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Highly potent and long-acting trimeric and tetrameric inhibitors of influenza virus neuraminidase

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Abstract—A set of trimeric and tetrameric derivatives **6**–**11** of the influenza virus neuraminidase inhibitor zanamivir **1** have been synthesized by coupling a common monomeric zanamivir derivative **3** onto various multimeric carboxylic acid core groups. These discrete multimeric compounds are all significantly more antiviral than zanamivir and also show outstanding long-lasting protective activity when tested in mouse influenza infectivity experiments.

Influenza A and B have two major surface glycoproteins, hemagglutinin (HA) and the enzyme neuraminidase (NA), which are both essential for infectivity. HA binds to the terminal sialic acid groups on cell surface glycoproteins and is used by the virus to attach to cells. There are typically several hundred trimeric HA units on the surface of each virus particle and, taking advantage of the potential for multivalent binding, several research groups have demonstrated that synthetic polymers bearing multiple copies of sialic acid can show greatly enhanced binding to influenza HA.[1] Such polymeric sialosides interfere with viral attachment and although many show high in-vitro activity their clinical potential is seriously limited by the variability of binding to different influenza strains.[2]

Influenza NA cleaves terminal sialic acids from cell surface glycoconjugates and is thought to be necessary for release of the virus from cell surfaces and thus for the movement of virus through mucus. Two small-molecule nanomolar inhibitors of NA have demonstrated clinical efficacy and have recently been approved for use against

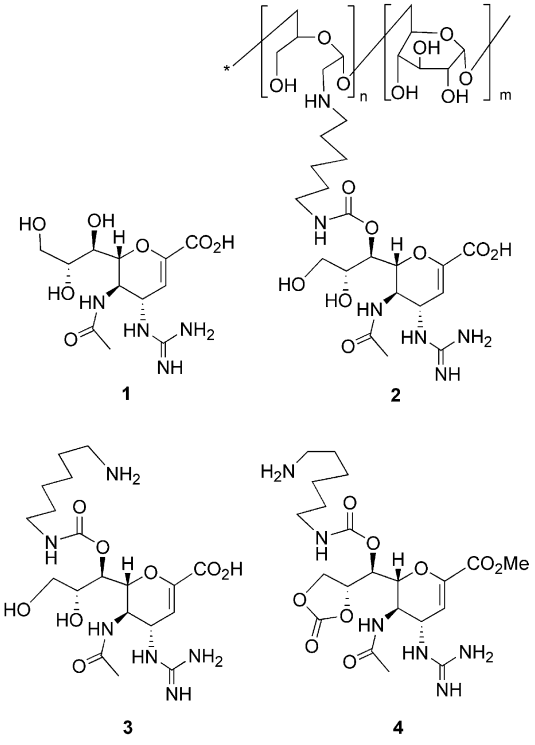

Figure 1.

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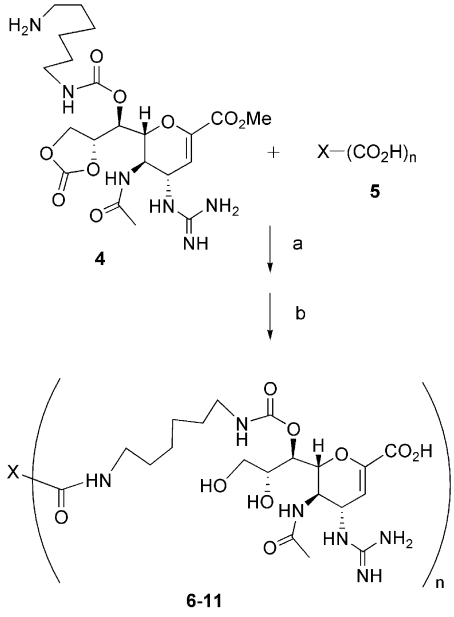
Table 1. Polymeric conjugate **2** shows long-acting inhibition of influenza infectivity[a]

Timing of compound dosing (h) prior to viral infection[b]	No. of virus free animals from zanamivir **1** dosing[c]	No. of virus free animals from polymer **2** dosing[c]
51	3/9	7/9
117	1/10	9/9
145	3/9	10/10
172	0/8	8/9
240	0/10	10/10

[a] Mice were dosed with compound and then later infected with influenza A/Singapore/1/57.
[b] A single prophylactic dose of zanamivir or compound **2** was administered intranasally at 12.5 mg/kg.
[c] Virus level in lungs of mice was measured 24 h after infection and scored No. virus free/Total No.

influenza. One of these NA inhibitors is zanamivir **1**,[3] the 4-guanidino analogue of 2,3-dehydro-sialic acid and an enzyme substrate mimic (Fig. 1). As there are about 50 tetrameric NA units on the surface of each influenza virion[4] we became interested in exploring the antiviral properties of multimeric zanamivir derivatives. We have previously found that polymers bearing multiple zanamivir molecules attached through the 7-position of the sialic acid, such as oxidised dextran conjugate **2**, are able to inhibit NA and show high in vitro anti-influenza activity.[5] A recent publication[6] describes related polymeric zanamivir conjugates and has prompted us to present this summary of our work on the synthesis and antiinfluenza activity of some trimeric and tetrameric zanamivir derivatives.

Our interest in multimeric NA inhibitors was heightened when we tested polymeric influenza inhibitors of type **2** in a mouse influenza infectivity model[7] and found that they show outstanding and long-lasting activity.

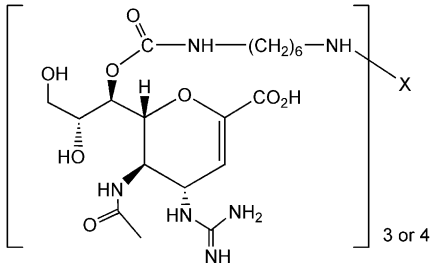


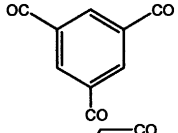
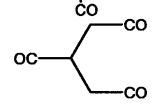
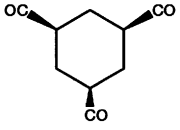
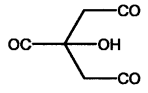
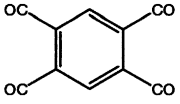
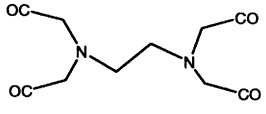
Scheme 1. Synthesis of multimers **6**–**11**. Reagents and conditions: (a) **4** (n + 0.5 equiv), **5** (1.0 equiv), BOP (3.5 equiv), DIPEA (20 equiv), DMF, 20 °C, 5 h, RP-HPLC purification; (b) MeOH/H_2O/Et_3N (10:10:3), 20 °C, 3 h, C18 RP-HPLC purification.

Thus polymeric compound **2** when dosed directly into the lungs is able to protect mice from influenza infection for up to 10 days compared to the less than 2 days of protection given by zanamivir (Table 1).

We believe that the prolonged in vivo activity of the polymeric conjugate **2** is related to the slow lung clearance of such high molecular weight (500 kD) compounds. It has previously been shown in animal experiments that the lung residence time of small molecules is directly proportional to their molecular weight and that smaller polar molecules are rapidly cleared by

Table 2. Structures and antiinfluenza activity of multimers **6**–**11** in a CPE assay[a]



Compd	Central multivalent group X	Influenza A/Sydney/5/97 EC_{50} (ng/mL)[b]	Influenza B/Harbin/7/95 EC_{50} (ng/mL)[b]
1 (Zanamivir)	–	32.5(5)	12.2(5)
3 (monomer)	–	438(3)	213(4)
6		0.82(4)	1.33(5)
7		0.4(1)	0.81(1)
8		0.3(1)	0.438(1)
9		0.159(1)	2.475(1)
10		0.53(3)	0.95(4)
11		1.07(1)	2.84(1)

[a] None of the compounds showed any cytotoxicity at concentrations up to 100 ng/mL.
[b] The EC_{50} values are the average from a number of separate assays (number in parenthesis).

passing through the tight junctions between cells.[8] The prolonged in vivo activity of polymer **2** raised the prospect of an antiinfluenza drug that could be used as a single dose for treatment of the disease or just once every week or more for prevention of infection. We were concerned however that the development path for a polymeric drug could be more problematic than for a small molecule and so we decided to investigate whether discrete lower molecular weight multivalent derivatives of zanamivir also possess high antiviral activity and prolonged in vivo activity.

The X-ray crystal structure of zanamivir bound in the influenza NA active site shows that the 7-hydroxy group is not involved in any significant interactions and in fact points out and away from the enzyme.[9] Consistent with this, derivatives of zanamivir functionalised at the 7-position, including the aminohexyl-7-carbamate derivative **3**, retain good activity against all tested influenza strains.[5,10] Therefore, as for the synthesis of the polymers **2**,[5] we have used compound **3** as a ligand, reacting it in the protected form **4** with a variety of multivalent carboxylic acid core groups **5** (Scheme 1), to give compounds such as the trimeric and tetrameric zanamivir conjugates **6–11** listed in Table 2.[11] When compounds **6–11** were tested in a standard[12] influenza cytopathic effect (CPE) assay they all showed significantly greater antiviral potency than zanamivir **1** (Table 2). Thus whilst the monomeric ligand **3** was found to be about ten-fold weaker than zanamivir, the multimers **6–11** were generally found to be 10 or even up to 100 times more active than **1** on representative influenza A and B test strains.

Next the efficacy of intranasally administered multimers **6–11** was compared with zanamivir **1** and the polymer **2** in the influenza virus infected mouse model. As above, the testing was carried out as has been described previously[7] and involved administering a single intranasal dose of compound 7 days prior to infection of the animals with influenza. The efficacy was measured 24 h after the viral infection by assaying the lung tissue for the level of virus and comparing to the level of virus in the lungs of untreated mice. Results for compounds **6–11** are shown in Tables 3a–b.

All of the trimeric and tetrameric compounds **6–11** show outstanding in vivo activity, being similar to, or more effective than, zanamivir at a fraction of the dose, with compounds **6** and **11**, e.g., being at least 40-fold more active than zanamivir.

The data show that the trimeric compounds **6–9** are just as active as the tetrameric compounds **10** and **11**, both in terms of antiviral potency and in vivo activity. It is also interesting to note that whilst the distance between the active sites within an influenza NA tetramer is at least 45 Å,[13] the linking group in compounds **6–11** is only 23 to 26 atoms in length and therefore not long enough to allow simultaneous binding of two zanamivir groups within the same NA tetramer. At this stage we have no definite explanation for the remarkable activity of the trimeric and tetrameric zanamivir derivatives.

Table 3a. Efficacy of compounds **6**, **10**, and **11** in a 7 day mouse influenza prevention assay

Compd	Compd dose (mg/kg)[a]	Mean virus titre ($logTCID_{50}$/mL)[b]	% Effectiveness[c]
Control	0	5.53–6.05	–
1 (Zanamivir)	1.6	3.93	97.03
1	0.4	5.54	0
2 (polymer)	0.1	5.05	90.00
2	0.01	5.63	62.42
6	0.1	4.63	96.24
6	0.01	5.53	70.15
10	1.0	2.30	99.68
10	0.1	4.20	74.88
11	0.1	4.70	95.53
11	0.01	5.55	68.38

[a] A group of 10 mice was used for each dose and for calculation of the mean virus titres.
[b] Influenza A/Singapore/1/57 was used.
[c] % Reduction in virus titre.

Table 3b. Efficacy of compounds **7–9** in a 7 day mouse influenza prevention assay

Compd	Compd dose (mg/kg)[a]	Mean virus titre ($logTCID_{50}$/mL)[b]	% Effectiveness[c]
Control	0	4.75–5.11	–
1 (Zanamivir)	1.0	4.45	81.76
7	1.0	3.35	98.24
7	0.1	4.60	68.78
8	1.0	2.98	98.32
8	0.1	3.50	94.38
9	1.0	3.05	99.11
9	0.1	4.25	85.88

[a] A group of 10 mice was used for each dose and for calculation of the mean virus titres.
[b] Influenza A/Victoria/3/75 was used.
[c] % Reduction in virus titre.

In summary, we have found that trimeric and tetrameric conjugates of zanamivir show remarkably potent antiviral activity and that it is not necessary to use complex and high molecular weight polymeric conjugates to achieve long-lasting in vivo antiviral activity.

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