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Bifunctional Polymeric Inhibitors of Human Influenza A Viruses

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Abstract

Purpose—New antiviral agents were prepared by attaching derivatives of sialic acid (**1**) and of the drug zanamivir (**2**) to poly(isobutylene-*alt*-maleic anhydride) (poly-(**1**+**2**)) or by mixing poly-**1** and poly-**2**, followed by assaying them against wild-type and drug-resistant influenza A Wuhan viruses.

Methods—Individually or together, **1** and **2** were covalently bonded to the polymer. The antiviral potencies of the resultant poly-**1**, poly-**2**, poly-(**1**+**2**), and poly-**1** + poly-**2**, as well as **1** and **2**, were assessed using plaque reduction assay.

Results—Attaching **1** to the polymer improved at best millimolar IC_{50} values over three orders of magnitude. While **2** exhibited micromolar IC_{50} values, poly-**2** was >100-fold even more potent. The IC_{50} of poly-(**1**+**2**) against the wild-type strain was >300-fold and ~17-fold better than of poly-**1** and poly-**2**, respectively. In contrast, the potency of poly-(**1**+**2**) *vs*. poly-**2** against the mutant strain merely doubled. The mixture of poly-**1** + poly-**2** inhibited both viral strains similarly to poly-**2**.

Conclusions—The bifunctional poly-(**1**+**2**) acts synergistically against the wild-type influenza virus, but not against its drug-resistant mutant, as compared to a physical mixture of the monofunctional poly-**1** and poly-**2**.

Keywords

drug-resistant mutant; influenza virus; polymeric antiviral agents; sialic acid; zanamivir

INTRODUCTION

Influenza A viruses cause a ubiquitous human infection: in the United States alone, up to 20% of the population contracts the disease in an average year, leading to over 200,000 hospitalizations and some 36,000 deaths (www.cdc.gov/flu.). Both neuraminidase inhibitor drugs currently recommended for treatment of influenza, zanamivir (ZA; Relenza®) and oseltamivir (Tamiflu®), leave much to be desired in terms of efficacy and side effects (1,2). Therefore, enhancing their potency (along with new approaches to preventing the spread of influenza viruses (3–5)) is an urgent and important challenge.

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A promising strategy for creating the next generation of anti-influenza drugs is covalently bonding numerous copies of them to a polymeric chain; the resultant multivalent agents typically dwarf the potency of their monovalent predecessors primarily because of entropically improved affinity for the viral surface proteins (6). Three such influenza A proteins—the lectin hemagglutinin (H), the enzyme neuraminidase (N), and the M2 ion channel protein—have all been considered as attractive drug targets. [The older drugs targeting the M2 ion channel protein (1,2), amantadine (Symmetrel®) and riman-tadine (Flumadine®), are currently not recommended to treat influenza due to a rapid emergence of strains resistant to them (www.cdc.gov/flu.).]

The power of multivalency was strikingly demonstrated by attaching numerous copies of *N*-acetylneuraminic acid (commonly termed sialic acid; SA) to various polymers (7–11). These water-soluble polymer-attached SA derivatives bind to influenza viruses far tighter than the monomeric SA parents due to many simultaneous interactions with hundreds of copies of hemagglutinin (essential for the docking to the host cells) present on a single viral particle (6). Likewise, the antiviral activities of the polymer-attached ZA derivatives have been found (12,13) to be much superior to those of their monomeric predecessors, presumably owing to simultaneous interactions with tens of copies of surface neuraminidase (essential for the propagation of the virus) per viral particle.

In the present study, we have endeavored to advance the multivalency concept (6) further by asking whether the anti-influenza potency can be enhanced even more if the multivalent compounds are *bifunctional*, i.e., either by attaching two distinct ligands to the same polymeric chain for targeting different viral surface proteins or by formulating the physical mixture of the two monofunctional polymer-attached agents.

MATERIALS AND METHODS

Materials

Poly(isobutylene-*alt*-maleic anhydride) (M_w=165 kDa), sialic acid, all other reagents and solvents were from Sigma-Aldrich. The polymer is listed as biocompatible by the vendor.

Synthesis and Characterization

The SA derivative **1**, (4-glycylamidobenzyl-5-acetamido-3,5-dideoxy-α-D-glycero-D-galactononulopyranoside)onic acid [$NH_2CH_2CONHC_6H_4CH_2$-SA] (14), and the ZA derivative **2**, (4S,5R,6R)-5-acetylamino-6-{1R-[(6-aminohexyl)-carbamoyloxy]-2R,3-dihydroxypropyl}-4-guanidino-5,6-dihydro-4H-pyran-carboxylic acid [$NH_2(CH_2)_6NHCO$-ZA] (15,16), were synthesized using combinations of published procedures. The subsequent polymer-attached SA and ZA derivatives poly-**1**, poly-**2**, and poly-(**1**+**2**) were synthesized as follows: **1** and/or **2** (0.08 mmol each) were added to a solution of poly(isobutylene-*alt*-maleic anhydride) (0.65 mmol on the monomer basis) in 10 ml of dry dimethylformamide containing 0.5 ml of pyridine. The reaction mixture was stirred at room temperature for 24 h, quenched with 10 ml of a 28% NH_4OH solution, stirred for another 24 h, dialyzed (dialysis membrane's molecular weight cutoff of 3,500 Da) against distilled water for 48 h, and then lyophilized to yield polymeric inhibitors. The **1** and **2** contents in the monofunctional and bifunctional polymeric derivatives prepared (the synthesis yields were in the 80–90% range) were quantified by ^{1}H-NMR.

The "bare polymer" (i.e., the polymer whose anhydride groups were quenched by ammonia) was synthesized by dissolving poly(isobutylene-*alt*-maleic anhydride) (0.65 mmol on the monomer basis) in 10 ml of dry dimethylformamide and reacting it with 10 ml of a 28% aqueous NH_4OH solution at room temperature for 24 h. The reaction mixture was then dialyzed (dialysis

membrane's molecular weight cutoff of 3,500 Da) against distilled water for 48 h and lyophilized.

The details and results of the ^{1}H-NMR analyses of all polymeric agents prepared in this study are presented below.

^{1}H-NMR (D_2O) δ for **1** (400 MHz): 1.66 (1H, t, H-3_{ax}), 2.02 (3H, s, NCOCH_3), 2.76 (1H, dd, H-3_{eq}), 3.56–3.85 (7H, m, H-4,5,6,7,8,9_a,9_b), 3.94 (2H, s, COCH_2NH_2), 4.46 and 4.69 (2H, 2d, PhCH_2), 7.37 and 7.43 (4H, 2d, aromatic).

^{1}H-NMR (D_2O) δ for **2** (600 MHz): 1.35 (4H, m, NHCH_2CH_2-(CH_2)$_2$-CH_2CH_2NH), 1.48 and 1.64 (4H, 2m, NHCH_2-CH_2-(CH_2)$_2$-CH_2-CH_2NH), 1.96 (3H, s, NCOCH_3), 2.96–3.09 (4H, m, NHCH_2-(CH_2)$_4$-CH_2NH), 3.50 (1H, dd, H-9_a), 3.66 (1H, d, H-9_b), 4.03 (1H, m, H-8), 4.15 (1H, t, H-5), 4.45 (1H, dd, H-4), 4.57 (1H, d, H-6), 4.95 (1H, d, H-7), 6.04 (1H, d, H-3).

^{1}H-NMR (400MHz, D_2O + MeOD) δ for poly-**1**: 0.75–1.25 (6H, m, 6H polymer), 1.25–1.75 (2H, m, H-3_{ax}, 1H polymer), 1.75–2.20 (4H, m, CH_3CON, 1Hpolymer), 2.20–2.60 (1H, m, 1H polymer), 2.60–2.90 (2H, m, H-3_{eq}, 1H polymer), 3.50–4.00 (9H, m, CH_2N, H-4,5,6,7,8,9_a,9_b), 4.40 (1H, m, CH_2Ph) and 7.20–7.50 (4H, m, aromatics).

^{1}H-NMR (400MHz, D_2O + MeOD) δ for poly-**2**: 0.75–1.25 (6H, m, 6H polymer), 1.25–1.75 (9H, 8H-linker, 1H polymer), 1.75–2.20 (4H, m, CH_3CON, 1H polymer), 2.20–2.55 (1H, m, 1H polymer), 2.55–3.10 (5H, m, 4H-linker, 1H polymer), 3.35–3.65 (2H, m, H-9_a,9_b), 3.90–4.15 (2H, m, H-5,8), 4.30–4.50 (2H, m, H-4,6) and 5.50–5.60 (1H, m, H-3).

^{1}H-NMR (400 MHz, D_2O + MeOD) δ for poly-(**1**+**2**): 0.75–1.25 (6H, m, 6H polymer), 1.25–1.75 (10H, m, H-3_{ax}-SA, 8H-linker-ZA, 1H polymer), 1.80–2.20 (7H, m, CH_3CON-SA, CH_3CON-ZA, 1H polymer), 2.20–2.60 (1H, m, 1H polymer), 2.50–3.10 (6H, m, H-3_{eq}-SA, 4H-linker-ZA, 1H polymer), 3.40–3.90 (11H, m, CH_2N, H-4,5,6,7,8,9_a,9_b-SA, H-9_a,9_b-ZA), 3.90–4.20 (2H, m, H-5,8-ZA), 4.30–4.50 (3H, m, CH_2Ph-SA, H-4,6-ZA), 5.50–5.60 (1H, m, H-3-ZA) and 7.20–7.50 (4H, m, aromatic-SA).

^{1}H-NMR (400MHz, D_2O + MeOD) δ for the bare polymer: 0.70–1.20 (6H, m, 2 CH_3), 1.30–1.70 (1H, m), 1.75–2.10 (1H, m), 2.15–2.50 (1H, m), 2.60–2.90 (1H, m).

Cells and Viruses

Madin-Darby canine kidney (MDCK) cells were obtained from the ATCC. They were grown at 37°C in a humidified-air atmosphere (5% CO_2/95% air) in Dulbecco's modified Eagle's (DME-hepes) medium (SAFC Biosciences) supplemented with 10% heat-inactivated fetal calf serum (GIRGO 614), 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM L-glutamine. Influenza viruses, A/Wuhan/359/95 (H3N2)-like wild type and its oseltamivir-resistant variant carrying Glu119Val mutation in the neuraminidase (NA) (17,18) were obtained from the U.S. Centers for Disease Control and Prevention (CDC).

Preparation of Viruses in Chicken Eggs

A 100-μl aliquot of a 10-fold diluted solution of the viruses was injected into the allantoic fluid of 10-day-old embryonated chicken eggs (5). The eggs were subsequently incubated at 37°C for 48 h and then at 4°C for 24 h. The allantoic liquid was collected and centrifuged at 1,000*g* at 4°C for 20 min, followed by passing the supernatant through a 0.45-μm syringe filter (low protein binding) and storing at −80°C.

Antiviral Activity

Plaque reduction assays to determine IC_{50} values (i.e., the compound concentrations required to reduce the number of plaques to 50% of the control one) were performed using a modified literature procedure (19). Specifically, solutions of the compounds in aqueous PBS were subjected to five serial 10-fold dilutions with the buffer; in the case of the control, the blank PBS solution was used instead. To 125 μl of each of the serially diluted solutions, the same volume of the virus solution (approximately 800 pfu/ml) in PBS was added and vortexed, and the resultant mixture was incubated for 1 h at room temperature. MDCK cells were seeded into six-well tissue culture plates and grown to confluence, followed by washing twice with 5 ml of aqueous PBS; the cells were subsequently incubated with 200 μl of the aforementioned virus plus compound mixtures at room temperature for 1 h. The cells were overlaid with 2 ml of plaque medium (two times F12 medium supplemented with 0.01% DEAE-dextran, 0.1% $NaHCO_3$, 100 units/ml penicillin G, 100 μg/ml streptomycin, 4 μg/ml trypsin, and 0.6% purified agar (L28 from Oxoid Co.)) containing appropriate compound concentrations. After a 3- to 4-day incubation at 37°C in humidified air (5% CO_2/95% air), the plaques formed were counted.

RESULTS AND DISCUSSION

To make sialic acid and zanamivir readily attachable to a polymer without destroying SA's and ZA's virus-binding properties, we synthesized their derivatives containing spacer arms ending with a primary amino group, namely **1** and **2**, respectively. The terminal NH_2 groups of **1** and **2** were then used to covalently bond them to either different or the same chains of poly(isobutylene-*alt*-maleic anhydride) (this polymer, aside from being commercially available and biocompatible, affords a single-step attachment of multiple copies of **1** and **2**). Remaining anhydride moieties of the polymer were subsequently quenched with an ammonium hydroxide solution to form the conjugates poly-**1**, poly-**2**, and poly-(**1**+**2**); in the first two (monofunctional) polymers, 8–10% of the monomeric units were derivatized with the ligand, and in the third (bifunctional) polymer, some 20% (an equimolar **1** to **2** ratio) (Fig. 1).

The target strains of human influenza A viruses employed (produced in chicken eggs (5)) were of the H3N2 subtype (20) afflicting people at least since the infamous 1968 "Hong Kong influenza" pandemic (1). [Based on the anti-genicity of these transmembrane glycoproteins, influenza A viruses cluster into sixteen H (H1 to H16) and nine N (N1 to N9) subtypes; five of these (H1, H2, H3, N1, and N2) had caused all human influenza pandemics of the last century.] To test the generality of our findings, we selected two different strains (A/Wuhan/359/95-like): wild-type and a corresponding oseltamivir-resistant mutant (containing a Glu119Val mutation in the neuraminidase enzyme) (17,18). Anti-influenza activities of all the compounds tested in this work were determined using a plaque reduction assay in six-well plates covered with a monolayer of the MDCK cells (19).

Compound **1**, like other SA derivatives (9–11), is a very poor inhibitor of both influenza viruses with no better than millimolar IC_{50} values. Using hemagglutinin inhibition (9–11) and fetuin-binding inhibition (7) assays, however, other researchers have demonstrated a great increase in the anti-influenza activity upon covalent attachment of SA to polymers due to the multivalent inhibition. In the present study, we observed the same phenomenon using the plaque reduction assay: one can see in the first two lines of Table I that poly-**1** is over 1,000-fold more effective against both the wild-type and mutant strains of influenza virus. Importantly, poly-**1** is approximately 6 times less potent in inhibiting the mutant *vs.* the wild-type virus, suggesting that neuraminidase modulates its activity, perhaps due to removal of the **1** moieties from the polymer and/or poorer binding of the polymer-attached **1** to the mutant neuraminidase.

It is also noteworthy that poly-**1** did not exhibit an appreciably enhanced inhibitory activity over the bare polymer backbone (with the anhydride moieties quenched with NH_3) against either the wild-type strain [IC_{50} values of (0.88±0.41) μg/ml for poly-**1** and (1.3±0.1) μg/ml for the polymer] or the mutant strain [IC_{50} of (4.9±1.7) μg/ml for poly-**1** and (5.3±1.4) μg/ml for the polymer].

One also can see in Table I that **2**, whose antiviral activity is similar to that of ZA itself and its other derivatives (16), is expectedly a much better inhibitor of influenza A viruses than **1**. Furthermore, consistent with earlier observations involving **2** bonded to other polymers (12, 13), attaching it to poly(isobutylene-*alt*-maleic anhydride) improves the antiviral activity against both the wild-type and oseltamivir-resistant strains by over two orders of magnitude due to the polyvalent inhibition (Table I).

Next, we explored the *bifunctional* polymeric inhibitors by attaching the two ligands (**1** and **2**) to the same polymer chain yielding poly-(**1**+**2**) (Fig. 1). The inhibitory activity of poly-(**1**+**2**) against the wild-type strain of the virus was found to be much greater than that of either monofunctional polymer: over two orders of magnitude compared to poly-**1** and over an order of magnitude compared to poly-**2** (Table I). The synergistic effect of **1** and **2** in the bifunctional polymeric inhibitor is likely caused by a strong affinity of the ZA moiety to the neuraminidase enzyme of the virus which, in turn, synergistically improves the binding of the SA moiety to the hemagglutinin (and/or neuraminidase) protein of the same virus.

The effect was qualitatively similar in the case of the drug-resistant strain (the Glu119Val mutation in NA), although only some 2-fold improvement was observed with poly-(**1**+**2**) compared to poly-**2** (Table I). The weaker inhibition of the mutant virus *vs.* its wild-type precursor by poly-(**1**+**2**) parallels the observed weaker inhibition of the mutant virus by poly-**1**, further supporting a critical role of the poly-(**1**+**2**) in mediating the observed synergistic effect.

We also explored an alternative way of creating bifunctional polymeric inhibitors, namely by formulating a 1:1 (*w*/*w*) physical mixture of the two monofunctional multivalent components, poly-**1** and poly-**2**. As can be seen in Table I, the poly-**1** + poly-**2** mixture, in contrast to poly-(**1**+**2**), inhibited both viral strains to the same extent as poly-**2** by itself. This difference in the antiviral action of the two types of bifunctional polymeric inhibitors further supports our foregoing interpretation of synergistic effect of poly-(**1**+**2**). Binding of the ZA moiety to the neuraminidase in poly-**2** should not synergistically improve the binding of the SA moiety attached to a different polymeric chain.

In conclusion, although the molecular mechanisms of the foregoing observations require further investigation, the effect of the bifunctionality is of definite interest if wide-spread. One reason for a possible superiority of the bifunctional agents is that they block not one but two distinct events in the life cycle of the virus, namely cell docking and propagation. Separately, it remains to be seen whether such anti-influenza potency enhancements due to the bifunctionality hold *in vivo*.

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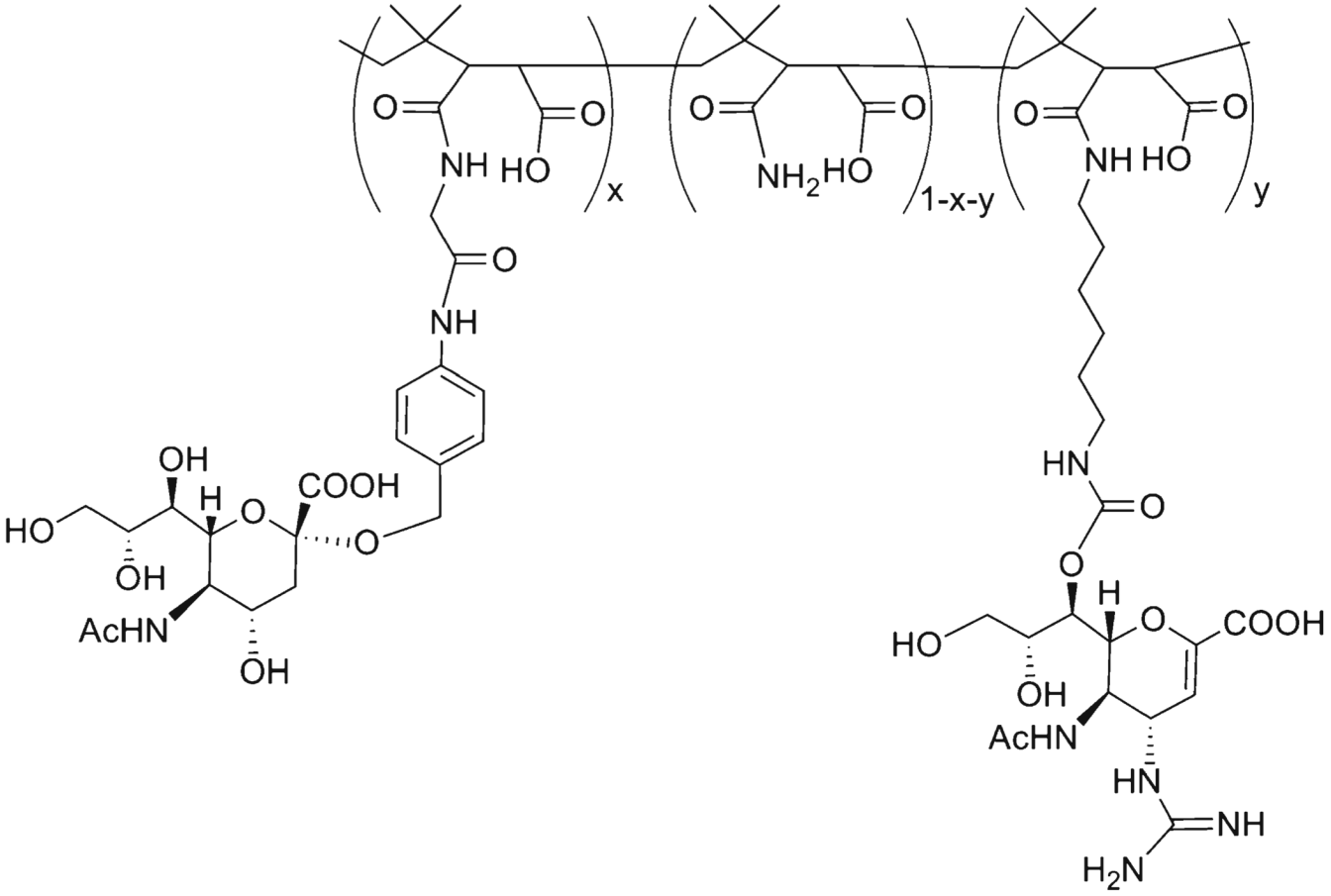

Fig. 1.

Chemical structure of a monomeric unit of **1** and/or **2** covalently bonded to poly (isobutylene-*alt*-maleic anhydride). Following the attachment of **1** and/or **2**, the remaining anhydride moieties were quenched with an ammonium hydroxide solution. In the depicted structure, *x* and *y* are the mole-fractions of **1** and **2**, respectively. For poly-**1**, *x*=0.1 and *y*=0; for poly-**2**, *x*=0 and *y*=0.08; for poly-(**1**+**2**), *x*=0.1 and *y*=0.1.

Table I

Antiviral Activities of Various Sialic Acid and Zanamivir Derivatives, Both Monomeric and Polymer-Attached, Against Human H3N2 (Wuhan) Influenza A Wild-Type and Oseltamivir-Resistant Strains[a]

	IC_{50} (nM)[a,b]	
Compound	**Wild-type strain**	**Mutant strain**
1	$(8.3\pm1.5)\times10^5$	$\gg10^6$
Poly-**1**	$(4.0\pm1.9)\times10^2$	$(2.3\pm0.8)\times10^3$
2	$(2.8\pm1.3)\times10^3$	$(7.4\pm4.4)\times10^3$
Poly-**2**	22 ± 14[c]	28 ± 7[d]
Poly-(**1**+**2**)	1.3 ± 0.9[c]	12 ± 1.5[d]
Poly-**1** + Poly-**2**	35±20	29±8

[a] All the data in the table arise from experiments carried out at least in triplicate with the mean IC_{50} values and standard deviations obtained shown. The IC_{50} values are expressed as the corresponding concentrations of **1** and/or **2**.

[b] To determine the IC_{50} values in the case of **1**, it and the virus were incubated before infecting the cells to prevent the initial viral attachment to host cells; **1** was not subsequently incorporated into the agar overlay. The rationale for this is that since **1** binds only very weakly to viral particles and shows little or no activity, its presence in the agar overlay (as in the case of the other inhibitors in the table) should not affect the propagation of infection and the number of plaques observed, and hence the IC_{50} values. The "≫" in the 1st line of the table signifies that no appreciable reduction of the number of plaques compared to the control was observed even at 1 mM **1**.

[c] The calculated Student's *t*-test *P* values were <0.01 for poly-**2** vis-à-vis **2** and <0.003 for poly-(**1**+**2**) vis-à-vis poly-**2**.

[d] The calculated Student's *t*-test *P* values were <0.02 for poly-**2** vis-à-vis **2** and <0.007 for poly-(**1**+**2**) vis-à-vis poly-**2**.