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Conjugation to polymeric chains of influenza drugs targeting M2 ion channels partially restores inhibition of drug-resistant mutants

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Abstract

By attaching multiple copies of the influenza M2 ion channel inhibitors amantadine (1) and rimantadine (2) to polymeric chains we endeavored to recover their potency in inhibiting drug-resistant influenza viruses. Depending on loading densities, as well as the nature of the drug, the polymer, and the spacer arm, polymer-conjugated drugs were up to 30-fold more potent inhibitors of drug-resistant strains than their monomeric parents. In particular, a 20% loading density and a short linker group on the negatively charged poly-L-glutamate resulted in some of the most potent inhibitors for 2’s conjugates against drug-resistant influenza strains. Although full recovery of the inhibitory action against drug-resistant strains was not achieved, this study may be a step toward salvaging anti-influenza drugs that are no longer effective.

Introduction

Influenza viruses are a commonly infect the respiratory tract in humans¹ and are a major cause of morbidity and mortality in the world.²,³ Two of the four FDA-approved small-molecule anti-influenza drugs — the adamantane-class M2 ion-channel inhibitors amantadine (1) and rimantadine (2) (Fig. 1) — are no longer recommended as therapeutics because nearly every circulating influenza A strain has evolved resistance to them.²,⁴,⁵ These drugs block the M2 ion channels on the surface of the virus,⁶-⁹ thereby preventing the flow of protons into the viral core (an essential step in the viral infection cycle).² Resistance to 1 and 2 is due to point mutations in the M2 ion channel protein, with the most common being the S31N in the interior of the channel.²

Because of the daunting challenges in discovering new anti-influenza drugs, it would be of great benefit to salvage older FDA-approved drugs that are impotent against newly emerged mutants. Previously, we have demonstrated that the attachment of multiple copies of the influenza neuraminidase inhibitor zanamivir to a flexible polymeric chain not only dramatically improves the potency against drug-sensitive strains, but also resurrects the inhibitory effect against zanamivir-resistant mutants.¹⁰,¹¹ This phenomenon appears to stem from two mechanisms. The first is multivalency, whereby several simultaneous interactions between polymer-attached zanamivir and its viral target result in a far greater avidity compared to the monomer’s binding constant,¹⁰,¹²,¹³ while also generating an increased drug concentration in the vicinity of the virus.¹³ The second contributor to the improved potency is a novel mechanism of inhibition, blocking earlier stages of the viral cycle, which monomeric zanamivir lacks.³ Herein we explore whether the approach of attaching multiple
copies of influenza drugs to polymeric chains can boost the adamantane inhibitors’ prowess against drug-resistant influenza mutants (as it did with zanamivir\(^\text{10}\)).

### Materials and Methods

#### Materials

Amantadine-HCl (1; here and henceforth the bold number equally applies to a free base/acid and its salt), rimantadine-HCl (2), 3-amino-1-adamantanecarboxylic acid (3), 3-(1-aminoethyl)adamantan-1-ol-HCl (7), poly-L-glutamate Na salt (MW of 50-100 kDa), carboxymethylcellulose Na salt (MW of \(\sim\)100 kDa) (CMC), poly(acrylic acid) (MW of \(\sim\)100 kDa), and all solvents and other reagents were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO) and used without further purification unless otherwise specified. N-Hydroxysulfosuccinimide (sulfo-NHS) was from Proteochem (Denver, CO), 5-azidopentanoic acid and 5-azidopentan-1-amine from Synthonix (Wake Forest, NC), and 11-azido-3,6,9-trioxaundecanoic acid from TCI America (Portland, OR).

#### Syntheses

**Synthesis of 1-linker-azide (6)—**

Linker addition to 3 was carried out as described by Wanka et al.\(^\text{14}\) Briefly, 300 mg (1.5 mmol) of 3 and 715 mg (6.7 mmol) of Na\(_2\)CO\(_3\) were suspended in a mixture of 10 mL of H\(_2\)O and 5 mL of acetone, followed by stirring and placing in an ice bath. Next, Fmoc-Cl (426 mg, 1.6 mmol) in 5 mL of acetone was added over 30 min with an addition funnel. The reaction mixture was incubated at room temperature (RT) overnight and then heated to 50°C for 2 h to evaporate acetone. To purify the product, the reaction was poured over ice (35 grams) and extracted thrice with diethyl ether. The aqueous layer was then acidified to pH 5 and extracted thrice with ethyl acetate. The ethyl acetate portions were then combined, washed with H\(_2\)O, and dried over Na\(_2\)SO\(_4\) to afford an off-white powder of Fmoc-3-amino-1-adamantanecarboxylic acid (4) (\(\sim\)40% yield). \(^1\)H NMR 4 (\([D_8]\)THF) \(\delta\) (400 MHz): 1.65 (2H, d, CH\(_2\)-1), 1.72 (2H, s, CH\(_2\)-1), 1.83 (4H, s, CH\(_2\)-1), 1.95 (4H, s, CH\(_2\)-1), 2.07 (H, s, CH-1), 2.14 (H, s, CH-1), 4.18 (1H, t, CH-Fmoc), 4.27 (2H, d, CH\(_2\)-Fmoc), 7.25 (2H, t, CH-aromatic-Fmoc), 7.3 (2H, t, CH-aromatic-Fmoc), 7.6 (2H, d, CH-aromatic-Fmoc), 7.8 (2H, d, CH-aromatic-Fmoc).

To synthesize 5, 4 (220 mg, 0.53 mmol) was dissolved in 5 mL of dry THF. To that, O-benzotriazole-N,N,N’,N’-tetramethyl-uronium-hexafluoro-phosphate (HBTU) (200 mg, 0.53 mmol) was added, followed by 65 \(\mu\)L (0.53 mmol) of 5-azidopentan-1-amine and 68 \(\mu\)L (0.5 mmol) of Hünig’s base. The reaction mixture was stirred at RT overnight and then heated to 60°C for 1 h. After cooling, 3 mL of brine was added, and the mixture was extracted with CHCl\(_3\) thrice. The organic phases were combined, washed with 1 M HCl, 5% NaHCO\(_3\), H\(_2\)O, and brine, and then further purified on a silica gel column with 10:1 (v/v) CH\(_2\)Cl\(_2\):methanol mobile phase to afford 5\(^\text{14}\) (\(\sim\)55% yield). \(^1\)H NMR 5 (CDCl\(_3\)) \(\delta\) (400 MHz): 1.35 (2H, m, CH\(_2\)-linker), 1.47(2H, m, CH\(_2\)-linker), 1.6 (4H, m, CH\(_2\)-1, CH\(_2\)-linker), 1.78 (4H, s, CH\(_2\)-1), 1.85 (2H, d, CH\(_2\)-1), 1.95 (2H, d, CH\(_2\)-1), 2.05 (2H, s, CH\(_2\)-1), 2.18 (2H, s, CH-1), 3.2 (2H, dd, CH\(_2\)-linker), 3.24 (2H, t, CH\(_2\)-linker), 4.18 (1H, t, CH-Fmoc), 4.3 (2H, d, CH\(_2\)-Fmoc), 7.25 (2H, t, CH-aromatic-Fmoc), 7.3 (2H, t, CH-aromatic-Fmoc), 7.6 (2H, d, CH-aromatic-Fmoc), 7.7 (2H, d, CH-aromatic-Fmoc).

To generate the deprotected final 1-linker-azide (6) for attachment to poly-L-glutamate, 5 (75 mg, 0.14mmol) was dissolved in 1.2 mL of dry acetonitrile and cooled to 0°C. Diethylamine (1.2 mL) was added, and the reaction mixture was stirred for 1 h at 0°C and RT for 24 h. The reaction mixture was then extracted with H\(_2\)O at pH 3, and the product (6)
was recovered from the aqueous phase\textsuperscript{14} (∼20% yield). R\textsubscript{f} on TLC silica plate of 0.12 in 10:1 (v/v) CH\textsubscript{2}Cl\textsubscript{2}:MeOH.\textsuperscript{1}H NMR \textsuperscript{6} (CDCl\textsubscript{3}) δ (400 MHz): 1.27 (2H, m, CH\textsubscript{2}-linker), 1.45 (2H, m, CH\textsubscript{2}-linker), 1.52 (2H, m, CH\textsubscript{2}-linker), 1.61 (2H, s, CH\textsubscript{2}-I), 1.7 (2H, d, CH\textsubscript{2}-I), 1.78 (4H, d, CH\textsubscript{2}-I), 1.83 (2H, d, CH\textsubscript{2}-I), 1.88 (2H, s, CH\textsubscript{2}-I), 3.1 (2H, t, CH\textsubscript{2}-linker), 2.25 (2H, s, CH-I), 3.25 (2H, t, CH\textsubscript{2}-linker).

Synthesis of 2-linker-azides (11 and 12)—To obtain an organic solvent soluble free base, 3-(1-aminoethyl)adamantan-1-ol·HCl was suspended in CH\textsubscript{2}Cl\textsubscript{2} and dried over Na\textsubscript{2}CO\textsubscript{3} and washed with 1 M NaOH. The resultant organic layer was rotary-evaporated, and the isolated white powder of 3-(1-aminoethyl)adamantan-1-ol (7) was Boc-protected for subsequent chemical modification. To this end, a solution of di-tert-butyl dicarbonate (327 mg, 1.5 mmol) in 25 mL of CH\textsubscript{2}Cl\textsubscript{2} was added to 5 mL of CH\textsubscript{2}Cl\textsubscript{2}:MeOH. The resultant organic layer was rotary-evaporated, and the isolated white powder of 7 (292 mg, 1.4 mmol). The reaction mixture was stirred at RT for 24 h after which it was extracted thrice with saturated Na\textsubscript{2}CO\textsubscript{3} and dried over Na\textsubscript{2}SO\textsubscript{4} to afford a white fluffy powder of 8\textsuperscript{15} (∼95% yield). R\textsubscript{f} on TLC silica plate of 0.63 in 10:1 (v/v) CH\textsubscript{2}Cl\textsubscript{2}:MeOH. \textsuperscript{1}H NMR \textsuperscript{8} (CDCl\textsubscript{3}) δ (400 MHz): 0.98 (3H, d, CH\textsubscript{3}-2), 1.33 (2H, d, CH\textsubscript{2}-2), 1.9 (1H, s, Boc, CH\textsubscript{2}-I), 1.47 (2H, s, CH\textsubscript{2}-2), 1.57 (2H, d, CH\textsubscript{2}-2), 1.62 (2H, d, CH\textsubscript{2}-2), 1.75 (1H, s, CH\textsubscript{2}-2), 2.15 (2H, s, CH\textsubscript{2}-2), 3.4 (1H, m, CH\textsubscript{2}-2), 4.4 (1H, m, CH\textsubscript{2}-2).

Next, the Boc-protected 3-(1-aminoethyl)adamantan-1-ol (8) was reacted with 5-azidopentanoic acid in a method similar to that of Saitoh et al.\textsuperscript{16} Briefly, 8 (134 mg, 0.5 mmol), 5-azidopentanoic acid (128 µL, 1.0 mmol), and 4-dimethylaminopyridine (DMAP) (6 mg, 0.03 mmol) were dissolved in 3 mL of chlorobenzene. To that mixture, di(2-pyridyl)thionocarbonate (DPTC) (230 mg, 1.0 mmol) was added, and the reaction mixture was refluxed for 1 h, concentrated, and purified on a silica gel column with 2:1 (v/v) hexane:ethyl acetate mobile phase to afford 9 (∼55% yield). R\textsubscript{f} on TLC silica plate of 0.54 in 2:1 (v/v) hexane:ethyl acetate. To prepare 10, 11-azido-3,6,9-trioxaundecanoic acid was used in place of 5-azidopentanoic acid and the reaction mixture was purified on a silica gel column with 7:1 (v/v) CHCl\textsubscript{3}:acetone as a mobile phase (∼65% yield). R\textsubscript{f} on TLC silica plate of 0.60 in 7:1 (v/v) CHCl\textsubscript{3}:acetone. \textsuperscript{1}H NMR \textsuperscript{9} (CDCl\textsubscript{3}) δ (400 MHz): 0.98 (3H, d, CH\textsubscript{3}-2), 1.35 (11H, s, Boc, CH\textsubscript{2}-2), 1.45 (2H, d, CH\textsubscript{2}-2), 1.57 (4H, m, CH\textsubscript{2}-linker), 1.75 (2H, m, CH\textsubscript{2}-2), 1.95 (3H, m, CH\textsubscript{2}-2, CH\textsubscript{2}-2), 2.05 (2H, d, CH\textsubscript{2}-2), 2.2 (4H, m, CH\textsubscript{2}-2, CH\textsubscript{2}-linker), 3.22 (2H, m, CH\textsubscript{2}-linker), 3.4 (1H, m, CH\textsubscript{2}-2), 4.35 (1H, m, CH\textsubscript{2}-2). \textsuperscript{1}H NMR \textsuperscript{10} (CDCl\textsubscript{3}) δ (400 MHz): 0.95 (3H, d, CH\textsubscript{3}-2), 1.35 (11H, s, Boc, CH\textsubscript{2}-2), 1.43 (2H, d, CH\textsubscript{2}-2), 1.54 (2H, d, CH\textsubscript{2}-2), 1.78 (2H, m, CH\textsubscript{2}-2), 1.93 (2H, d, CH\textsubscript{2}-2), 2.03 (2H, d, CH\textsubscript{2}-2), 2.09 (1H, s, CH\textsubscript{2}-2), 2.17 (2H, s, CH\textsubscript{2}-2), 3.3 (2H, t, CH\textsubscript{2}-linker), 3.6 (1H, m, CH\textsubscript{2}-linker, CH\textsubscript{2}-2), 3.93 (2H, s, CH\textsubscript{2}-linker, CH\textsubscript{2}-2), 4.35 (1H, m, CH\textsubscript{2}-2).

Deprotection of the Boc group was performed with 4 M HCl in dioxane, as described previously,\textsuperscript{17} to yield the final 2-linker-azide conjugates (11 and 12) (∼90% yield). R\textsubscript{f} on TLC silica plate of 0 in 2:1 (v/v) hexane:ethyl acetate. \textsuperscript{1}H NMR \textsuperscript{11} (CDCl\textsubscript{3}) δ (400 MHz): 1.2 (3H, d, CH\textsubscript{3}-2), 1.5 (8H, m, CH\textsubscript{2}-2, CH\textsubscript{2}-linker), 1.65 (1H, d, CH\textsubscript{2}-2), 1.75 (4H, s, CH\textsubscript{2}-2), 2.05 (2H, s, CH\textsubscript{2}-2), 2.15 (2H, t, CH\textsubscript{2}-linker), 2.2 (2H, s, CH\textsubscript{2}-2), 2.95 (1H, t, CH\textsubscript{2}-2), 3.2 (2H, t, CH\textsubscript{2}-linker), 4.3 (1H, d, CH\textsubscript{2}-2). \textsuperscript{1}H NMR \textsuperscript{12} (CDCl\textsubscript{3}) δ (400 MHz): 1.3 (3H, d, CH\textsubscript{3}-2), 1.5 (2H, d, CH\textsubscript{2}-2), 1.6 (2H, s, CH\textsubscript{2}-2), 1.72 (1H, d, CH\textsubscript{2}-2), 1.92 (4H, m, CH\textsubscript{2}-2), 2.1 (2H, d, CH\textsubscript{2}-2), 2.25 (2H, s, CH\textsubscript{2}-2), 3.0 (1H, s, CH\textsubscript{2}-2), 3.33 (2H, t, CH\textsubscript{2}-linker), 3.62 (11H, s, CH\textsubscript{2}-linker, CH\textsubscript{2}-2), 3.95 (2H, s, CH\textsubscript{2}-linker).

Because of the more facile nature and higher yields of the 2-linker-azide synthesis, we utilized 2 for our extensive structure-activity relationship of the drug-polymer conjugates.
Polymer activation—Polymers were activated with propargylamine to incorporate a terminal alkyne for subsequent conjugation reactions with the aforementioned azide-containing inhibitors 6, 11, and 12. As a representative example, poly-L-glutamate Na salt (100 mg) was dissolved in 30 mL of H2O. To that was added 300 mg of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) (1.1 mmol) and 7 μL (0.15 mmol) of propargylamine to afford a ~10% derivatized polymer. The reaction mixture was stirred for 5 h and purified as described previously.18,19 The amount of propargyl amine was varied to tune the % of derivatization of the polymer used. The % of derivatization was verified by 1H NMR where the integration of one propargylamine H peak (the alkyne singlet hydrogen) was compared to that of the integration of one known polymer H peak (the singlet hydrogen from the poly-L-glutamate backbone).

For compounds 21 and 22, poly-L-glutamate was reacted as described above with propargylamine and approximately 0.2 mol-eq. of either benzylamine or 2,2-dimethyl-1-propanime, respectively.

To generate a poly-L-glutamine backbone for compound 23, the purified polymer pre-derivatized to have ~20% of its monomeric units activated with propargylamine was dissolved at 10 mg/mL in 0.1 M 2-N-morpholinoethanesulfonate (MES) buffer, pH 6, containing 0.5 M NaCl. To that mixture was added 1.2 mol-eq. of both 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and sulfo-NHS. The reaction mixture was stirred for 30 min at RT, after which an excess of saturated aqueous NH4OH was added. Following an overnight incubation, the product was purified for subsequent small molecule derivatization.18

Polymer conjugation—Once the propargylamine-derivatized polymers were purified and quantified, they were reacted in a Cu2+-catalyzed [3+2] azide-alkyne cycloaddition with the azide containing amantadine or rimantadine derivatives (6, 11, and 12) as previously described.18 1’s or 2’s % of derivatization for each polymer-conjugate was determined by 1H NMR by comparing the integrated peak for the newly formed triazole hydrogen to that for the singlet hydrogen from the poly-L-glutamate backbone. An example 1H NMR breakdown is described. 1H NMR 16 (D2O) δ (600 MHz): 1.2 (3H, d, CH3-2), 1.5 (8H, s, CH2-linker), 2.0 (2H, m, CH2-polymer, 8H CH2-2, CH-2), 2.3 (2H, s, CH2-polymer, 4H, s, CH2-2, CH2-linker), 3.1 (1H, s, CH-2), 4.3 (1H, s, CH-polymer, 4H, d, CH2-2, CH2-propargylamine), 8.0 (1H, s, CH-triazole). Efficiency for the azide-alkyne cycloaddition ranged from approximately 70% to 100% for the small molecules. Representative 1H NMR spectra are presented in the supporting information (Figures S1-S4).

Cells, viruses, and antiviral assays—Madin-Darby canine kidney (MDCK) cells for use in plaque reduction assays were from the American Type Culture Collection and maintained as described previously.11,20 The human wild-type influenza strains A/Wuhan/359/95 (H3N2) and A/PR/8/34 (H1N1) were obtained from the U.S. Centers for Disease Control and Prevention (Atlanta, GA) and Charles River Laboratories (North Franklin, CT), respectively. The influenza strain A/WSN/33 (H1N1) was a gift from Dr. Peter Palese of Mount Sinai School of Medicine (NY, NY). Viruses were stored in a −80°C freezer and diluted in phosphate-buffered saline (PBS) for use in assays.

Plaque reduction assays with MDCK cells were carried out to determine the half-maximal inhibitory concentration (IC50) of both monomeric and polymer-attached compounds as described before.10,11,18 When analyzing polymer conjugates, the IC50 values were calculated based on the concentration of the small-molecule inhibitors. In investigating the effect of the inhibitors’ presence for the complete viral cycle (denoted herein as “In Agar”),
equal concentrations of inhibitors were used for both pre-incubation with the virus and in the nutrient agar overlay.18

Results and Discussion

Syntheses

To covalently attach multiple copies of amantadine (1) and rimantadine (2) to polymeric chains without chemically modifying their amine moieties (revealed by protein X-ray crystallography to be oriented toward interior of the virion upon binding6 and hence presumably important), we sought structural analogs of 1 and 2 with readily functionalizable groups on the opposite side of the molecules. Since commercially available 3-amino-1-adamantanecarboxylic acid (3) and 3-(1-aminoethyl)adamantan-1-ol (7) fit this description, we employed them as starting points.

Both 3 and 7 were first derivatized for polymer attachment through a series of chemical reactions depicted in Figures 2 and 3. Since our previous studies (albeit with another influenza inhibitor) demonstrated the benefits of a linker (spacer arm) between the drug and the polymeric backbone in reducing steric hindrances,18 we also decided to insert a linker between 3 or 7 and the polymers used. As seen in Figs. 2 and 3, first the drugs' amino groups were protected by Fmoc and Boc groups, respectively, followed by linker attachment and amine deprotection to afford 1 and 2 with azide terminating linkers to be subsequently used for conjugation to polymers. Our initial studies herein utilized poly-L-glutamate as a polymer because it is benign, freely water-soluble, biodegradable, and non-immunogenic.10

Amantadine (1) and its polymer conjugates

To verify which strains were resistant to the adamantane class of inhibitors, we determined the IC50 values for monomeric 1 against three representative influenza strains using the plaque reduction assay. They were A/Wuhan/359/95 (herein denoted as “Wuhan”), a human strain with no known resistance to the adamantane class of influenza inhibitors; A/PR/8/34 (herein denoted as “PR8”), a human strain with documented resistance to the adamantanes;21 and A/WSN/33 (herein denoted as “WSN”), a laboratory-adapted human strain also with known resistance to the adamantanes.21 As seen in the 1st line of Table 1 (the first three data columns), the non-resistant Wuhan strain was indeed quite sensitive to 1 with an IC50 of 60 ± 24 μM. In contrast, the drug-resistant PR8 and WSN strains were both far less sensitive toward the inhibitor with much poorer IC50 values of 2.2 ± 0.66 mM and 3.4 ± 0.2 mM, respectively, thus illustrating why 1 is no longer recommended for therapeutic use.

When 6 was attached to poly-L-glutamate at a ∼7% loading (i.e., ∼7% of all the monomeric units on the polymeric chain were drug-decorated), the inhibition for the resultant compound 13 (Figure 4) was nearly the same for the Wuhan and PR8 strains; for WSN, however, 13 was some 4-fold better inhibitor than 1 (Table 1, the first three data columns). When the degree of loading was roughly doubled (to yield compound 14 in Fig. 4), the inhibition for both the PR8 and WSN strains improved a few fold. Note that the original 1’s precursor 3 could not be tested as a monomer in the plaque reduction assay to determine the effect of adding a –COOH to the structure because of its poor solubility in aqueous PBS, which is the medium used in our antiviral assays.

We then investigated whether a further improvement in anti-influenza potency of the polymer conjugates 13 and 14 over the monomeric 1 could be attained by lengthening the time of their contact with the viruses during the assay. In our standard plaque reduction assay (designated as “Not in Agar” in Table 1), compounds are incubated with the viruses

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for 1 h prior to infection and during the subsequent 1-h infection period. After that, the inhibitors are removed, and fresh nutrient agar replaces them for the duration of the assay (while plaque formation occurs). Therefore, in the next series of experiments, we decided to keep the inhibitors present not only during those initial steps of the assay but also in an equal concentration in the nutrient agar overlay to allow them to act on all subsequent steps in the viral cycle.

As seen in Table 1 (the “In Agar” data columns), including the inhibitor for the entire plaque reduction assay improved IC$_{50}$ values in most instances. With 13 and 14, we observed no improvement in IC$_{50}$ values over 1 for the Wuhan strain but several-fold improvements for the PR8 and WSN strains. Thus although the inclusion in the agar results in net lower IC$_{50}$ values for most of the compounds tested, a greater magnitude of the improvement over the monomer (1) was seen when polymer conjugates were present only at initial steps of viral infection. Overall, however, these data show a marginal recovery in inhibitory potency toward drug-resistant strains when multiple copies of 1 are attached to poly-L-glutamate.

Rimantadine (2) and its polymer conjugates

We next explored how the other FDA-approved M2 ion channel inhibitor, 2, as well as its multiple copies attached to poly-L-glutamate, behaved against the aforementioned influenza strains. As in the case of 1, the Wuhan strain was very sensitive to 2 with an even better IC$_{50}$ value of 2.7 ± 1.4 μM, while both the PR8 and WSN strains were far less sensitive with IC$_{50}$s well into the low-single-millimolar range (Table 2), again confirming the reported drug resistance patterns. We then tested the effect of the insertion of an OH group into 2’s scaffold (to yield 7, which was used in subsequent derivatization (Fig. 3)). This small group greatly diminished the inhibitory potency against the Wuhan strain raising the IC$_{50}$ some ~90-fold (Table 2, first data column, 2nd line). The addition of an OH group also deleteriously, albeit less drastically, affected the already sub-optimal inhibition for the resistant strains (Table 2, second and third data columns, 2nd line). Previous studies have also demonstrated that the addition of a linker group to a small-molecule inhibitor can negatively affect its inhibitory potency. (This decline can be overcome, however, though multivalency.) That even a small addition, such as an OH group, has this deleterious effect demonstrates how subtle the structure-activity relationship is in this case.

Covalent attachment of 11 to poly-L-glutamate at a ~10% loading (compound 15, Fig. 4) afforded a significant improvement for all three viral strains over 7 (Table 2, the first three data columns, 3rd line). When compared to the native inhibitor (2), however, the polymer conjugation did not fully overcome the negative effects of the OH for the Wuhan strain leaving the IC$_{50}$ value at 24 ± 5 μM, i.e., some 10-fold inferior to 2’s. The attachment to poly-L-glutamate at a ~10% loading did little for the inhibitory effect against the PR8 strain but improved the IC$_{50}$ for WSN some 4-fold over 2.

Effect of the degree of loading on rimantadine-polymer conjugates

Since our initial studies with 1 yielded an improvement when the loading of the inhibitor on the polymeric chain was increased (Table 1), we explored whether 2 also followed this trend. As seen in Table 2 (4th line, the first three data columns), increasing the loading to ~20% (compound 16, Fig. 4) afforded some of the most potent poly-L-glutamate conjugates: although the IC$_{50}$ for 16 was still 10-fold worse than 2’s for the Wuhan strain, for PR8 and WSN we saw 8-fold and 30-fold improvements over 2, respectively. Interestingly, further increasing the degree of loading to ~30% and then to ~43% (compounds 17 and 18, respectively, Fig. 4) gave little additional improvement or even yielded worse inhibitors (Table 2).
When testing the polymeric inhibitors 15, 16, 17, and 18 for the duration of the plaque reduction assay (Table 2, the “In Agar” columns), patterns similar to those in the experiments with 1 were observed: the net IC50 was improved in most cases, but the magnitude of improvement over the monomer (2) became smaller. The optimal degree of loading also became less clear for the PR8 strain because all compounds had nearly the same IC50 values.

**Effect of linker’s properties on rimantadine-polymer conjugates**

We next determined whether a slightly longer and more hydrophilic spacer arm between 2 and poly-L-glutamate would be of benefit. Figure 3 depicts the synthetic route to compound 12 which contains a hydrophilic, poly(ethylene glycol), linker and six extra atoms between the drug and the polymeric chain. As seen in Table 2, when 12 is attached to poly-L-glutamate at a ∼10% loading to generate compound 19 (Fig. 4), there was little or no improvement in inhibition over 2 (some 70-fold worse for the Wuhan strain, no change for PR8, and approximately a 3-fold improvement for WSN). When the loading was increased to ∼20% (compound 20), which was optimal for the shorter and more hydrophobic linker, the inhibition was improved compared to 19: only a 18-fold worse inhibition for the Wuhan strain and a 13-fold improvements for both PR8 and WSN over 2 (Table 2).

When assaying 19 and 20 for the duration of the assay (Table 2, “In Agar”), no sizable benefit to the longer incubation was observed again and, in fact, 19 exhibited toxic effects in this modality. Since the slightly longer and more hydrophilic linker failed to generate major improvements compared to the shorter and more hydrophobic one, we decided to continue our studies with the latter.

**Effect of polymer’s structure on rimantadine-polymer conjugates**

To determine whether increasing hydrophobicity of the polymeric backbone by inserting extra aromatic or aliphatic hydrophobic moieties into it would improve inhibition, perhaps due to extra interactions between the polymer and the viral surface,12 we synthesized 21 containing ∼20% of benzyl rings in addition to a ∼15% loading of 2, as well as 22 containing ∼25% of tert-butyl substituents in addition to ∼20% of 2 moieties (Fig. 5). When conjugate 21 was tested against all three influenza strains in the plaque reduction assay, we observed similar improvements over 2 compared to that afforded by poly-L-glutamate with ∼20% loading and no additional hydrophobic moieties (16) for the Wuhan and PR8 strains, and not as marked improvements for the WSN strain: some 18-fold weaker inhibition than the monomer for the Wuhan strain and 16/17-fold improvements for WSN and PR8 (Table 2). Similarly, for the tert-butyl-derivatized poly-L-glutamate conjugate 22, we saw the same 18-fold decline in improvement over 2 for the Wuhan strain and some 10- and 22-fold improvements over 2 for PR8 and WSN, respectively. Therefore, although the addition of hydrophobic groups to the polymer in some cases did lead to a modest improvement over the 20%-derivatized plain poly-L-glutamate, there was no compelling reason for their inclusion.

We also examined the role of polymer’s characteristics in the conjugate’s inhibitory potency. To this end, first the charge of the poly-L-glutamate backbone was abolished by transforming it into the neutral poly-L-glutamine (compound 23, Fig. 6). As seen in Table 2, the neutralization of the polymer resulted in no improvements and actually in over 20-fold weaker inhibition than with the monomeric 2 for the Wuhan strain. For PR8 and WSN, 23 afforded 2- and 12-fold improvements, respectively, over 2, which is worse than 23’s negatively charged counterpart, 16. When incubated with the viruses for the duration of the assay, 23 exhibited improvements over the monomer for the PR8 and WSN strains, but not marked ones (Table 2, “In Agar”), Thus, putative electrostatic repulsions between the virus
and polymer are not a significant factor in viral inhibition for our system (in contrast to observations in previous studies\textsuperscript{10,12}); also, the neutralization of the poly-L-glutamate backbone to form poly-L-glutamine generated less potent inhibitors for drug-polymer conjugates at the same degree of small-molecule loading.

To investigate how new, structurally unrelated polymeric backbones would affect the inhibition of drug-resistant influenza viruses by polymer-conjugated 2, the dissimilar polymers poly(acrylic acid Na salt) and carboxymethylcellulose Na salt (CMC) were employed. Since each of these polymers, like poly-L-glutamate, contained carboxylate moieties, the same conjugation chemistry could be carried out.

Polyacrylate with \( \sim \)10\% of its monomeric units derivatized with 2 (compound 24, Fig. 6) was superior to all other conjugates tested against the Wuhan strain: it overcame all negative effects from an OH addition to 2 with an IC\(_{50}\) equal to that of the commercial drug itself (Table 2). However, against the other two influenza strains 24 did not possess the same potency with IC\(_{50}\) values of 120 \( \pm \) 50 and 280 \( \pm \) 50 \( \mu \)M for PR8 and WSN, thus resulting in some 11- and 8-fold improvements, respectively, over 2 for these drug-resistant strains (Table 2). Compound 24 was, however, markedly better than other 10\%-modified polymer-conjugates (15 and 19), suggesting that the polyacrylate backbone is a promising lead for multivalent inhibitors.\textsuperscript{12,13}

Attachment of 2 to CMC at a \( \sim \)20\% loading (25, Fig. 6) drastically curtailed inhibition of the Wuhan strain, with an IC\(_{50}\) of 760 \( \pm \) 160 \( \mu \)M (Table 2). Similarly poor IC\(_{50}\) values were obtained against PR8 and WSN (1,200 \( \pm \) 800 and 780 \( \pm \) 90 \( \mu \)M, respectively) suggesting that the inhibition afforded by these compounds might not even be caused by 2’s action per se but be due to CMC’s intrinsic weak antiviral activity.

In closing, in this study we have attached multiple copies of influenza M2 ion-channel inhibitors to a variety of polymers in an attempt to ameliorate, if not salvage, their ability to inhibit adamantane-resistant influenza strains. Our previous studies have shown that for another influenza virus inhibitor, zanamivir, polymer conjugation to form multivalent inhibitors could completely overcome resistance in zanamivir-resistant strains.\textsuperscript{10} However, zanamivir binds to its target neuraminidase on the outermost solvent-exposed portion of the enzyme\textsuperscript{2,22} which is presumably readily accessed to the drug-polymer conjugate. In contrast, the M2 ion channel imbedded within the viral membrane could be more difficult to access, thereby hindering multivalency. Additionally, the neuraminidase’s greater abundance on the viral surface (about 100 copies per virion compared to some 20 copies for the M2 ion channel)\textsuperscript{22} may also play a role in our incomplete potency recovery. Nonetheless, a progress toward our goal of recovering the adamantane inhibitors has been made by preparing 2-polymer conjugates up to 30-fold more potent than their monomeric precursors against some drug-resistant influenza strains.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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References


Figure 1. Chemical structures of both FDA-approved adamantane-class M2 ion channel influenza A inhibitors still in therapeutic use: amantadine·HCl (1) and rimantadine·HCl (2).
Figure 2.
Synthetic route to generating the 1-linker-azide compound 6 for subsequent covalent attachment to poly-L-glutamate. Reagents employed: (a) Fmoc-Cl, Na$_2$CO$_3$, H$_2$O/acetone; (b) HBTU, 5-azidopentan-1-amine, Hünig's base, THF; and (c) anhydrous acetonitrile/diethylamine. See Methods for details.
Figure 3.
Synthetic route to generating the 2-linker-azide compounds 11 and 12 for subsequent covalent attachment to poly-L-glutamate and other polymers. Reagents employed: (a) di-tert-butyl dicarbonate, dichloromethane; (b) 5-azido-pentanoic acid or 11-azido-3,6,9-trioxaundecanoic acid, DPTC, DMAP, chlorobenzene (reflux); (c) 4 M HCl in dioxane. See Methods for details.
Figure 4.
Chemical structures of poly-L-glutamate-attached conjugates of 1 and 2 at various degrees of loading (% of derivatization).
Figure 5.
Chemical structures of poly-L-glutamate derivatized with \( \sim 15\% \) of 2 of plus \( \sim 20\% \) of benzylamine (21) and with \( \sim 20\% \) of 2 plus \( \sim 25\% \) of 2,2-dimethyl-1-propamine (22).

\[
\]
Figure 6.
Chemical structures of poly-L glutamine, poly(acrylic acid Na salt), and CMC derivatized with 2.
Table 1

The IC$_{50}$ values for both monomeric 1 and its poly-L-glutamate conjugates against the Wuhan, PR8, and WSN strains of influenza A virus.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Wuhan</th>
<th>PR8</th>
<th>WSN</th>
<th>Wuhan</th>
<th>PR8</th>
<th>WSN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(6.0 ± 2.4) × 10$^1$</td>
<td>(2.2 ± 0.6) × 10$^1$</td>
<td>(3.4 ± 0.2) × 10$^1$</td>
<td>(3.8 ± 0.7) × 10$^1$</td>
<td>&gt;5 × 10$^2$</td>
<td>(1.8 ± 0.1) × 10$^2$</td>
</tr>
<tr>
<td>13</td>
<td>(8.5 ± 4.7) × 10$^1$</td>
<td>(1.4 ± 0.2) × 10$^1$</td>
<td>(7.8 ± 3.5) × 10$^2$</td>
<td>(3.3 ± 0.7) × 10$^1$</td>
<td>(1.2 ± 0.1) × 10$^2$</td>
<td>(8.3 ± 0.3) × 10$^1$</td>
</tr>
<tr>
<td>14</td>
<td>(4.4 ± 0.1) × 10$^1$</td>
<td>(8.9 ± 1.7) × 10$^1$</td>
<td>(8.6 ± 1.8) × 10$^1$</td>
<td>(4.9 ± 1.4) × 10$^1$</td>
<td>(2.2 ± 0.3) × 10$^2$</td>
<td>(7.7 ± 1.9) × 10$^1$</td>
</tr>
</tbody>
</table>

$a$The plaque reduction assay experiments were run in triplicate; the calculated mean and standard deviation values are presented in the table. All IC$_{50}$ values are expressed based on the concentration of 1. Our previous studies have demonstrated that the IC$_{50}$ value of bare poly-L-glutamate far exceeds 1 mM. Therefore, no inhibition can be attributed to the polymer itself.

$b$“Not in Agar” refers to an inhibitor present only in initial steps of infection, i.e., during a 1-h pre-incubation and subsequent viral binding to MDCK cells.

$c$“In Agar” refers to an inhibitor present during all stages of viral infection, i.e., during a 1-h pre-incubation, subsequent viral binding to MDCK cells, and the rest of the 72-h plaque reduction assay.
The IC\textsubscript{50} values for monomeric 2 and 7, as well as for the latter’s various polymer conjugates against the Wuhan, PR8, and WSN strains of influenza A virus.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Wuhan (\textmu M)</th>
<th>PR8 (\textmu M)</th>
<th>WSN (\textmu M)</th>
<th>Wuhan (\textmu M)</th>
<th>PR8 (\textmu M)</th>
<th>WSN (\textmu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.7 ± 1.4</td>
<td>(1.3 ± 0.09) \times 10^3</td>
<td>(2.4 ± 1.2) \times 10^3</td>
<td>1.4 ± 0.5</td>
<td>(3.0 ± 0.6) \times 10^2</td>
<td>(1.9 ± 0.8) \times 10^2</td>
</tr>
<tr>
<td>7</td>
<td>(2.4 ± 0.5) \times 10^2</td>
<td>(3.5 ± 0.8) \times 10^3</td>
<td>&gt;4.3 \times 10^3</td>
<td>(1.2 ± 0.8) \times 10^2</td>
<td>(8.6 ± 1.1) \times 10^2</td>
<td>(5.0 ± 0.9) \times 10^2</td>
</tr>
<tr>
<td>15</td>
<td>(2.8 ± 0.4) \times 10^1</td>
<td>(1.5 ± 0.4) \times 10^3</td>
<td>(6.4 ± 1.0) \times 10^2</td>
<td>(8.0 ± 3.5) \times 10^1</td>
<td>(1.1 ± 0.4) \times 10^2</td>
<td>(4.2 ± 0.3) \times 10^1</td>
</tr>
<tr>
<td>16</td>
<td>(2.5 ± 0.5) \times 10^1</td>
<td>(1.5 ± 0.8) \times 10^2</td>
<td>(7.7 ± 0.7) \times 10^1</td>
<td>(4.8 ± 3.2) \times 10^1</td>
<td>(1.4 ± 0.4) \times 10^2</td>
<td>(5.0 ± 1.3) \times 10^1</td>
</tr>
<tr>
<td>17</td>
<td>(1.4 ± 0.1) \times 10^2</td>
<td>(9.3 ± 2.4) \times 10^2</td>
<td>(3.9 ± 0.6) \times 10^2</td>
<td>(1.5 ± 0.09) \times 10^2</td>
<td>(1.7 ± 0.1) \times 10^2</td>
<td>(1.8 ± 0.09) \times 10^2</td>
</tr>
<tr>
<td>18</td>
<td>(8.0 ± 1.7) \times 10^2</td>
<td>&gt;1.4 \times 10^1</td>
<td>&gt;1.4 \times 10^1</td>
<td>(1.5 ± 0.3) \times 10^2</td>
<td>(1.2 ± 0.5) \times 10^2</td>
<td>(1.8 ± 0.4) \times 10^2</td>
</tr>
<tr>
<td>19</td>
<td>(1.9 ± 0.7) \times 10^2</td>
<td>(1.1 ± 0.4) \times 10^3</td>
<td>(9.2 ± 4.1) \times 10^2</td>
<td>Tox &gt;125\textsuperscript{d}</td>
<td>Tox &gt;125\textsuperscript{d}</td>
<td>Tox &gt;125\textsuperscript{d}</td>
</tr>
<tr>
<td>20</td>
<td>(4.9 ± 0.5) \times 10^1</td>
<td>(1.0 ± 0.5) \times 10^2</td>
<td>(1.8 ± 1.1) \times 10^2</td>
<td>(7.3 ± 1.8) \times 10^1</td>
<td>(1.1 ± 0.9) \times 10^2</td>
<td>(8.8 ± 1.3) \times 10^1</td>
</tr>
<tr>
<td>21</td>
<td>(4.7 ± 0.8) \times 10^1</td>
<td>(7.6 ± 2.9) \times 10^1</td>
<td>(1.5 ± 0.5) \times 10^2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>22</td>
<td>(4.9 ± 0.2) \times 10^1</td>
<td>(1.3 ± 0.5) \times 10^2</td>
<td>(1.1 ± 0.5) \times 10^2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>23</td>
<td>(6.4 ± 1.6) \times 10^1</td>
<td>(6.0 ± 3.6) \times 10^2</td>
<td>(2.0 ± 0.7) \times 10^2</td>
<td>(1.2 ± 0.3) \times 10^2</td>
<td>(9.9 ± 0.5) \times 10^1</td>
<td>(5.0 ± 1.6) \times 10^1</td>
</tr>
<tr>
<td>24</td>
<td>2.5 ± 0.4</td>
<td>(1.2 ± 0.5) \times 10^2</td>
<td>(2.8 ± 0.5) \times 10^2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>25</td>
<td>(7.6 ± 1.6) \times 10^2</td>
<td>(1.2 ± 0.8) \times 10^2</td>
<td>(7.8 ± 0.9) \times 10^2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The plaque reduction assay experiments were run in triplicate; the calculated mean and standard deviation values are presented in the table. All IC\textsubscript{50} values are expressed based on the concentration of 2; n.d. stands for “not determined”. Our previous studies have demonstrated that neither poly-L-glutamate nor poly-L-glutamine possesses any intrinsic anti-influenza properties with IC\textsubscript{50} values far exceeding 1 mM.\textsuperscript{10,18} Therefore, no inhibition can be attributed to the polymers themselves.

\textsuperscript{b}“Not in Agar” refers to an inhibitor present only in initial steps of infection, i.e., during a 1-h pre-incubation and subsequent viral binding to MDCK cells.

\textsuperscript{c}“In Agar” refers to inhibitor present during all stages of viral infection, i.e., during a 1-h pre-incubation, subsequent viral binding to MDCK cells, and the rest of the 72-h plaque reduction assay.

\textsuperscript{d}The IC\textsubscript{50} value could not be determined because concentrations under 125 \textmu M were below the IC\textsubscript{50} and concentrations above were toxic to the MDCK cells (i.e., no plaques could be visualized).