Monomeric inhibitors of influenza neuraminidase enhance the hemagglutination inhibition activities of polyacrylamides presenting multiple C-sialoside groups

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Background: Influenza viruses use hemagglutinin (HA) arrays to bind to sialic acid moieties on the surface of cells; crosslinking of erythrocytes by this mechanism leads to hemagglutination. A number of synthetic polymers containing multiple sialic acid (Neu5Ac) groups as side chains are potent inhibitors of this process. Inhibition may be due to two mechanisms: polyvalent binding of the inhibitor's multiple Neu5Ac side chains to multiple HA sites on the viral surface, or steric stabilization of the viral particle by a layer of the adsorbed, water-swollen polymer, which prevents adhesion to the erythrocyte. The balance between these two effects is not yet known.

Results: Polyacrylamides with multiple C-sialosides (PA(Neu5Ac)) were 2–20 fold more effective as inhibitors of virally mediated hemagglutination when assayed in the presence of Neu2en-NH_2, a potent monomeric inhibitor of influenza neuraminidase (NA). The ability of monomeric inhibitors of NA to enhance the inhibition of hemagglutination in this assay correlated with the affinity of the monomer for NA.

Conclusions: We propose that inhibitors of NA act by competing with the C-sialosides of PA(Neu5Ac) for binding to the active sites of the NA. Competitive displacement of Neu5Ac causes an expansion of the layer of polymeric gel adsorbed to the virus, enhancing its inhibitory effect. This study provides an example of synergy between two ligands directed toward the active sites of two different proteins, and reinforces the conclusion that steric stabilization is important for the activity of polyvalent inhibitors.

Chemistry & Biology February 1996, **3**:97–104

Key words: hemagglutination inhibitors, influenza virus, neuraminidase inhibitors, polyvalency, steric stabilization

Introduction

Influenza virus A is the causative agent of a serious disease in humans [1,2]. The virus initiates infection by attaching to multiple sialyl (*N*-acetylneuraminic acid, Neu5Ac) moieties on the surface of mammalian epithelial cells in the upper respiratory system, via hemagglutinin (HA) on the viral surface (Fig. 1) [3–5]. HA exists as a symmetric trimer ($[HA]_3$), and extends ~135 Å from the lipid membrane of the viral surface. HA is expressed at a density of ~200–300 $[HA]_3$ units per virion, comprising ~80–90 % of all the proteins on the surface. Viral neuraminidase (NA), another surface glycoprotein, is a hydrolytic enzyme that catalyzes cleavage of the α-O-glycosidic linkage of Neu5Ac; this bond connects Neu5Ac to a variety of aglycons on the surface of the target cell [6,7]. NA exists as a tetramer ($[NA]_4$), which extends ~60 Å from the surface, and each virion contains ~20–40 copies of $[NA]_4$. Thus, *in vivo*, α-glycosides of Neu5Ac are both substrates for NA and ligands for HA. The action of NA is curiously opposite to that of HA; the former releases the virus from the cellular surface, whereas the latter attaches it. The enzymatic action of NA may be important in facilitating the penetration of virus to the target cell through the mucous layer, which is rich in a variety of Neu5Ac-containing mucins, as well as in promoting release of newly formed virions from the surface of infected cells [7].

An important consideration in designing strategies to block adhesion of influenza virus to cells is that the virus binds to the cell strongly, although individual viral surface receptors bind individual cell-surface ligands weakly. Influenza virus attaches to the surface of an erythrocyte using multiple interactions between clusters of HA and sialyl residues on the surface of the erythrocyte with an apparent K_d^{HA} of $< 10^{-12}$ M [8–10], while bromelain-cleaved HA (HA that has been cleaved from the surface of the virus; BHA) binds to α-methyl sialosides (Neu5Ac-OMe in Fig. 1b) more weakly by a factor of ~10^9 ($K_d^{HA} = 2.5 \times 10^{-3}$ M) [11,12]. This large difference is due to polyvalent binding of viral receptors to cellular ligands.

To inhibit this polyvalent interaction between the virus and cell, we have designed and synthesized a number of polyvalent inhibitors [13], in particular polymers that present multiple Neu5Ac groups on side chains. These polyvalent inhibitors are more effective inhibitors of hemagglutination than structurally analogous monovalent derivatives by a factor of 10^4–10^7 (per Neu5Ac ligand) [8,10,13–16], and are currently the most potent of all synthetic or natural inhibitors [13].

There are at least two mechanisms that might, in principle, underlie the remarkable effectiveness of these

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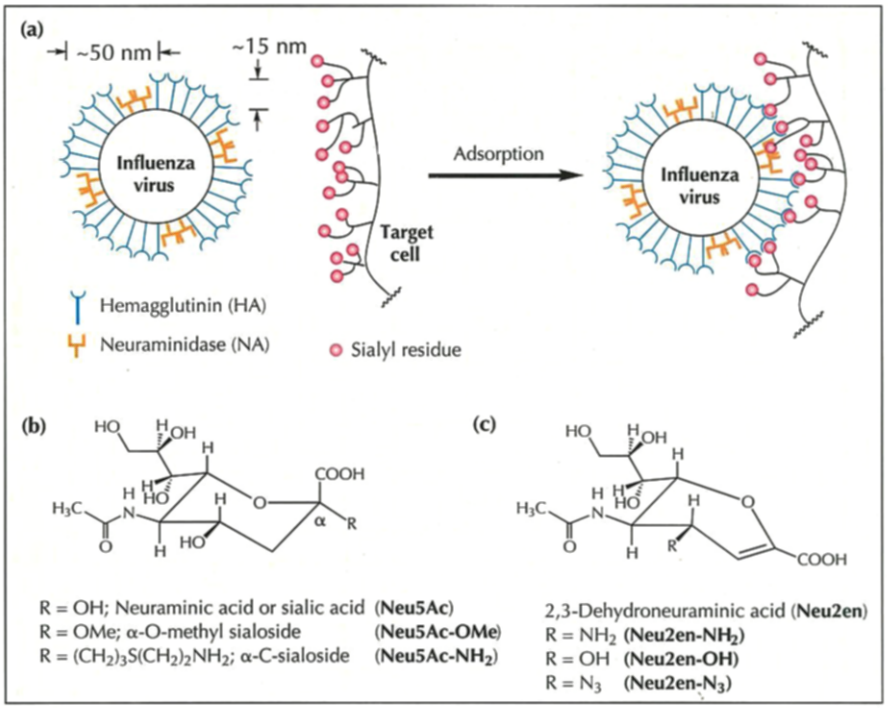

Fig.1. The biological system and the compounds used in this study. **(a)** A schematic model showing adsorption of influenza virus to a target cell, which is the initial, necessary step of viral infection [4]. The attachment occurs through interactions between viral HA and sialyl moieties on the surface of the target cell. **(b)** Structure of Neu5Ac (*N*-acetylneuraminic acid; sialic acid) and analogs. **(c)** Structures of Neu2en (2,3-dehydroneuraminic acid) and analogs. These compounds are potent inhibitors of influenza NA.

polymeric species as inhibitors of the adhesion of virus to cells. The first mechanism involves high affinity resulting from polyvalent binding; the Neu5Ac groups on the side chains of the polymer might bind cooperatively to the HA on the surface of virus. Alternatively, the polymeric inhibitors may work by steric stabilization of the virus against association with the erythrocyte; parts of the polymer not involved directly in binding to HA may make the surface of the virus sterically inaccessible to the surface of the target cell. These two mechanisms might operate independently or additively, but yield the same effect — enhanced inhibition of hemagglutination. The two mechanisms are almost certainly countered, to some extent, by steric interference of binding of the Neu5Ac to HA by the backbone of the polymer; a single ligand attached to a polymer might bind less tightly to a receptor than the same unattached ligand [14].

Here we describe the influence of monomeric inhibitors of influenza NA on the ability of polymers presenting multiple Neu5Ac groups to inhibit the agglutination of erythrocytes by influenza virus. We use these data to clarify the modes of action of the polymers. First, we demonstrate that the effectiveness of certain polymers can be enhanced by factors of 2–20 when assayed in a system that includes Neu2en-NH_2 [17], which is a potent competitive inhibitor of NA (K_i^{NA} = 50 nM) but a poor ligand for HA ($K_d^{HA} \geq 50$ mM). The enhancement results from a combined use of two classes of inhibitors; the action of either inhibitor alone cannot account for the observed activities. Second, the inhibition constants of three different monomeric inhibitors of NA (K_i^{NA}) correlated with the concentrations of these inhibitors required to enhance the hemagglutinin inhibition (HAI) activities of the polymers. This correlation suggests strongly that the enhancement of inhibition of hemagglutination by the polymers is a result of specific binding of monomeric inhibitors of NA to the active sites of NA.

Results and discussion

Preparation of inhibitors of influenza virus

We prepared derivatives of polyacrylamide having α-C-glycosidic Neu5Ac groups (Neu5Ac-NH_2; Fig. 1b) as side chains. These polymers inhibited the agglutination of erythrocytes by influenza virus [13]. We used an α-C-glycosidic linkage rather than an α-O-glycosidic linkage between the Neu5Ac group and the linking side chain to prevent the hydrolytic cleavage of α-O-glycosidic Neu5Ac, which is catalyzed by NA (the value of the Michaelis constant, K_m, of Neu5Ac(α2,3)-lactose is 0.2 mM for NA from influenza virus) [18]. This instability to NA-catalyzed cleavage has been a major drawback of polymeric species containing α-O-sialoside groups as inhibitors of hemagglutination (e.g., the naturally occurring inhibitors of infection by influenza virus, such as α_2-macroglobulin, have this limitation). The polymers containing an α-C-glycosidic linkage are inert to hydrolysis by NA. Neu5Ac groups coupled via α-C-glycosidic linkages are believed to bind to HA (and NA) in the same manner as the α-O-sialoside, based on the crystal structures of the complexes of HA and NA with their ligand [3,12,19,20], in which the oxygen atom of the α-O-linkage does not interact with the active-site residues significantly.

The α-O-sialosides bind weakly to NA. By contrast, some 2,3-dehydroneuraminic acids (Neu2en; Fig. 1c) bind tightly to NA [17,21] . The molecular basis of this tight interaction is probably the similarity in structure

between Neu2en and the transition state for the NA-catalyzed hydrolysis of α-O-sialosides. Neu2en is the core of a class of potent inhibitors of influenza NA that mimic the hydrolytic transition state, such as 4-guanidinoNeu2en (K_i^{NA} = 0.2 nM), Neu2en-NH_2 (K_i^{NA} = 50 nM), Neu2en-OH (K_i^{NA} = 1 μM), and Neu2en-N_3 (believed to be the least potent analog as judged from K_i for NA from other sources, although the value of K_i for influenza NA is not available) [17,20,22,23]. We synthesized these analogs of Neu2en using methods developed by von Itzstein *et al.* [22].

The synthesis of polyacrylamides presenting α-C-glycosidic Neu5Ac groups as side chains [13,16] is summarized in Figure 2. We first prepared a polymer (poly(*N*-acryloyloxysuccinimide), pNAS) that has carboxylic acids preactivated for reaction with amine-containing nucleophiles by radical-initiated polymerization of *N*-acryloyloxysuccinimide [13]. We allowed this homopolymer to react with χ equivalents of Neu5Ac-NH_2 (χ = 0.05, 0.2, 0.35, 0.6 or 1.0 molar equivalents per mole of activated ester on the polymer); here χ denotes the mole fraction of Neu5Ac in the polymers and is defined as the number of side chains containing Neu5Ac divided by the total number of side chains. Subsequent reaction with excess ammonia converted unreacted *N*-hydroxysuccinimide esters to primary amide groups. These two reactions yielded polyacrylamide with pendant C-sialosides. For most of the polymers, the experimental value of χ^{Neu5Ac} (obtained from analysis of both integrations of ^{1}H-NMR spectra and sulfur/nitrogen ratios following complete oxidative combustion) was generally about 10 % lower than that predicted theoretically [13]. For simplicity in nomenclature, we refer to these polymeric C-sialosides as PA(Neu5Ac, χ = 0.05, 0.2, 0.35, 0.6, 1.0). This synthetic strategy, which uses the modification of a preformed, reactive homopolymer to make a (formal) copolymer having two (or more) types of side chains, enabled us to prepare a wide range of polymers having the same polydispersity and polytacticity, but with controlled densities of biologically-active functional groups along the backbone of the polymer [13,24].

Effect of Neu2en-NH_2 on HAI activity of PA(Neu5Ac)

Using previously established methods [13,25–27], we assayed the HAI activities of the set of polymers PA(Neu5Ac, χ = 0.05, 0.2, 0.35, 0.6, 1.0) against virally-induced agglutination of chicken erythrocytes. The HAI activities were assayed using PA(Neu5Ac) alone or using PA(Neu5Ac) in the presence of various concentrations of the NA inhibitor Neu2en-NH_2 (Fig. 3a). A plot of K_i^{HAI} (defined as the lowest concentration of an inhibitor required to prevent hemagglutination) of polymers against χ^{Neu5Ac} is shown in Figure 3b. HAI activity of the polymer increased rapidly with increasing χ^{Neu5Ac}, reached a maximum at $\chi^{Neu5Ac} \approx 0.4$–$0.5$, and then gradually decreased. The molecular basis of this variation of activities with changing χ^{Neu5Ac} is not completely defined, but appears to be a consequence of a combination of high affinity, polyvalent binding of Neu5Ac groups to HA, steric stabilization of the virus by the adsorbed layer of polymer [13,14] and inefficient use of Neu5Ac groups at high values of χ.

The dependence of the value of K_i^{HAI} of PA(Neu5Ac, χ) on the concentration of Neu2en-NH_2 for each value of χ^{Neu5Ac} (0.05, 0.2, 0.35, 0.6 and 1.0) is plotted in Figure 3a. The presence of Neu2en-NH_2 in the system, at concentrations greater than ~0.5 mM, increased the effectiveness of PA(Neu5Ac, χ) in inhibiting hemagglutination by 2–20 fold (Fig. 3b,c). PA(Neu5Ac, χ = 1.0) showed the largest enhancement (~20 fold) in HAI activity among tested polymers. There is a sigmoidal relationship between the measured value of K_i^{HAI} and concentrations of the monovalent NA inhibitor (Fig. 3a), with the midpoints centered around 0.07–0.2 mM of the NA inhibitor.

We suggest two hypotheses to rationalize the synergism between polymeric sialosides and monomeric inhibitors of NA in inhibiting hemagglutination (Fig. 4). The synergy might be due to the release of Neu5Ac moieties on the polymer from active sites of NA (Mode a) and/or from binding sites of HA (Mode b) by competitive binding of Neu2en-NH_2. In either case, releasing Neu5Ac from the surface of the virus would both

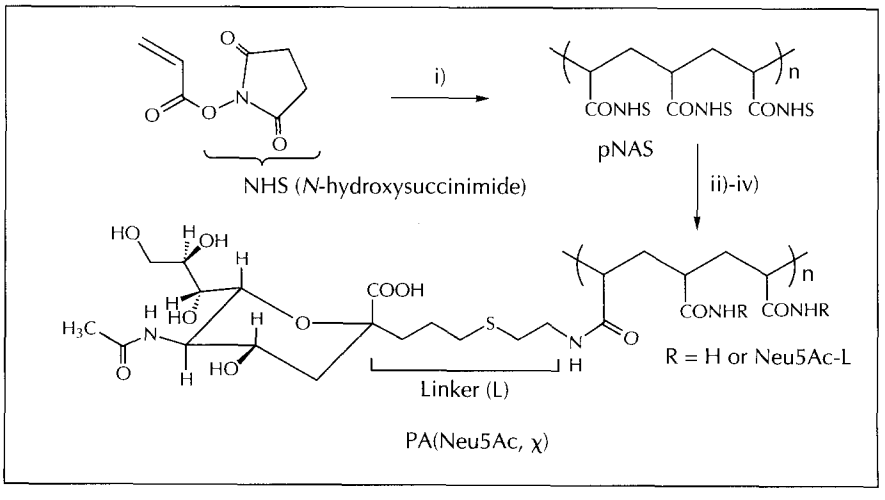

Fig. 2. Preparation of PA(Neu5Ac), polyacrylamides with side chains containing multiple copies of α-C-sialosides [13]. The reaction conditions are: i) 2,2'-azobis-iso-butyronitrile (AIBN), tetrahydrofuran (THF), 60 °C, ii) χ molar equivalents of Neu5Ac-NH_2 (per mole of activated ester on the polymer), triethylamine (Et_3N), N,N-dimethylformamide (DMF), room temperature, iii) excess ammonia, iv) dialysis against distilled water. By varying the number of equivalents of Neu5Ac-NH2 (χ), five polymers, PA(Neu5Ac, χ = 0.05, 0.2, 0.35, 0.6, 1.0), were prepared, where χ denotes the mole fraction of Neu5Ac in the polymers and is defined as the number of side chains containing Neu5Ac divided by the total number of side chains.

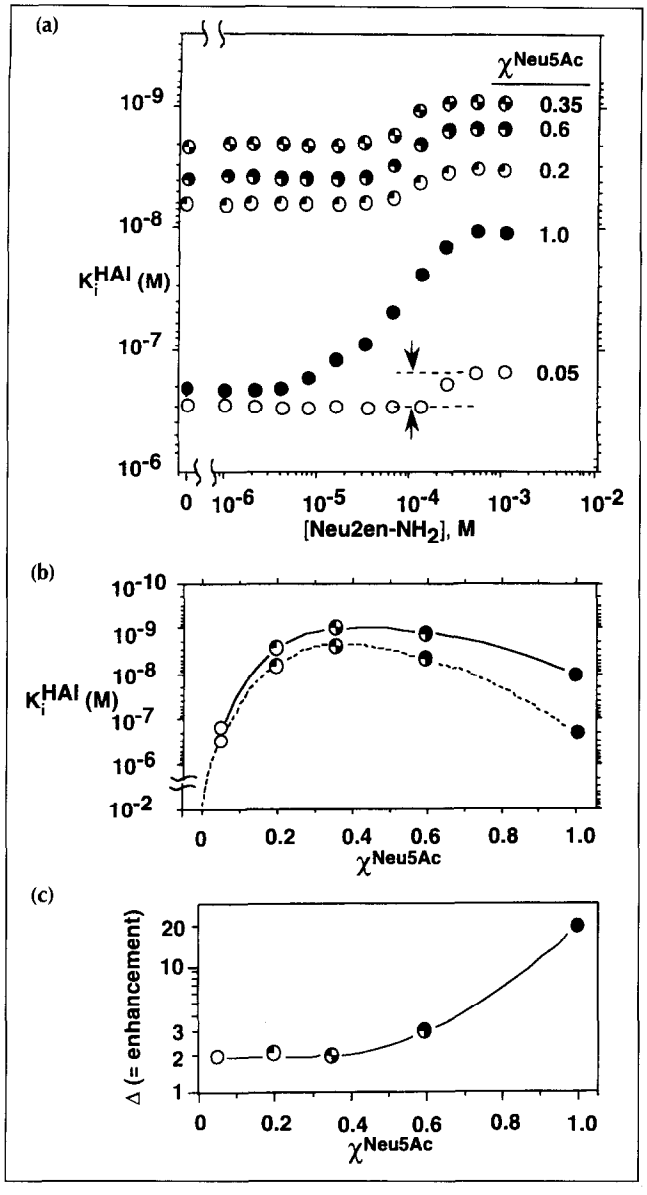

Fig. 3. HAI activity of PA(Neu5Ac) varies with χ, and is increased by the addition of the NA inhibitor Neu2en-NH_2. **(a)** The values of K_i^{HAI} of PA(Neu5Ac, χ = 0.05, 0.2, 0.35, 0.6, 1.0) are plotted as a function of the concentration of Neu2en-NH_2. The arrows indicate the value of the maximum enhancement ($\Delta = K_i^{HAI}{}_{[Neu2en]=0} / K_i^{HAI}{}_{[Neu2en]}$) in HAI activity of PA(Neu5Ac, χ = 0.05) by Neu2en-NH_2. **(b)** The value of K_i^{HAI} of PA(Neu5Ac, χ) varied with the mole fraction of Neu5Ac (χ^{Neu5Ac}); the detailed shape of the curve depended on the concentration of added Neu2en-NH_2 (0 M, dotted line or 10^{-3} M, solid line). **(c)** The maximum enhancements in HAI activities of PA(Neu5Ac, χ) by Neu2en-NH_2 are plotted. Hemagglutination inhibition assays (at 20 °C) of PA(Neu5Ac, χ) were performed in the presence of various concentrations of Neu2en-NH_2 according to standard procedures [24,25] using erythrocytes from 2-week-old chicks and influenza A virus (strain X-31). Each reported value of K_i^{HAI} is the average of at least five independent trials; the experimental uncertainty in each value is approximately ± 25 %.

increase the number of free Neu5Ac groups near the surface of the virus and decrease the number of contact points between the virus and the polymer. We consider the influence of these two effects in turn.

(i) Increased concentration of Neu5Ac

The concentrations of HA and NA used in the assay system are estimated to be ~2 x 10^{-10} M and ~2 x 10^{-11} M, respectively, based on the concentrations of virus (~ 0.06 μg total protein ml^{-1} corresponding to ~2 x 10^{-13} mole of HA ml^{-1}). The increase in concentration of Neu5Ac by release from binding sites on NA or HA is small compared to the total amount of Neu5Ac in the system (10^{-6}–10^{-9} M; Fig. 3); release from NA sites could increase the concentration of Neu5Ac groups by at most only 1 %. We do not believe that this effect, by itself, is sufficient to explain the increase in HAI activities of the polymer in the presence of Neu2en.

(ii) Decreased number of contacts between the polymer and the virus

Displacing Neu5Ac moieties of the polymer from either HA or NA sites would decrease the number of contact points between the polymer and the virus. We propose that this decrease in the number of attachment points will have two effects. First, the average size of the loops of polymer formed on the surface may increase. This increase is effectively an expansion of the thickness of the polymeric gel layer adsorbed to the surface of the virus. Such expansion may increase the steric stabilization of the surface of the virus, enhancing the inhibition of hemagglutination by the polymer. Second, the affinity of the polymer for the viral surface may decrease, which may reduce the effectiveness of the polymer. These opposing consequences of competitive displacement of Neu5Ac from sites on NA or HA may yield different net effects depending on the polymer.

We suggest that displacement of Neu5Ac moieties from the NA binding sites (Fig. 4, mode a) is more important than displacement from the HA binding sites (Fig. 4, mode b) on the basis of two observations. First, although Neu2en-NH_2 has a high affinity for NA (K_i^{NA} = 50 nM) [17], it exhibited no HAI activity even at K_i^{HAI} > 50 mM under the same assay conditions. Since, for monomeric inhibitors, the value of K_i^{HAI} closely matches the dissociation constant of the complex between HA and the inhibitor (K_d^{HA}) [9,11], this result suggests that the binding of Neu2en-NH_2 to HA sites (Mode b) is weak (K_d^{HA} > 50 mM) and therefore is not significant. Second, monomeric α-O-sialosides, which bind to HA with $K_i^{HAI} \approx$ 2.5 mM (more tightly than Neu2en-NH_2) as well as to NA with $K_d^{NA} \approx$ 0.1 mM (much less tightly than Neu2en-NH_2), did not affect the HAI activities of polymeric sialosides.

A non-linear behavior in the factor of maximum enhancement of HAI activities (Δ, which is equal to $K_i^{HAI}{}_{[Neu2en]=0} / K_i^{HAI}{}_{[Neu2en]}$) of PA(Neu5Ac) is indicated in Figure 3b,c. The enhancement is greater at high values of χ^{Neu5Ac} (0.6–1.0) than at low values of χ^{Neu5Ac} (0–0.6). The reason for this behavior is not yet completely clear, but may reflect the possible mechanisms underlying the variation of HAI activities of

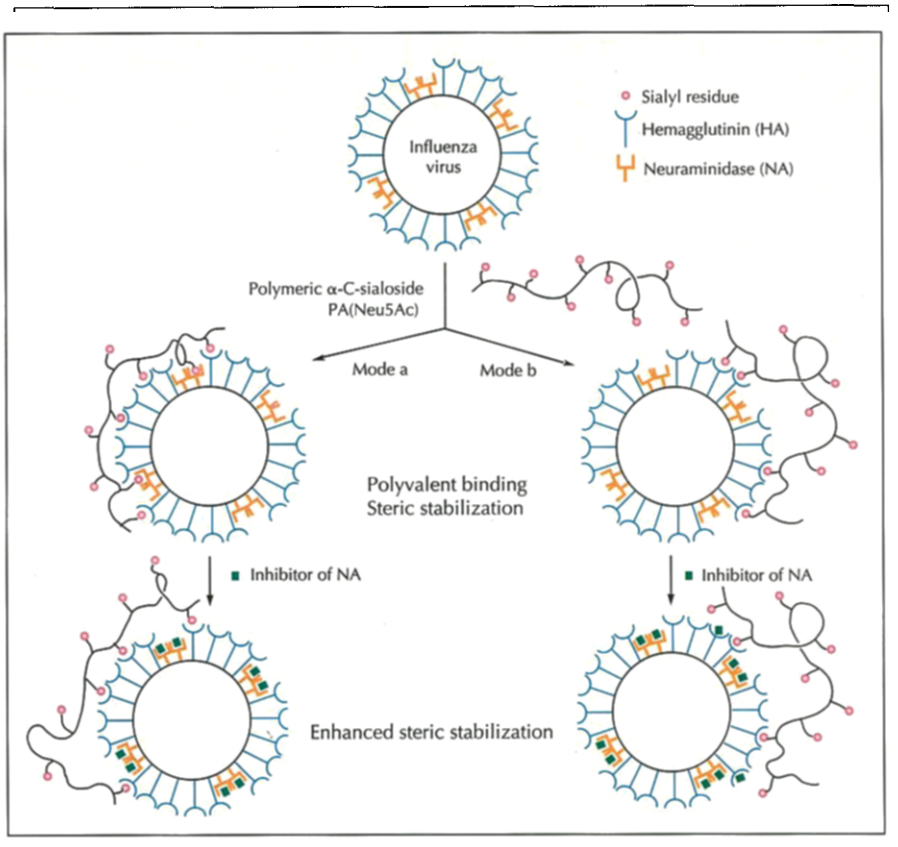

Fig. 4. Hypothetical mechanisms, describing the possible modes of action of PA(Neu5Ac) in inhibition of virally induced hemagglutination, and the effect of inhibitors of NA on HAI activities of PA(Neu5Ac). Polymers inhibit virus by a combination of polyvalent binding of Neu5Ac moieties to HA sites as well as steric stabilization of the surface of virus. The latter effect may be caused by adsorbed layers of water-swollen polymers. The presence of NA inhibitor is believed to release Neu5Ac from active sites of NA (mode a) and/or HA sites (mode b), which may subsequently lead to expansion of gel layers.

PA(Neu5Ac) with χ^{Neu5Ac}. Increasing χ^{Neu5Ac} of the polymer may lead to a positive contribution to HAI activities from improved polyvalent binding of the polymer to the surface of virus, and to a negative contribution from a reduced ability of the polymer to stabilize sterically the viral surface (due to an increased persistence length and the greater rigidity of the increasingly negatively charged polymer) [13,14]. A linear increase of χ^{Neu5Ac} of PA(Neu5Ac) led to a non-linear decrease in the HAI activities of the polymer in the range of $\chi^{Neu5Ac} > \sim 0.5$ (Fig. 3b, dotted curve). We hypothesize that, as the value of χ^{Neu5Ac} increases (especially beyond 0.5), the increase in the affinity of the polymer for the surface of the virus is marginal, and the decrease in the ability of the polymer to stabilize sterically the viral surface is significant. The monomeric inhibitors of NA, according to this hypothesis, reduce the negative impact of a decreasing ability of the polymers with high values of χ^{Neu5Ac} to stabilize sterically the viral surface, while marginally reducing the affinities of these polymers for the viral surface (Fig. 3b, solid curve).

$[Neu2en]_{1/2\Delta}$ correlates with K_d^{NA} for inhibitors of NA

The concentrations of Neu2en that gave half-maximal enhancement of the HAI activity of all PA(Neu5Ac) (referred to as $[Neu2en]_{1/2\Delta}$) were ~70–200 μM (Fig. 3a). We hypothesize that the value of $[Neu2en]_{1/2\Delta}$ is the concentration of monomeric inhibitor required to displace a certain fraction of the Neu5Ac groups of the polymer bound at the active sites of NA. We assayed three inhibitors of influenza NA: Neu2en-NH_2 (K_i^{NA} = 50 nM), Neu2en-OH (K_i^{NA} = 1 μM) and Neu2en-N_3 ($K_i^{NA} > 1$ μM, [23]). The values of K_i^{HAI} for PA(Neu5Ac, χ = 1.0) at different concentrations of Neu2en-NH_2, Neu2en-OH or Neu2en-N_3 are plotted in Figure 5a. The values of $[Neu2en]_{1/2\Delta}$ are 70 μM, 300 μM and ≥ 2 mM for Neu2en-NH_2, Neu2en-OH, and Neu2en-N_3, respectively.

How tightly does the polymeric Neu5Ac bind to NA? Although the data are limited, there seems to be a correlation between the values of $[Neu2en]_{1/2\Delta}$ and those of K_i^{NA} (Fig. 5b). The values of $[Neu2en]_{1/2\Delta}$ for Neu2en-NH_2 and Neu2en-OH (70 and 300 μM) are ~800 (±400)-fold higher than the corresponding values of K_i^{NA}. Furthermore, monomeric sialosides bind weakly to influenza NA ($K_M \approx 10^{-4}$ M) in comparison with the inhibitors of NA used here ($K_i^{NA} \approx K_d^{NA} \approx 10^{-6}$–$10^{-8}$ M). These results indicate that sialosides on the polymer are difficult to displace from sites on NA even with tight binding inhibitors of NA; we interpret the observed discrepancy between K_i^{NA} and $[Neu2en]_{1/2\Delta}$ as evidence for polyvalent, high affinity binding of polymeric Neu5Ac to NA sites on the surface of virus.

In summary, we have demonstrated that HAI activities of polyacrylamides presenting C-sialosides could be enhanced by factors of 2–20 by co-application of monomeric inhibitors of influenza NA. In addition, our results suggest that the potency of a monomeric inhibitor of NA in enhancing HAI activity is inversely proportional to the value of K_i^{NA} for that inhibitor.

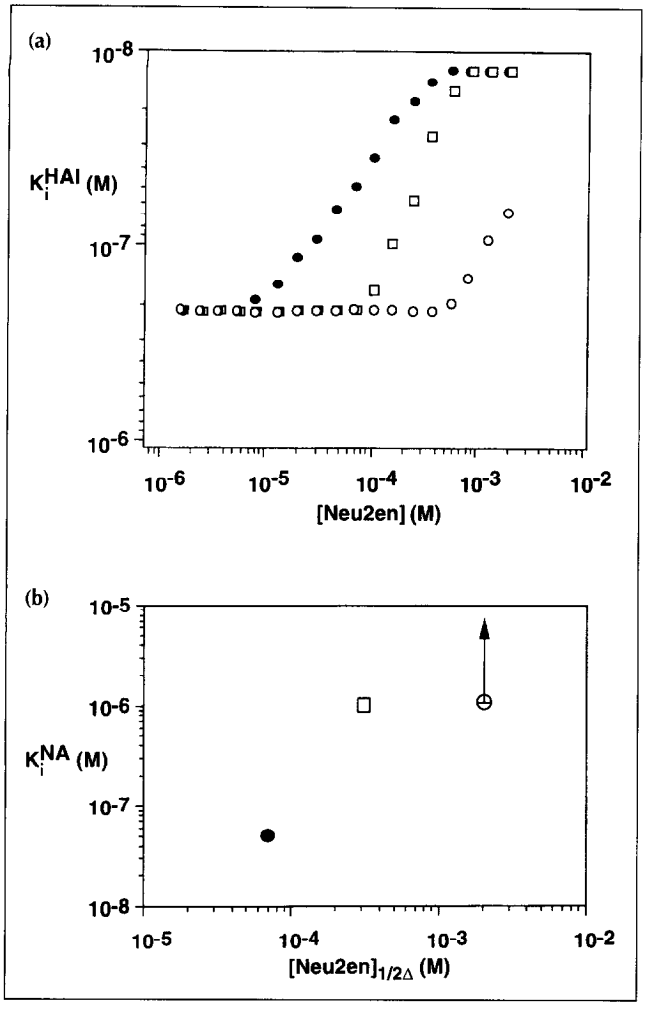

Fig. 5. The concentrations of Neu2en that give half-maximal enhancement of the HAI activity of PA(Neu5Ac) ($[Neu2en]_{1/2\Delta}$) correlate with the values of K_i^{NA} for the NA inhibitors. **(a)** The values of K_i^{HAI} for PA(Neu5Ac, $\chi = 1.0$) were measured as a function of the concentrations of inhibitors of NA (Neu2en-NH_2, ●; -OH, □; and -N_3, ○). **(b)** A plot showing the relationship between $[Neu2en]_{1/2\Delta}$ and K_i^{NA} for these inhibitors of NA. The value of K_i^{NA} for Neu2en-N_3 is a lower limit; this value has not been determined precisely. The uncertainties are approximately ± 25 %.

Significance

There are a number of approaches, in principle, to inhibiting infection by influenza virus. These include the induction of anti-influenza antibodies and influenza-specific lymphocytes by vaccination [28–30], the use of inhibitors of either of the two surface proteins of the virus (HA and NA) [13–17,25,31–35], and the use of inhibitors that block the viral M2 protein (a proton channel) [36]. Antibodies act by binding to the surfaces of HA or NA [28–30]; this action may prevent the virus from attaching to cells by competitively occupying HA sites, and/or by sterically preventing the surface of the target cell from coming into close contact with the virus [29]. Due to frequent antigenic drift and occasional antigenic shift of viral proteins, the effectiveness of antibodies induced from even relatively recent strains of the virus is limited in the prevention and treatment of influenza [30].

Glycopolymers containing Neu5Ac represent a class of agents that can block the attachment of influenza to its target cell by binding to the Neu5Ac binding sites on HA. Polyvalent inhibition may be a general strategy to block interaction of complementary biological surfaces, which themselves interact polyvalently. This mode of action is not used in current antiviral pharmaceuticals, and is therefore a potential new entry into this difficult area. The practical value of this approach depends on issues of toxicity, effectiveness and delivery *in vivo*, and has not been evaluated.

The unsaturated analogs of Neu5Ac (Neu2en) belong to another class of agents that may prevent the viral infection by inhibiting the action of NA. The present study, which demonstrates the synergy of simultaneous application of these two classes of inhibitors, widens our understanding regarding the molecular basis of interactions between polyvalent ligands and clusters of receptors on a biological surface. We conclude that the observed HAI activities reflect both polyvalent binding of Neu5Ac at HA (and NA) sites, and steric stabilization of the virion by adsorbed polymer. To our knowledge, this result represents the first example of synergy in the action of a monovalent inhibitor directed toward one receptor and a polyvalent, polymeric inhibitor directed toward a second receptor. This combination strategy may be applicable to other viruses that are structurally and functionally similar to influenza virus [37–39].

Materials and methods

Materials

All common chemicals used in this study were purchased from chemical suppliers, and were used as received without further purification. Polyacrylamides containing α-C-sialosides (PA(Neu5Ac, χ)) were synthesized according to the scheme in Figure 2 by following detailed procedures developed in this laboratory [13]. The monomers Neu2en-NH_2 and Neu2en-N_3 were synthesized as described previously [22], and Neu2en-OH was purchased from Boehringer Mannheim. The synthetic compounds were characterized by ^{1}H-NMR and fast atom bombardment mass spectroscopy.

Phosphate-buffered saline (PBS) used in the HAI assay was prepared by dissolving 80 g NaCl, 2 g KCl, 11 g Na_2HPO_4, and 2 g KH_2PO_4 in 1 l of deionized H_2O, then diluting this stock solution 10-fold in deionized H_2O, and finally adjusting the pH of the diluted solution to 7.2 with 1 N NaOH. Erythrocytes from two week old chicks were purchased from Spafas Inc. An aliquot of erythrocytes (5 ml), supplied as a suspension in a storage buffer (5 % by volume), was diluted to 50 ml with PBS (pH 7.2), followed by centrifugation at 2000 rpm for ~7 min at 4 °C. After centrifugation,

the supernatant was gently decanted and the pellet was resuspended in PBS (50 ml) followed by further centrifugation; this procedure was repeated two more times. At the conclusion of the washing steps, the red pellet was resuspended in PBS (50 ml) and was used immediately in the assay. Influenza virus A (strain X-31) was a gift from Professor John J. Skehel at the Division of Virology, National Institute of Medical Research, UK. For storage, a stock solution of virus was kept at 4 °C at ~15 mg of protein per ml. Before use, the suspension was diluted by a factor of 5000 with PBS, after which the diluted solution was sonicated for ~30 s. Monomeric inhibitors of NA (Neu2en-NH_2, -OH and -N_3) were dissolved in PBS at a concentration of 400 mM. The polymers were also dissolved in PBS to give a final concentration of Neu5Ac of 0.1–0.01 mM.

Hemagglutination inhibition assay

The titer of the prepared PBS solution of X-31 influenza virus (~3 μg ml^{-1}) was determined by two-fold serial dilution of 50 μl of the virus solution through 12 wells of an 8 x 12-well microtiter plate with conically-shaped bottoms [25]. Another 50 μl of PBS was added to each well, followed by a suspension of chicken erythrocytes in PBS (100 μl). The solution was mixed and incubated at 20 °C for 1 h. The end point of hemagglutination is defined as the last well in which a sufficient amount of virus remains to agglutinate the erythrocytes. This titration was used as the basis of a hemagglutination inhibition (HAI) assay [13,25–27].

HAI assays examining the action of polymer or NA inhibitors alone

A stock solution of polymer (50 μl of 0.01 mM) or inhibitor of NA (50 μl of 400 mM) was two-fold serially diluted through 12 microtiter wells containing 50 μl of PBS. After preparation of serially diluted solutions of polymer or NA inhibitor, 50 μl from each well was mixed with 50 μl of a suspension of X-31 virus (0.25 μg ml^{-1}). After 30 min of incubation at 20 °C, 100 μl of a suspension of chicken erythrocytes (0.5 %) was added to each well followed by gentle agitation and incubation for 1 h at 20 °C. The end point of HAI is the last well in which an agglutinated pellet is observed. This end point (K_i^{HAI}) is defined as the lowest concentration of inhibitor in solution that inhibited the agglutination of erythrocytes by influenza virus. The values of K_i^{HAI} were calculated on the basis of four or five independent trials.

HAI assays examining the cooperativity between polymers and NA inhibitors

Each of 192 (96 x 2) microtiter wells (2 plates) was filled with 50 μl of PBS. An NA inhibitor was then serially diluted in each column, and the polymer serially diluted in each row, to achieve all possible combinations of concentrations. A solution (100 μl) of an inhibitor of NA (typically, 20 mM) was loaded in the 12 top wells of one plate (1–12; row A). After thorough mixing, an aliquot (100 μl) from each of the wells in row A was transferred to the adjacent well in row B. We continued this serial (1.5-fold) dilution over 16 rows. The last aliquot (100 μl) in row P was discarded. After this serial dilution, a solution (50 μl) of a polymer (0.01 mM) was loaded in each of the 16 (8 x 2) wells of the two plates under column 1, which contained 50 μl of the serially diluted solutions of monomeric inhibitor. After thorough mixing, an aliquot (50 μl) from each of the 16 wells was taken out, and transferred to the adjacent well in column 2. We continued this perpendicular, serial (2-fold) dilution over 12 columns. The last aliquot (50 μl) was discarded. After preparation of cross-serially diluted solutions of polymer and NA inhibitor, 50 μl of a suspension of X-31 virus (0.25 μg ml^{-1}) and 100 μl of chicken erythrocytes (0.5 %) were added to the 50 μl of solution already in each well. The results of this assay were read after 1 h, and yielded values of K_i^{HAI} for a polymeric inhibitor at 16 different concentrations of monomeric inhibitor.

Acknowledgements: The study was supported by the NIH (grant number GM 30367). M.M. thanks Eli Lilly for a predoctoral fellowship. We are grateful to Professor John J. Skehel for his generous gift of influenza virus A (strain X-31), and George Sigal for useful discussions. We thank personnel in charge of NMR and mass spectroscopy facilities in the Department of Chemistry at Harvard University; the instruments have been supported by funds from the NSF (CHE88-140195/CHE-9020043) and the NIH (SIO-RR06716/1-SIO-RR04870-01).

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Received: **11 Jan 1996**. Accepted: **29 Jan 1996**.