inhibitor was evaluated in an infectious focus reduction microneutralization assay.

TABLE 3

Binding affinity (K_{aff}, μM Sia (I)) and virus-inhibiting potency (ED_{50}, μM Sia (II)) of fractions of $P_{12.1}$[a]

Mean molecular mass of fraction, kDa	Weight % of fraction in $P_{12.1}$	Activities against			
		A/Philippines/2/82		A/USSR/90/77	
		I	II	I	II
20	13	4	10	10	>10
70	16	1.5		2	
200	26	0.3		1.2	
500	30	0.04	0.15	0.8	2.5
>1000	15	0.01	<0.1	0.5	0.6
Unfractionated $P_{12.1}$	100	0.15	0.1	1.2	1.4

[a] Polymeric sialoside $P_{12.1}$ was fractionated as described in Materials and Methods section. Each fraction was assayed for the binding by the virus (I), and some fractions were tested for inhibition of virus infectivity in MDCK cells (II).

MDCK cells were seeded in 96-well microplates and incubated at 37°C in 5% CO_2. After the cultures had become confluent, growth medium was withdrawn and the cell monolayers were washed twice with serum-free maintenance medium (MM). Serial 2-fold dilution of inhibitor in MM or control diluent were mixed with an equal volume of influenza virus adjusted to give a final control count of 50–200 infected cells per microscopic field (250–1000 infected cells per well). The mixtures were incubated for 1 h at room temperature and were added to the cultures, 0.02 ml per well, at least 3 wells per dilution. After incubation for 1 h at 20°C, 0.1 ml MM was added to each well and the plates were incubated at 35°C in a 5% CO_2 atmosphere. After 14 to 18 h incubation, the cells were fixed with 5% formalin in MM (0.5 h at room temperature). For the detection of infected cells, cultures were successively treated with polyvalent rabbit anti-influenza HA serum (1:1000), peroxidase conjugated sheep anti-rabbit antibody (1:1000, the Gamaleya Institute of Microbiology, Moscow), and standard substrate solution (0.5 mg/ml 3-amino-9-ethylcarbazole, 0.005% H_2O_2, pH 5.5). Intervening washes were done with PBST. The infected cells stained for the presence of viral glycoproteins on their surface were counted under an inverted microscope. The neutralizing activity of inhibitors (ED_{50}) was expressed as the concentration of Sia groups in the highest dilution of the compound that reduced the number of infected cells to 50% of that of the virus control. The ED_{50}s were estimated to the nearest $\log_2$ value. Standard deviations of the mean values of $\log_2 ED_{50}$ calculated from the three replicates performed on the same tissue culture microplate were usually in the range 0–0.6. The validity of the assays performed on different days were checked by using the reference virus strain A/Philippines/2/82 and the reference inhibitor $P_{12.1}$. Results from assays showing more than 4-fold difference from the standard ED_{50} value (0.2 μM Sia) were disregarded.

To determine possible effects of polymeric sialosides on cellular proliferation, approximately 5×10^3 MDCK-cells were seeded per well of the microplate and incubated in growth medium containing 2-fold dilutions of the compound (8 wells per dilution) until formation of confluent monolayers (3–4 days). Cells in experimental wells and in noninhibitor-exposed control wells were counted under an inverted microscope.

Virus neutralization assay in embryonated eggs

Serial dilutions of an inhibitor in PBS sterilized by ultrafiltration or PBS alone (control) were mixed with an equal volume of A/Philippines/2/82 virus adjusted to about 200 EID_{50}/0.2 ml. The mixtures were incubated for 1 h at 20°C, and were inoculated in 0.2 ml aliquots into the allantoic cavity of 9- or 10-day-old embryonated eggs (6–8 eggs per dilution). After incubation at 35°C for 2 days the allantoic fluid of each egg was tested for the presence of hemagglutinating virus.

Results

Susceptibility of influenza A and B virus strains to inhibition by polymeric sialoside $P_{12.1}$

Susceptibility testing was performed by measuring the ability of the sialoside to bind to virus receptor-binding sites in a competitive solid phase assay and to inhibit the virus infectivity in cell culture. Equine α_2-macroglobulin (EM), the known potent γ-inhibitor from horse serum (Pritchett and Paulson, 1989), was tested for comparison. Table 1 summarizes the results obtained.

As can be seen, the antiviral effect of $P_{12.1}$ assayed in cell culture was dependent on the virus strain. In particular, H3 subtype strains evolved in humans after 1975 were inhibited most effectively; H1 strains were less susceptible, while no inhibition was observed for the earlier H3 viruses and for the type B strains at the highest concentration of $P_{12.1}$ used. The antiviral effect of the polymeric sialoside correlated with its affinity for the corresponding virus strain in the binding inhibition assay ($r = 0.61$), and also with the binding affinity of the virus for the monomeric ligand Neu5Ac2Bn ($r = 0.31$). No influence of the carrier itself ($P_{0.1}$) on the virus infectivity was detected under these assay conditions.

In general, the strain specificities of the viruses as compared to equine macroglobulin and to $P_{12.1}$ were similar. It could be noted, however, that the difference between the early and the later H3 strains in the susceptibility to the inhibition by EM was not as remarkable as found for the synthetic inhibitor. With susceptible strains, the sialylglycoprotein was about one order of magnitude more potent than polymeric sialoside $P_{12.1}$, when effective concentrations of sialic acid moieties of the two compounds were compared. However, the weight effective concentrations of the two inhibitors were comparable, as at equimolar concentrations of the Sia groups, the concentration of $P_{12.1}$ in μg/ml was about 10-fold lower than that of EM.

To check for possible cellular toxicities of the polymeric sialoside and the carrier, their effects on the growth of MDCK cells were studied. Neither compound revealed an inhibition of cell growth at the highest concentrations tested (50 μg/ml for $P_{0.1}$, and 60 μg/ml (50 μM Sia) for $P_{12.1}$). On this basis, the selectivity index of $P_{12.1}$ with respect to most susceptible H3 strains, estimated as a ratio of the cytostatic dose to ED_{50}, was greater than 100.

Inhibition of influenza virus multiplication in embryonated eggs

Neutralization tests were performed utilizing $P_{12.1}$, EM and one of the susceptible virus strain, A/Philippines/2/82. The mean ED_{50} value for $P_{12.1}$ evaluated from three separate experiments corresponded to about 10 μM Sia in the inoculum, or to approximately 0.3 μM final concentration of Sia in the allantoic sac of the embryo. The mean ED_{50} value for the equine α_2-macroglobulin (two experiments performed on separate days) was about 5 μM Sia in the inoculum. Thus, the difference between the antiviral potency of the two inhibitors assayed in ovo was lower than that observed in MDCK cells.

This might be explained by a higher susceptibility of the natural inhibitor to inactivation under the more stringent in ovo conditions.

Structure-antiviral activity relationships of synthetic inhibitors

In an effort to improve the virus-inhibiting potency of the compounds, a number of different polymeric sialosides were prepared and tested for their binding to A/Philippines/2/82 and for their antiviral activity in MDCK cells.

The affinity of the conjugates for the virus receptor-binding sites and, concomitantly, their antiviral potency were found to depend on both the density of sialic acid moieties, and the nature of the polymeric carrier (Table 2). The polyacrylic acid copolymers of the $P_{X.1}$ series (see Fig. 1a) contained from 8 to 20 residues of the Sia-GAB ligand per 100 monomeric units of the main chain of the polymer. As can be seen from Table 2, 8–12 mol % of sialic acid residues provided the maximum affinity of the polymeric sialoside for the virus and the maximum antiviral activity of the conjugate in tissue culture.

In the $P_{12.n}$ series (Fig. 1b), modifications of the polyacrylate carrier were made by using the same intermediate polymer bearing 12 mol % of Sia-GAB groups. Neither substitution of the carboxylic groups of the carrier increased the binding affinity or antiviral activity of the inhibitor as compared to the parental $P_{12.1}$. The three polymeric sialosides $P_{12.6}$, $P_{12.7}$, and $P_{12.8}$, in which all side chain carboxylic groups of the carrier were modified, were substantially less potent than $P_{12.1}$. This effect was probably due to a steric interference of the neighboring bulky substituents with a proper accommodation of the ligand in the virus receptor-binding site. Modifications of each tenth monomeric unit of the carrier in $P_{12.3}$, $P_{12.4}$, and $P_{12.5}$ had no significant effect on virus-inhibiting activity. In contrast, even 1 mol % of the highly hydrophobic *n*-cetyl substituent ($P_{12.2}$) decreased the inhibitory potency of the conjugate. It may be suggested, that this substituent lead to a more compact globular conformation of the macromolecule and thus to a poorer exposition of the ligand.

In the Sia-GAB-dextran series, $D70_4$ (4 mol % of Sia) had a 2-fold lower affinity for the virus and a 10-fold lower inhibitory potency than $D70_{17}$ (17 mol % Sia). The activity of inhibitors in this series was increased 10-fold when going from $D70_4$ (70 kD) to $D500_4$ (500 kD). Both $D70_{17}$ and $D500_4$ were, however, slightly less potent than $P_{12.1}$.

To evaluate a possible influence of the molecular mass of $P_{12.1}$ on the inhibitory activity, an initially polydisperse preparation of $P_{12.1}$ was fractionated, and the fractions obtained were tested for the binding to viruses and for antiviral activity. It was found (Table 3), that the higher molecular mass of the polymeric sialoside afforded a higher activity, the heaviest fraction being superior to the unfractionated $P_{12.1}$. The lowest molecular mass fraction (20 kDa) was far less active than unfractionated $P_{12.1}$. However, it still exhibited one order of magnitude higher binding affinity for the viruses compared to the monovalent ligand (Neu5Ac2Bn).

Discussion

In the present study, the ability of synthetic polymeric inhibitors of hemagglutination to inhibit virus infectivity in vitro and in ovo was demonstrated.

The antiviral potency of polymeric sialosides correlated well with their affinity for the virus receptor-binding sites. This finding suggests that the main effect of these compounds may be the inhibition of the virus attachment to target cells. As for natural inhibitors (Gottschalk et al., 1972; Krizanova and Ratnova, 1969; Paulson, 1985), significant type-, subtype-, and strain-specific variation in virus susceptibility to inhibition by synthetic glycylamidobenzyl sialoside-co-polyacrylic acid was observed. This effect could be explained, at least partly, by the differences in the virus affinity for the ligand. Bearing in mind the variability of the fine architecture of the receptor-binding sites of influenza A and B viruses (Matrosovich et al., 1991, 1993), it may be suggested, that different ligands may be required to obtain potent polymeric inhibitors of distinct receptor-binding viruses. In addition to the nature of the ligand, some other parameters should obviously affect the potency of an inhibitor. Our previous study of polyacrylic acid-based sialosides (Matrosovich et al., 1990) revealed that the polymeric sialoside with an average 10 mol % of Sia-GAB residues ($P_{10.1}$, see Fig. 1a) was a significantly better inhibitor than the polymers with either 5, or 20 mol % of Sia groups. To specify further the optimal density of the ligand, polymers with a narrower range of the Sia-GAB content were prepared and studied. 10–12% of sialic acid residues were found to be optimal.

Among a number of modifications of the polyacrylic acid carrier tested, none was found to be beneficial for the binding affinity and the activity of the inhibitors against the A/Philippines/2/82 strain. This carrier probably confers the maximally extended conformation of the inhibitor, the best exposition of the ligand, and/or some favorable electrostatic interactions with the surface of the virus particle. Since the latter effect can depend on the particular virus strain tested, additional studies with different viruses are needed for a further optimization of the carrier.

For both dextran and polyacrylic acid conjugates, significant influence of the molecular mass on the inhibitory activity was observed. In general, the shorter polymers were less potent.

To provide efficient binding to the virus particles, the sialic acid moieties of the inhibitor should be properly presented to the virus receptor-binding sites. Evidently, this would require the optimization of the spacer group between Sia and the carrier. In the present study, this question was not addressed and the same spacer (-Gly-4-amidobenzyl-) was used in all conjugates prepared. It may be speculated, however, that a longer spacer could be more useful, especially in the case of type B influenza viruses, as the B receptor-binding site appears to be deeper than that of the type A hemagglutinin (Matrosovich et al., 1993).

In summary, our data seem to indicate that the rational design of artificial polyvalent sialoside inhibitors of influenza virus receptor-binding sites could provide preparations with significant antiviral potency. Further studies are needed, however, to evaluate the prospects of this approach for the development of new anti-influenza virus drugs.

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