Synthetic polymeric sialoside inhibitors of influenza virus receptor-binding activity

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Anomeric aminobenzylglycosides of Neu5Ac were coupled with the polyacrylate carrier and a number of synthetic polyvalent sialosides obtained were investigated as inhibitors of influenza virus attachment. The inhibitory activity of polymeric sialosides is highly dependent upon the Neu5Ac residue content and the nature of the carrier. The polyacrylic acid based polymer bearing 10 mol% of Neu5Ac is 3 orders of magnitude more potent inhibitor than the corresponding monovalent benzylsialoside and considerably more active than fetuin.

Influenza virus; Virus attachment inhibitor; Inhibitor of hemagglutination; Sialic acid; Fetuin; Antiviral drug

1. INTRODUCTION

Influenza virus infection is initiated by the specific attachment of virus to the cellular receptors mediated by the viral HA; the RBS of HA recognizes sialic acid residues of the host cell glycoproteins or glycolipids (reviewed in [1,2]).

Hemagglutination and in some cases infection by influenza viruses could be inhibited by natural sialylglycoproteins [1-3]. Some low molecular mass sialic acid derivatives have been tested [3-5], but their inhibitory activity was too low to be of practical interest. Considering the multivalent cooperative nature of the virus–cell interaction, some authors supposed that the development of potent artificial inhibitors of influenza virus attachment must include the design of polyvalent structures [6-9]. Roy et al. [10] reported protein conjugates with Neu5Ac to be active in HAI test. Totally synthetic polymers containing Neu5Ac were also obtained [11], but nothing was reported on their inhibitory activity.

We have synthesized water-soluble polymers differing in Neu5Ac content and demonstrated the existence of the sharp optimum for Neu5Ac density on the carrier to reveal maximal inhibition of the influenza virus receptor-binding activity.

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Abbreviations: FBI, fetuin-binding inhibition; HA, hemagglutinin; HAI, hemagglutination inhibition; HRP, horseradish peroxidase; Neu5Ac, 5-*N*-acetylneuraminic acid; RBS, receptor-binding site; SAα2,6Lac, α2,6 sialyllactose

2. MATERIALS AND METHODS

2.1. *Materials*

The synthesis of monosialosides (Table 1A) will be described elsewhere (Byramova et al.). Poly(4-nitrophenylacrylate) was a gift from Dr A.E. Ivanov (Shemyakin Institute of Bioorganic Chemistry). SAα2,6Lac, Twin-80, HPR were obtained from Serva, bovine fetuin from Fluka. All the other reagents were of analytical grade. Buffers: PBS = 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.3; PBST = PBS containing 0.01% Twin-80.

2.2. *Viruses*

Seed stocks of influenza viruses were obtained from the Virus Collection of the Ivanovsky Institute of Virology, Moscow. Viruses were grown in 9-day-old embryonated chicken eggs and purified by sucrose density gradient centrifugation [12].

2.3. *Synthesis of polymeric sialosides*

3.5 μmol SA$\alpha(\beta)$GAB in 200 μl of DMF were mixed with 70 μl of 10% solution of poly(4-nitrophenylacrylate) in DMF (35 μmol with respect to monomeric units). 20 μl of triethylamine were then added and the mixture was kept at 20°C for 48–60 h until monovalent sialoside depletion as judged by TLC. The resulting copolymer was further modified by addition of 2 ml of either 0.1 N aqueous NaOH or 10% ethanolamine in DMF: 48–72 h later 4-nitrophenol was removed by gel-filtration on Sephadex LH-20 column (1 × 25 cm; MeCN/H_2O, 1:1). In the first case the copolymers designated as $P_{\alpha\,10.1}$ or $P_{\beta.10.1}$, in the second as $P_{\alpha.10.2}$ (Table 1B) were obtained with 85–90% yield. To obtain $P_{\alpha.5.1}$, $P_{\alpha.20.1}$ and $P_{\alpha.30\,1}$ (Table 1B), 3.5 μmol of SAαGAB in 200 μl of DMF were mixed with 140, 35, or 23 μl of 10% poly(4-nitrophenylacrylate) in DMF, respectively, the unreacted groups being hydrolyzed by NaOH. A control polymer, polyacryl(2-hydroxyethyl)amide (P), was obtained by treatment of 14 mg of poly(4-nitrophenylacrylate) with 2 ml of 10% ethanolamine in DMF. Its average molecular mass determined by gel-filtration using dextrans as standards was approximately 100 kDa.

The SA$\alpha(\beta)$GAB content in polymeric sialosides was assessed spectrophotometrically at 248 nm using SAαAAB as a standard.

2.4. *Hemagglutination inhibition assay*

HAI test was carried out by the microtiter method [12] using PBS as a diluent.

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Table I

Mono- (A) and polyvalent (B) sialosides studied

(A) Common formula	R	Designation
Neu5Acα(or β)2→$OCH_2C_6H_4NH$-R	$COCH_3$	SAα(or β)AAB
	$COCH_2NHCOOC(CH_3)_3$	SAα(or β)BGAB
	$COCH_2NH_2$	SAα(or β)GAB

(B) Common formula	X	Mole fraction of Neu5Ac (%)	Y	Designation
$-[-CH_2-CH-]_n-CH_2-CH-$ (C=O under each CH; Y on the first, X on the second)	SAαGAB	5	ONa	$P_{\alpha.5.1}$
	SAαGAB	10	ONa	$P_{\alpha.10.1}$
	SAβGAB	10	ONa	$P_{\beta.10.1}$
	SAαGAB	10	$NH(CH_2)_2OH$	$P_{\alpha.10.2}$
	SAαGAB	20	ONa	$P_{\alpha.20.1}$
	SAαGAB	30	ONa	$P_{\alpha.30.1}$
	$NH(CH_2)_2OH$	0	$NH(CH_2)_2OH$	P

2.5. *Fetuin-binding inhibition assay*

FBI test (A.S. Gambaryan and M.N. Matrosovich, in preparation) was carried out as follows. Fetuin-HRP conjugate was synthesized by the periodate method [13]. The microtiter plate wells (Linbro, Flow, USA) were coated with 100 μl of fetuin solution (10 μg/ml) in PBS at 4°C overnight. The plate was washed with PBST, water and air-dried. The virus (0.2 μg) in 100 μl of PBST was allowed to bind to the immobilized fetuin for 2 h at 4°C then the plate was washed with PBST. The PBS-dissolved inhibitors in various concentrations or PBS alone (50 μl) were added for 10 min followed by 50 μl of fetuin-HRP conjugate (1 μg/ml in respect to fetuin). After incubation for 2 h at 4°C the plate was washed, filled with *o*-phenylendiamine and further operations were carried out as usual.

3. RESULTS AND DISCUSSION

To produce potent artificial influenza virus attachment inhibitors, polyvalent sialic acid-containing macromolecules were synthesized.

Pritchett et al. [4] showed Neu5Ac α-benzylglycoside to be the most potent inhibitor of A/Memphis/102/72 (H3N2) influenza virus adsorption to erythrocytes among a number of synthetic monovalent sialosides examined. Taking this into account and intending to bind Neu5Ac residues to the polymeric carrier, aminobenzylglycosides of Neu5Ac (Table 1A) were synthesized. Polyvalent sialosides were obtained by condensation of SAαGAB or SAβGAB with poly(4-nitrophenylacrylate) followed by modification of the resulting polymer with aqueous alkali or ethanolamine (Table 1B).

The studies on inhibitors of influenza virus attachment are usually based on an assessment of their ability to block virus-mediated hemagglutination or adsorption of the virus to erythrocytes (2–4,9], but monovalent sialosides were found to compete poorly with red blood cells for virus binding [4]. To compare relative inhibitory activity of mono- and polyvalent sialosides, an alternative assay has been explored here. This solid-phase enzyme-linked assay is based on the competition for specific binding by the viral HA between the sialoside under study and sialylglycoprotein fetuin labeled with HPR.

The competition curves (Fig. 1) show that binding of the fetuin conjugate by the A/Bangkok/1/79 (H3N2) virus could be totally inhibited by monovalent SAαAAB, SAαBGAB and their polymeric analogs. β-Anomers SAβAAB and SAβBGAB, corresponding to polymer $P_{\beta.10.1}$, and polymer carrier alone (P; see Table II) are inactive. These results indicate that binding of fetuin-HRP to the immobilized virus is specifically inhibited by mono- and polyvalent α-benzylglycosides of Neu5Ac.

The activity of substances tested has been expressed as the concentration of sialic acid residues required to inhibit fetuin-HRP binding to virus by 50%. Comparing a number of mono- and polyvalent inhibitors (Table II), we came to the following conclusions. Monovalent sialosides SAαAAB and SAαBGAB are approximately 5-fold more active inhibitors of BK/79 and TX/77 (H3N2) influenza viruses than SAα2,6Lac in accordance with the data of Pritchett et al. [4]. The coupling of the monovalent receptor analog with the polymeric carrier increases markedly its ability to block viral

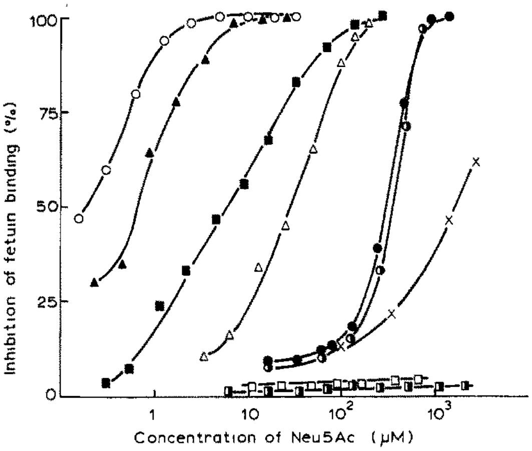

Fig. 1. Inhibition of the A/Bangkok/1/79 binding of the fetuin conjugate by SAα2,6Lac (×), SAαAAB (◑), SAαBGAB (●), SAβBGAB (◧), $P_{\beta.10.1}$ (□), $P_{\alpha.5.1}$ (Δ), $P_{\alpha.10.1}$ (○), $P_{\alpha.20.1}$ (▲), $P_{\alpha.30.1}$ (■).

Table II

Inhibition of A/Bangkok/1/79 (BK/79) and A/Texas/1/77 (TX/77) by synthetic and natural sialosides

Sialoside	FBI test				HAI test	
	Concentration for 50% inhibition[a]		Relative potency[b]		Minimum concentration for HAI[a]	
	BK/79	TX/77	BK/79	TX/77	BK/79	TX/77
SAα2,6Lac	2100	1500	0.2	0.2	ND[c]	ND
SAαAAB	450	300	1	1	ND	ND
SAαBGAB	470	310	1	1	ND	ND
$P_{\alpha\ 10.2}$	141	4.5	3.3	70	>400[d]	>400[d]
$P_{\alpha.5.1}$	31	10.4	15	30	>100[d]	100[d]
$P_{\alpha.10.1}$	0.23	0.23	2 040	1350	1.8 (2.5)	3.6 (4.8)
$P_{\alpha.20.1}$	0.6	1.7	783	182	4.8 (4.4)	17 (16)
$P_{\alpha.30.1}$	5.8	3.7	81	84	32 (24)	167 (125)
P	(>900)[d]	(>900)[d]			(>250)[d]	(>250)[d]
Fetuin	7	14	67	22	10(51)	49(250)

[a]αM Neu5Ac (values in parentheses represent weight concentration in μg/ml) [b]Inhibitory potency in respect to SAαBGAB [c]ND = not determined [d]There was no inhibition at the highest concentration used

receptor-binding activity, $P_{\alpha.5.1}$ being 15–30-fold, and $P_{\alpha.10.1}$ being 3 orders of magnitude more active inhibitors of BK/79 and TX/77 viruses than monovalent sialosides. Most likely, $P_{\alpha.10.1}$ has an optimal composition for interaction with the multivalent viral HA, since an additional increase of the Neu5Ac residue content in the polymers ($P_{\alpha.20.1}$ and especially $P_{\alpha.30.1}$) lowered inhibitory activity substantially. It should be clarified whether the linear distance between the neighboring Neu5Ac residues, the distinctions in the shape of polymeric sialosides differing on Neu5Ac density, or other factors play a crucial role in the observed effect.

The influence of the macromolecular carrier on the inhibitory activity is demonstrated by comparison of $P_{\alpha.10.1}$ and $P_{\alpha.10.2}$ polymers with the same Neu5Ac content. The activity of $P_{\alpha.10.1}$ bearing ionizable $-COOH$ residues against TX/77 and BK/79, respectively, is 20- and 1000-fold higher than that for $P_{\alpha.10.2}$ which contains $-CONHCH_2CH_2OH$ moieties. The higher activity of $P_{\alpha.10.1}$ polysialoside may be caused by the imitation with polyanionic carrier of negatively charged cell membrane, or by the higher conformational rigidity of the charged polymer macromolecule.

The relative potency of polyvalent inhibitors in the FBI test correlates with their activity in the HAI assay (Table II). $P_{\alpha.10.1}$, the most potent inhibitor synthesized, blocks hemagglutination of BK/79 and TX/77, respectively, 6 and 13 times stronger than fetuin (20 and 50, respectively, if the weight concentrations of both inhibitors are compared). As far as we know, this is the first example of a totally synthetic and comparatively potent inhibitor of influenza virus-mediated hemagglutination.

Since individual influenza viruses are known to vary considerably in their receptor-binding properties, the inhibition of different viral strains by synthesized compounds has been checked. Monovalent sialoside SAαAAB, when tested in the FBI assay, as well as the corresponding polyvalent sialoside $P_{\alpha.10.1}$ in both FBI and HAI tests inhibits only H3 subtype human influenza A viruses BK/79, TX/77, A/Aichi/2/68, A/Philippines/ 2/82, while being inactive as inhibitors of avian H3 subtype virus A/duck/Ukraine/63 and all the other A and B viruses studied (A/Chile/1/83, A/Taiwan/1/86 (H1), A/Singapore/1/57 (H2), B/Lee/40, B/Hong Kong/8/73 and B/USSR/100/83; data not shown). It seems that Neu5Ac α-benzylglycosides mimic the Neu5Acα2,6Gal receptor determinants, recognized by human H3 subtype viruses [1,2].

In the present study, a new principle of designing artificial influenza virus attachment inhibitors has been evaluated. The results obtained may be interesting in the development of inhibitors of influenza virus infection.

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