Chemoenzymatic synthesis, characterization, and application of glycopolymers carrying lactosamine repeats as entry inhibitors against influenza virus infection

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Received on April 8, 2008; revised on June 17, 2008; accepted on July 9, 2008

Keywords: chemoenzymatic synthesis/entry inhibitor/glycopolymer/influenza virus/lactosamine repeat

Introduction

Sialic acid-containing glycoconjugates act as receptors for various types of influenza viruses (Suzuki 2005). Influenza viruses infect host cells through the binding of viral hemagglutinins (HAs) to sialo-glycoconjugates as receptors on the host cell surface. Influenza viruses found in humans and other animals appear to have originated in wild birds (Webster et al. 1992; Wright and Webster 2001; Ha et al. 2002; Fouchier et al. 2005). When avian and human viruses simultaneously infect an intermediate host, such as pigs, genetic recombination can occur between these viruses. This event could lead to the emergence of new viruses that may give rise to a flu pandemic, such as the Asian flu in 1957 and the Hong Kong flu in 1968 (Scholtissek et al. 1978; Kawaoka et al. 1989). In 1997, avian H5N1 virus was transmitted directly into humans. Since 1997, other avian virus subtypes, H7N7 and H9N2, have also emerged. Interspecies transmission, especially both initial avian to human infection and outbreaks of pandemic viruses in the human population, has been rare to date. The major reason for restriction of interspecies transmission is thought to be limited adaptation of avian viruses to humans (Claas et al. 1998; Lin et al. 2000; Lipatov et al. 2004; Palese 2004; Horimoto and Kawaoka 2005). Hemagglutinin is a trimeric protein expressed on viral particles. This protein comprises two subunits, HA1 and HA2, which are cleaved by the action of proteases derived from host cells. Such posttranslational processing is essential for influenza virus infection. A globular head domain in the HA1 subunit containing a receptor-binding site (RBS) mediates virus attachment to host cells, while the HA2 subunit is involved in virus–host membrane fusion mediated through a fusion peptide at the N-terminus. Sialic acid-containing carbohydrate molecules expressed on the host cell surface are critical determinants for both interspecies transmission and epidemics in a specific host. Several lines of evidence indicate that the influenza viruses recognize specific linkages of sialic acids in the receptors, such as sialyl lactosamine (sialyl LacNAc) structures, Neu5Acα2-3Galβ1-3GlcNAcβ1-R, and Neu5Acα2-3Galβ1-4GlcNAcβ1-R. Human influenza A viruses, which have been isolated from humans over the last three decades, preferentially recognize Neu5Acα2-6 residues. In contrast, avian influenza viruses bind predominantly to Neu5Acα2-3 residues (Ito et al. 1997; Miller-Podraza et al. 2000; Matrosovich et al. 2004; Suzuki 2005; Gambaryan, Yamnikova, et al. 2005). Recently, histochemical studies using lectins specific for sialic acid linkages demonstrated that the characteristic distribution of sialo-glycoconjugates is associated with avian to human

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viral transmission (Shinya et al. 2006; Thompson et al. 2006). In addition, the amino acid residues responsible for recognition of terminal sialic acid linkages were determined on HA of an avian H5 virus (Yamada et al. 2006). Previous studies isolated and characterized carbohydrate determinants that are predominantly recognized by avian and human viruses, such as Neu5Acα2-3nLc4 for avian viruses and Neu5Acα2-6nLc4 for human viruses. However, receptor carbohydrate molecules with high affinity for humans and other species have yet to be defined in host cells. Very recently, we determined the relative binding affinities of sialic acid-containing carbohydrate molecules for both avian and human viruses on lipid bilayers using newly established surface plasmon resonance (SPR) assays (Hidari et al. 2007). The binding affinity of receptor carbohydrates was remarkably distinct between avian and/or human influenza viruses. Human influenza viruses recognize not only specific linkages of sialic acids but also core carbohydrates containing LacNAc repeats in the receptors. In contrast, avian viruses bind to a short core carbohydrate containing Neu5Acα2-3 residues with higher affinity than elongated carbohydrate chains.

Compounds that effectively inhibit virus entry could be ideal tools to address the molecular mechanisms of the virus–host cell interaction. In addition, such entry inhibitors are potentially very useful for investigation and control of influenza virus infection. Based on previous observations regarding receptor carbohydrate structures for human and other influenza viruses, synthetic glycopolymers, such as polyacrylamide or α-polyglutamic acid (α-PGA) carrying multivalent sialyl LacNAc residues, have been applied for inhibition of influenza virus infection (Totani et al. 2003; Gambaryan, Boravleva, et al. 2005). These compounds carry a multivalent or a single sialyl oligosaccharide chain attached to the polymer backbone, contributing to augmentation of inhibitory activity.

In the present study, we synthesized, characterized, and applied alternative glycopolymers carrying multivalent sialyl LacNAc oligosaccharides for further investigation of the molecular mechanisms underlying the recognition of different species by influenza viruses with specific carbohydrate structures.

### Results

**Synthesis of glycopolymers carrying lactosamine repeats**

Tetrasaccharide glycoside containing two LacNAc repeats, \((\text{LacNAc})_2\beta\_p\)-NP, was synthesized by the alternative addition of \(\beta\_1-3\)-linked GlcNAc and \(\beta\_1-4\)-linked Gal to LacNAcβ-pNP using two glycosyltransferases. GlcNAc and Gal were further added to the obtained tetrasaccharide glycoside in a similar manner to generate \((\text{LacNAc})_3\beta\_p\)NP. Both products were then reduced to p-aminophenyl (pAP) glycosides. Amino functional groups of LacNAcβ-pAP, \((\text{LacNAc})_2\beta\_p\)-AP, and \((\text{LacNAc})_3\beta\_p\)-AP were coupled with the carboxyl group of α-PGA as a backbone (Figure 1) in the presence of the condensation reagents...
Glycopolymers with LacNAc repeats as influenza virus inhibitor

Table I. Inhibitory effect of Neu5Aca2-6glycopolymers on influenza virus infection to MDCK cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>Subtype</th>
<th>IC50 (nM)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Single</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H1N1</td>
</tr>
<tr>
<td>Human</td>
<td>A/WSN/33</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>A/Aichi/2/68</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>A/Shizuoka/75/2003</td>
<td>N.E.</td>
</tr>
<tr>
<td></td>
<td>A/Shizuoka/64/2004</td>
<td>N.E.</td>
</tr>
<tr>
<td></td>
<td>A/Shizuoka/214/2005</td>
<td>900</td>
</tr>
<tr>
<td>Swine</td>
<td>A/Swine/Hokkaido/10/85</td>
<td>H3N2</td>
</tr>
<tr>
<td>Avian</td>
<td>A/Duck/HongKong/24/76</td>
<td>H3N2</td>
</tr>
<tr>
<td></td>
<td>A/Duck/HongKong/333/4/76</td>
<td>H5N3</td>
</tr>
<tr>
<td>Equine</td>
<td>A/Equine/Tennessee/5/86</td>
<td>H3N8</td>
</tr>
</tbody>
</table>

N.E., no effect; means that inhibitory effect was not observed up to 1000 nM of glycopolymers.

Inhibitory activity was determined by a focus-forming assay as described in Materials and methods.

Table I. Inhibitory effect of Neu5Aca2-6glycopolymers on influenza virus infection to MDCK cells

BOP and HOBT, as described previously (Zeng et al. 2000). Introduction of pAP-glycosides into α-PGA with the degree of polymerization (DP) of 467 was adjusted to 36–40% (degree of substitution; DS) for the generation of nonsialyl glycopolymers designated as single, tandem, and triplet, respectively (Figure 1). The three glycopolymers were then sialylated to nonreducing terminal residues using α2,6-ST. The structures of all synthesized glycopolymers were confirmed by both 1H- and 13C-nuclear magnetic resonance (NMR) analyses as described previously (Zeng et al. 2000; Totani et al. 2003).

Inhibition of infection of MDCK cells by influenza virus by administration of glycopolymers

To determine the inhibitory effects of glycopolymers on infection of MDCK cells by influenza virus, virus and test agents were inoculated concurrently. The inhibitory activity was determined by a focus-forming assay as described in Materials and methods.

Single, Tandem, and Triplet means single, tandem and triplet repeats of LacNAc residues, respectively.

Protection of mice by intranasal administration of glycopolymers from death induced by A/WSN/33 infection

We examined the protective effects of Neu5Aca2-6glycopolymers with single and triplet LacNAc repeats in a murine model of respiratory tract viral infection with A/WSN/33 strain. In order to determine a minimum lethal dose, mice were infected by several doses of the virus. When 5-week-old mice were treated intranasally with 50 µL of viral suspension containing more than 2.0 × 10^4 pfu/mL, they all died within 14 days. On the other hand, 0.5 × 10^4 pfu/mL of the virus did not kill any mice until day 14 postinfection. Thus, mice were challenged by 2.0 × 10^4 pfu/mL of A/WSN/33. A previous study showed that administration of 1.5–5 nmol of a sulfated glycopolymers as a total dose received by each animal protected mice from lethal challenge of influenza viruses (Gambaryan, Boravleva, et al. 2005). The Neu5Aca2-6glycopolymer with a triplet LacNAc repeat inhibited viral infection to MDCK cells with approximately 10-fold stronger extent than the previous sulfated glycopolymer (Table I). We planned to administer glycopolymers to mice at two total doses, about 0.1 and 1 nmol. The calculated doses were corresponded to 4.5 and 45 nmol/kg of Neu5Aca2-6glycopolymer with a triplet LacNAc repeat. Nonsialyl glycopolymers were used as controls to examine influenza infection. Groups of mice were concurrently administered glycopolymers. Administration of the glycopolymers at 4.5 nmol/kg did not affect mortality of mice caused by A/WSN/33 infection (data not shown). Statistical analysis (log-rank test on Kaplan–Meier survival curves) demonstrated that Neu5Aca2-6glycopolymer with a triplet LacNAc repeat, administered at a dose of 45 nmol/kg, significantly reduced mortality below 20% on day 17 postinfection as compared to administration of nonsialyl glycopolymers (P-value < 0.05) (Figure 2A). In contrast, either nonsialyl glycopolymer or Neu5Aca2-6glycopolymer with a single LacNAc, administered at the same dosage, did not affect lethality in mice caused by A/WSN/33 infection (Figure 2A).

In mice treated with Neu5Aca2-6glycopolymers with a triplet LacNAc repeat, but not either Neu5Aca2-6glycopolymers with a single LacNAc or nonsialyl glycopolymers at a dose of 45 nmol/kg, we also observed improvement in the weight gain of mice surviving to day 17 postinfection (Figure 2B). Thus, sialyl glycopolymers with an elongated LacNAc chain as a core structure showed a significant protective effect in this murine model of influenza virus infection. The protective effect of this glycopolymer is comparable to that observed in vitro (Table I). Glycopolymer-related toxicity was not observed in any of the groups examined.

In order to determine the effect of glycopolymers on virus propagation in the lung, we set additional three mice in groups treated with nonsialyl or Neu5Aca2-6glycopolymers. Mice were sacrificed on day 3 postinfection and lungs were collected for virus titration in MDCK cells. Figure 3 shows virus titers in respectively. A swine virus strain also showed high susceptibility to Neu5Aca2-6glycopolymers. The number of foci was reduced by treatment with Neu5Aca2-6glycopolymers in a core carbohydrate length-dependent manner. In contrast, cellular infection by avian and equine viruses was not inhibited by treatment with any Neu5Aca2-6glycopolymer carrying LacNAc repeats. Taken together, in vitro infection experiments strongly suggested that the elongated core carbohydrate structure may be a critical determinant for human and swine virus recognition.
Fig. 2. Effects of glycopolymers on influenza virus (A/WSN/33) infection in mice. Influenza virus infection in mice was performed as described in Materials and methods. Graph (A) indicates Kaplan–Meier survival curves (*P < 0.05, Log-rank test). Mice were intranasally inoculated with viral suspension and nonsialyl (rhombus) or Neu5Acα2-6 (circle) glycopolymer at a dose of 45 nmol/kg. Value (n) means the number of mice tested in each group. Graph (B) shows body weight dynamics of mice. Lines and dotted lines indicate mice intranasally inoculated with either Neu5Acα2-6 or nonsialyl glycopolymer-treated virus, respectively.

Evaluation of binding affinities of glycopolymers to influenza virus hemagglutinin

To investigate the mechanisms of the inhibitory effects of core carbohydrate structures on viral infection, we first examined the effects of glycopolymers on virus-mediated hemagglutination. Erythrocyte hemagglutination is mediated by the interaction of viral HA with sialic acid-containing glycoconjugates on the erythrocyte surface. Following incubation of erythrocytes with influenza virus in the presence of glycopolymers at the indicated concentrations, inhibition of virus-mediated hemagglutination was observed (Table II). In human and swine viruses, but not avian viruses, Neu5Acα2-6glycopolymers carrying elongated LacNAc repeats showed competitive inhibition in erythrocyte hemagglutination with much higher activity than a single LacNAc. This result was consistent with in vitro and in vivo infection experiments, suggesting that enhancement of the binding affinity of glycopolymers with viral HA is accompanied with the extension of LacNAc repeats as the core structure. Nonsialyl glycopolymers as controls did not interfere with virion attachment to the host cell surface (data not shown).

For further investigation, we directly determined the binding activity of glycopolymers to human and avian viruses by a solid-phase virus binding assay. Previously, we established a direct binding assay with synthetic glycopolymers for avian influenza viruses (Yamada et al. 2006). In this study, we applied the assay for determination of direct binding dynamics of glycopolymers carrying elongated core carbohydrate structures to human and avian viruses. As glycopolymers are highly water soluble, they
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Fig. 3. Virus titers in the lungs of mice intranasally inoculated with glycopolymer-treated virus. On the third day postinfection, three mice from each group were killed humanely. Their lungs were collected, and viral titers were determined by a plaque-forming assay as described in Materials and methods. The results represent the means of triplicate determinations. Asterisk (*) indicates statistical significance (P < 0.05, t-test). The dose of each glycopolymer administered to mice was 45 nmol/kg.

Table II. Effect of Neu5Acα2-6glycopolymers on hemagglutination induced by influenza virus

<table>
<thead>
<tr>
<th>Virus</th>
<th>Subtype</th>
<th>IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Single</td>
</tr>
<tr>
<td>Human A/WSN/33</td>
<td>H1N1</td>
<td>25</td>
</tr>
<tr>
<td>A/Aichi/2/68</td>
<td>H3N2</td>
<td>18.8</td>
</tr>
<tr>
<td>A/Shizuoka/214/2005</td>
<td>H3N2</td>
<td>375</td>
</tr>
<tr>
<td>Swine A/Swine/Hokkaido/10/85</td>
<td>H3N2</td>
<td>9.4</td>
</tr>
<tr>
<td>Avian A/Duck/HongKong/313/4/78</td>
<td>H5N3</td>
<td>N.E.</td>
</tr>
</tbody>
</table>

N.E., no effect; means that inhibitory effect was not observed up to 1000 nM of glycopolymers.

Single, Tandem, and Triplet means single, tandem and triplet repeats of LacNAc residues, respectively.

cannot be immobilized on polystyrene surfaces by hydrophobic interactions. Finally, they were immobilized on the surface by UV crosslinking. The quantity of virus binding was evaluated by neuraminidase activity of viral particles captured on the glycopolymer-immobilized polystyrene surface. The binding signals were detected when Neu5Acα2-6glycopolymers, but not nonsialyl glycopolymers, were used as ligands (data not shown).

As the concentration of glycopolymers with LacNAc repeats increased, the signals from human, but not avian, viruses bound to glycopolymers increase in a dose-dependent manner regardless of the core carbohydrate structure (Figure 4). The direct binding experiment demonstrated that there are significant differences in binding properties between glycopolymers carrying three types of the core carbohydrate structure. First, Neu5Acα2-6glycopolymers carrying LacNAc repeats bound directly to human influenza viruses. Second, Neu5Acα2-6glycopolymers carrying tandem or triplet LacNAc repeats bound to the virus with much higher binding activity than to that carrying a single copy. These results were comparable with those obtained by in vitro and in vivo infection experiments. Taken together, the human influenza viruses bind exclusively to Neu5Acα2-6glycopolymers carrying elongated core carbohydrates with higher affinity.

Sequencing and modeling analyses of human H3 clinical strains

Of human clinical isolates, A/Shizuoka/214/2005 was highly susceptible to glycopolymer with triplet LacNAc repeats. The inhibitory effect of triplet LacNAc glycopolymer on A/Shizuoka/214/2005 infection was more than 1000-fold, which was greater than that on infection by A/Shizuoka/75/2003 (Table I). The other strain, A/Shizuoka/64/2004, showed the intermediate susceptibility to triplet LacNAc glycopolymer. To elucidate the molecular basis of susceptibility to glycopolymers, the nucleotide sequences of viral HA were examined. The amino acid sequences of HA were deduced by
sequencing analysis in two clinical and A/Aichi/2/68 strains. Alignment of the amino acid sequences distinct from A/Aichi/2/68 strain is summarized in Table III. Seven amino acid substitutions were identified in the HA region between the two clinical strains, A/Shizuoka/75/2003 and A/Shizuoka/214/2005. Amino acid residues at positions 138, 226, and 227 compose part of the RBS. Among these amino acid residues, substitution of serine to proline at position 227 may affect the conformation of RBS, resulting in susceptibility of clinical strains to triplet LacNAc glycopolymer. Locations of amino acid residues other than in the RBS were estimated in homology-modeled structures of two clinical strains, A/Shizuoka/75/2003 and A/Shizuoka/214/2005, generated on the basis of the HA structure of A/Aichi/2/68 (PDB accession number 1HGG). Figure 5 shows the globular head domain structures of the HA of each clinical strain. The overall structures of both domains are very similar. However, the capacity of RBS in A/Shizuoka/214/2005 appears to be less than that in A/Shizuoka/75/2003. Particularly, Ser138 and Ile226 in A/Shizuoka/214/2005 are closer to the sialic acid residue than Ala138 and Val226 in A/Shizuoka/75/2003. These substitutions may contribute to the preferable association of sialic acid with RBS. The side chain of Lys145 proximal to the right side of RBS extends to this site. On the other hand, Asn145 does not occupy the space covering the right side of the RBS. Thus, substitution of Lys to Asn at position 145 may also affect accessibility of sialic acid-containing carbohydrate chains to RBS. Taken together, there is a strong possibility that some amino acid residues not only in the RBS but also outside the RBS contribute directly to the susceptibility of human H3-subtype influenza viruses to triplet LacNAc glycopolymer.

Discussion

A number of virus-entry inhibitors have been developed. An antiviral peptide that targets viral attachment to host cells inhibits influenza virus infection in mice (Jones et al. 2006). In addition, a polyacrylamide-based glycopolymer with Neu5Acα2-6 LacNAc residues protects mice against infection by influenza virus (Gambaryan, Boravleva, et al. 2005). These compounds effectively prevent influenza virus infection in mice, and thus virus attachment to host cells is a favorable target for development of antiviral agents, such as neuraminidase inhibitors (von Itzstein et al. 1993; Sidwell et al. 1998). However, these compounds reported previously required repeated administration to mice over a long period to be effective.

In this study, we newly synthesized and tested glycopolymer carrying multivalent sialyl LacNAc repeats as virus-entry inhibitors. Alpha-PGA was used as the backbone of each glycopolymer as it showed very low toxicity and high reactivity with pAP-oligosaccharide glycosides (Totani et al. 2003). Tandem- and triple-repeat LacNAc glycosides were synthesized by the addition of GlcNAc and Gal to LacNAcβ-pNP as the starting substrate. We reported previously that (LacNAc)β-p-PAP was an effective chromogenic substrate for endo-β-galactosidase (Murata et al. 2003). Nonsialylated glycopolymer carrying single- to triple-repeat LacNAc were next synthesized by a two-step procedures involving the reduction of the p-nitrophenyl group and introduction of the resulting pAP-oligosaccharide glycoside to α-PGA. In this study, DS of pAP-oligosaccharides into α-PGA was strictly adjusted to 36–40% by controlling the coupling reaction. Sialylation using ST6Gal I resulted in an efficient and linkage-specific yield of sialyl glycopolymer.

Several lines of evidence reported to date indicated that clustering, ionic charge, and molecular weight of oligosaccharide chains on glycopolymers affect the inhibitory activity of sialyl glycopolymers against influenza virus infection (Lees et al. 1994; Reuter et al. 1999; Totani et al. 2003; Makimura et al. 2006). Particularly, clustering of oligosaccharide chains predominantly contributes to enhancement of binding affinity of glycopolymers to viruses. To examine the effects of core carbohydrate structures attached to a polymer on inhibitory activity, we used α-PGA glycopolymers with LacNAc repeats in the present study. The in vitro infection experiments demonstrated that Neu5Acα2-6glycopolymers effectively inhibited cellular infection by human laboratory strains A/WSN/33 (H1N1) and A/Aichi/2/68 (H3N2). Viral infection was markedly reduced by repeating LacNAc residues. These results strongly suggest that elongation of the core carbohydrate (LacNAc) chain attached to α-PGA enhances the inhibitory activity of glycopolymers. Similar effects on viral infection were observed when human clinical strains were treated with Neu5Acα2-6glycopolymers carrying LacNAc repeats (Table I). Thus, glycopolymers with elongated core carbohydrates are expected to inhibit all types of human influenza virus, regardless of the manner of propagation. Neu5Acα2-6glycopolymers also prevented infection of

**Table III.** Alignment of amino acid residues of H3N2-subtype influenza virus HA1

<table>
<thead>
<tr>
<th>Virus</th>
<th>Position of amino acid residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Aichi/2/68</td>
<td>V  A  S  S  Q  L  S</td>
</tr>
<tr>
<td>A/Shizuoka/75/2003</td>
<td>D  A  K  Y  S  V  S</td>
</tr>
<tr>
<td>A/Shizuoka/214/2005</td>
<td>G  S  N  F  N  I  P</td>
</tr>
</tbody>
</table>

Amino acid residues at the position of 138, 226, and 227 indicated in italic compose part of the receptor-binding site (RBS).
host cells by a swine influenza virus, but not those of other animals, such as avian or equine viruses. These results were in good agreement with the recognition profiles of influenza viruses with receptor sialo-glycoconjugates with Neu5Aca2-6 or Neu5Aca2-3 linkages specific for host animals as described previously (Shinya et al. 2006).

To examine the inhibitory activity of α-PGA glycopolymers with LacNac repeats, we administered glycopolymers to mice as an infection model. After inoculation with A/WSN/33, virus invades and propagates in various tissues, particularly in the lung and brain. As a result, mice develop mortality and typical morbidity, such as loss of movement and food consumption, resulting in loss of weight. Neu5Aca2-6-glycopolymers with triplet LacNac repeats, but not with a single unit, effectively protected mice from lethal challenge of A/WSN/33. At 17 days postinfection, only mice administered 45 nmol/kg of Neu5Aca2-6-glycopolymers with triplet LacNac repeats survived. This observation was consistent with the results of in vitro infection experiments. No toxic effect was observed in mice inoculated with the same concentration of glycopolymers alone. Simultaneous administration of Neu5Aca2-6-glycopolymer with triplet LacNac repeats reduced mortality and loss of weight in mice caused by lethal challenge with influenza virus. In addition, Neu5Aca2-6-glycopolymer with triplet LacNac repeats, but not with a single LacNac, significantly inhibited virus propagation in the lung. These observation results strongly suggested that the reduction of virus growth by the glycopolymer may result in protection of mice from lethal challenge by the virus. Taken together, it was suggested that the protective effect of the glycopolymer is due to the prevention of virus attachment to the host cell surface.

Hemagglutinin inhibition and solid-phase binding assays clearly showed that elongation of core carbohydrate portion markedly enhances binding affinity of Neu5Aca2-6-glycopolymer with influenza viruses. This is consistent with the findings of in vitro and in vivo infection experiments. Our results indicated that modification of core carbohydrate chains contributes to enhancement of the binding affinity of sialyl oligosaccharide with influenza viruses as described recently (Hide et al. 2007; Chandrasekaran et al. 2008). The addition of LacNac residues may affect clustering of sialyl sugar chains attached to α-PGA.

To address the mechanisms on enhancement of inhibitory activity by elongation of core carbohydrates, we analyzed HA sequences and modeled structures of human clinical strains (Figure 5). Previous studies using co-crystallization and microarray technologies indicated that interactions of HA with host not only anomeric linkages of sialic acid residues but also the core carbohydrate length to the interaction of influenza virus HA with Neu5Aca2-6 carbohydrate chain, which efficiently places sialic acid into the RBS. As the length of the oligosaccharide (LacNac repeats) critically influences HA binding contacts in the umbrella-like topology, some amino acids are predicted to be involved in interactions with this topology. Based on our modeled structures, three other substitutions at positions 145, 159, and 189 are located close to the RBS. There is a strong possibility that substitution of Lys145 in the 2003 strain for Asn145 in 2005 enhances the accessibility of the α-6-linked sialic acid residue to the nonreducing terminal of the elongated carbohydrate chain. Two other amino acid residues may interact directly with LacNac repeats.

In this study, we synthesized, characterized, and applied the Neu5Aca2-6-glycopolymer with triplet LacNac repeats for influenza virus infection as a virus-entry inhibitor. In vitro and in vivo infection experiments indicated that the core carbohydrate portion, such as the LacNac repeats of receptor sialylglycoconjugates, significantly affects viral recognition of host cells. These experiments also demonstrated the efficacy of α-PGA carrying elongated carbohydrate chains in the prevention of viral infection; a single dose of Neu5Aca2-6-glycopolymer with triplet LacNac repeats significantly protected mice from lethal challenge by A/WSN/33. Alpha-PGA glycopolymer has several advantages: it is highly soluble in water, has very low immunogenicity (Totani et al. 2003), and is highly stable. In addition, this glycopolymer did not show any toxic effects on cell culture in the present study. Taken together, the observations reported here indicate that not only modification of sialyl linkage but also introduction of LacNac residues into the core carbohydrate portion is highly effective for the design and development of virus-entry inhibitors as anti-influenza agents.

Materials and methods

Materials

Alpha-polyglutamic acid (α-PGA) sodium salt (MW 70500) was purchased from Sigma-Aldrich (St. Louis, MO). Bovine milk β-1,4-galactosyltransferase (β4GalT) and α2,6-(N)-sialyltransferase (α2,6-ST, rat recombinant, Spodoptera frugiperda) were purchased from Calbiochem-Novabiochem (San Diego, CA). β-1,3-N-Acetylgalcosaminyltransferase (β3GnT) was prepared as described previously (Kato et al. 2003). CMP-Neu5Ac and UDP-GlcNAc were supplied from Yamasa Corporation. Para-nitrophenyl-β-N-acetyllactosamine (LacNacβ-pNP) was synthesized by our method described previously (Usui et al. 1993; Zeng et al. 2000). All other chemicals were of the highest quality and commercially available.

Viruses

The human, swine, equine, and avian influenza virus strains used in this study were propagated and purified as described previously (Suzuki et al. 1985, 1986).
Synthesis of oligosaccharides containing LacNAc repeats

(LacNAc)\(_2\)β-pNP was synthesized by two glycosyltransferases (Murata et al. 2003). LacNAcβ-pNP (260 mg, 0.52 mmol) and UDP-GlcNAc (672 mg, 1.03 mmol) were dissolved in a 150 mM Tris–HCl buffer, pH 6.8 (34 mL), containing MnCl\(_2\) (82 mg) and 1% (w/v) NaN\(_3\) (0.8 mL), followed by the addition of 340 µM of β3GalT. The mixture was incubated for 48 h at 37°C. UDP-Gal (629 mg, 1.03 mmol) and 2 U of β4GalT were added to the mixture and further incubated for 148 h at 37°C. After sedimentation, the supernatant was loaded onto a Toyopearl HW-40S column pre-equilibrated with MeOH/water (25:75, by vol). The eluate was monitored by measuring the absorbance at 300 nm (ρ-nitrophenyl group). Aliquots from fractions 199–214 were collected. After concentration, samples were loaded onto a Biogel-P2 column and resolved with water. Aliquots from fractions 44–54 were concentrated and lyophilized. (LacNAc)\(_2\)β-pNP was obtained in a total yield of 86% (386 mg) based on the acceptor substrate (LacNAc)\(_2\)β-pNP. (LacNAc)\(_3\)β-pNP was synthesized by the alternative addition of GlcNAc and Gal to (LacNAc)\(_2\)β-pNP in a similar manner. (LacNAc)\(_3\)β-pNP was obtained in a total yield of 75% (120 mg) based on the acceptor substrate (LacNAc)\(_2\)β-pNP. (LacNAc)\(_2\)β-pAP was prepared by our method described previously (Zeng et al. 2000). (LacNAc)\(_3\)β-pAP was obtained in a total yield of 82% (83 mg) based on the acceptor substrate (LacNAc)\(_2\)β-pNP. In a similar manner, (LacNAc)\(_3\)β-pAP was obtained in a total yield of 87% (102 mg) based on the acceptor substrate (LacNAc)\(_3\)β-pNP.

Synthesis of α-PGA carrying LacNAc repeats

The amino functions of LacNAcβ-pAP-repeat oligosaccharide glycosides were coupled with the carboxyl group of α-PGA by a condensation reaction. Degrees of substitution (DS) of carbohydrate residues in glycopolymer were adjusted; the 36–40%. Poly[(LacNAc)\(_2\)β-pAP/Gln-co-Glu] was synthesized as follows. DS values in the glycopolymer were calculated as percentages from the relative intensities of the \(^1\)H-NMR signal areas of phenolic protons, peptide β- and γ-methylene protons, and acetyl protons. Alpha-PGA (6.5 mg) was dissolved in 0.5 mL of dimethyl sulfoxide. Benzo[triazol-1-yl]oxytris(dimethylamino)phosphonium hexafluorophosphate (74.5 mg) and 1-hydroxybenzotriazole hydrate (8.4 mg) were added to the α-PGA solution and stirred for 15 min at room temperature. (LacNAc)\(_2\)β-pAP (52.5 mg) was further added and reacted for 24 h. The reaction mixture was loaded onto a Sephadex G-25M PD-10 column. The high molecular weight fraction (20.3 mg) was collected, dialyzed against distilled water, and then lyophilized.

Sialylation of α-PGA carrying LacNAc repeats

Poly[(LacNAc)\(_3\)β-pAP/Gln-co-Glu] was enzymatically sialylated to obtain Neu5Acα2,6-α-PGAs. A mixture containing Poly[(LacNAc)\(_3\)β-pAP/Gln-co-Glu], 20 mM CMP-α-Neu5Ac, 50 µM/mL of α2,6-ST, 2.5 mM MnCl\(_2\), 0.1% BSA, and 10 U/mL of calf intestine alkaline phosphatase in a 50 mM MOPS buffer (pH 7.4) was incubated at 37°C for 48 h in a total volume of 0.47 mL. The reaction mixtures were loaded onto a Sephadex G-25M PD-10 column. The high molecular weight fraction was collected, dialyzed, and lyophilized.

Focus-forming assay

Virus titers were determined by a focus-forming assay using Madin–Darby canine kidney (MDCK) cells, which were seeded at 1.5 × 10^6 cells/well in 96-well plates and cultured in MEM supplemented with 10% fetal bovine serum at 37°C. After removal of medium, virus solutions serially diluted with serum-free DMEM were inoculated onto the plates, and the cells were incubated for 30 min at 34.5°C. After removing the virus solution, an overlay medium (DMEM containing 0.5% (w/v) tritrancah ym (Wako, Osaka) and 0.2% (w/v) bovine serum albumin) was added, and plates were incubated at 34.5°C for 20–24 h. The cells were fixed with methanol. Infectious foci were detected with mouse anti-nucleaseprotein (NP) monoclonal antibody (ATCC; clone H16L-10-4R55) as the primary antibody and HRP-conjugated goat anti-mouse immunoglobulin as the secondary antibody. Virus infectivity was determined as focus-forming units (FFU).

Inhibition of virus infection by glycopolymer

Influenza viruses were mixed on ice with or without glycopolymer at the indicated concentrations. The virus–glycopolymer mixtures (50 µL) were then inoculated for 30 min at 34.5°C onto MDCK cells grown in 96-well plates. After washing three times with serum-free DMEM, the overlay medium was added and plates were incubated at 34.5°C for 20–24 h. Infectious foci of cells were then visualized by a focus-forming assay as described above and counted under a light microscope. The optimal titer of inoculated virus was predetermined such that more than 80–100 foci appeared per well. Each experiment was performed in triplicate wells.

Plaque assay

The assay measuring human influenza virus infection to MDCK cells was performed as previously described (Suzuki et al. 1983). Viral infection was quantified by counting the plaques formed on the infected cells. Briefly, after viral inoculation, cells were washed twice in serum-free MEM. The cells were then overlaid with serum-free MEM containing 0.8% agarose, 8.3 mg/mL bovine serum albumin, and 10 µg/mL trypsin. Cells were cultured further for 48 h at 34.5°C. The cells were then fixed for 1 h with ethanol/acetic acid (5:1, by vol). Fixed cells were stained with a 0.5% amide black 10B solution. Each experiment was performed in duplicate wells.

Influenza virus infection in mice

Influenza A/WSN/33 virus was used in this study to evaluate glycopolymer antiviral effects. Briefly, groups of six to nine female BALB/c mice (5 weeks old, 15–18 g) were anesthetized with pentobarbital and inoculated intranasally with 50 µL of premixes containing viral suspension (2.0 × 10^6 pfu/mL) and sialyl glycopolymer at the indicated concentrations. Control mice were treated intranasally with 50 µL of viral suspension (2.0 × 10^6 pfu/mL) and nonsialylated glycopolymer at the indicated concentrations. Mice were observed for 17 days after infection. Anti-influenza agent efficacy was assessed by prevention of loss of body weight and protection against lethality caused by influenza virus. The significance of differences in the ratios of survival mice between groups treated with nonsialyl and Neu5Acα2-6glycopolymer was analyzed by the log-rank test (Peto et al. 1977) on Kaplan–Meier survival curves (Kaplan
and Meier 1958). Additional three mice in groups treated with nonsialyl or Neu5Ac2-6glycopolymer were killed on the third day postinfection. Virus titer in the mouse lung was also assessed by a plaque-forming assay as described above. The significance of differences in the means of virus titers in the lung between groups treated with nonsialyl and Neu5Ac2-6glycopolymer was analyzed by the t-test. Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Shizuoka.

**Hemagglutination inhibition (HAI) assay**

A hemagglutination inhibition (HAI) assay was carried out as described previously (Watanabe et al. 1995). Briefly, virus suspension (2° hemagglutination units (HU)) was incubated for 1 h at 4°C with agents serially diluted 2-fold with 0.01% gelatin-containing phosphate-buffered saline (PBS) in 96-well microtiter plates. After the addition of 0.5% (v/v) of guinea pig gelatin-containing phosphate-buffered saline (PBS) in 96-well substrate solution containing 40°FFU, focus-forming unit; HA, hemagglutinin; HAI, hemagglutination inhibition; HAU, hemagglutination unit; IC50, 50% inhibitory concentration; LacNAc, N-acetyllactosamine; MDCK, Madin-Darby canine kidney; NMR, nuclear magnetic resonance; pAP, para-amino phenyl; pNP, para-nitrophenyl; RBS, receptor-binding site; SPR, surface plasmon resonance; ST, sialyltransferase.

**Solid-phase binding assay**

The assay measuring direct binding activity of influenza viruses to glycopolymers was performed by the method described previously (Yamada et al. 2006) with slight modifications. Briefly, glycopolymers were immobilized on polystyrene Universal-BIND microplates (Corning) by ultraviolet irradiation. After blocking with PBS containing 2% bovine serum albumin, the plates were incubated in solutions containing influenza viruses in PBS (2°HAU of A/WSN/33, 2°HAU of A/Aichi/2/68, and 2°HAU of A/Duck/HongKong/313/4/76) at 4°C overnight. After five washes with ice-cold PBS, the plates were incubated in a substrate solution containing 40 μM 2-(4-methylumbelliferyl)-α-D-N-acetyleneuraminic acid in PBS at 37°C for 1 h. The reactions were terminated by the addition of a 500 mM carbonate buffer (pH 10.2). Fluorescence was measured at 355 nm (excitation) and 460 nm (emission). The direct virus-binding activity was determined from the quantity of 4-methylumbelliferon released by viral neuraminidases.

**Sequencing of cRNA of human influenza viruses**

A fragment of the HA gene covering the HAI region (nucleotides 29–1060) was reverse-transcribed and amplified from RNA extracted from the purified viruses using Taq DNA polymerase with forward and reverse primers, 5′-GCAAAAGCAGGGGATAATTTCT-3′ and 5′-TTTCTCTGGATACATTGCCAT-3′, respectively (GenBank accession number M55059). The PCR products were directly sequenced.

**Homology modeling**

The crystal structures of HA from A/Aichi/2/68 (H3N2) (Sauer et al. 1992) were used as templates for simulation of HA binding to SA2,3Lactose (Protein Data Bank (PDB), accession number 1HGG). In homology modeling, HAs of clinical human virus isolates were three-dimensionally aligned via the SWISS-MODEL server (Schwede et al. 2003) using PDB accession number 1HGG. VMD (Humphrey et al. 1996) tools running on UNIX were used to visualize all figures.

**Funding**


**Conflict of interest statement**

None declared.

**Abbreviations**

α-PGA, alpha-poliglutamic acid; DS, degree of substitution; FFU, focus-forming unit; HA, hemagglutinin; HAI, hemagglutination inhibition; HAU, hemagglutination unit; IC50, 50% inhibitory concentration; LacNAc, N-acetyllactosamine; MDCK, Madin-Darby canine kidney; NMR, nuclear magnetic resonance; pAP, para-amino phenyl; pNP, para-nitrophenyl; RBS, receptor-binding site; SPR, surface plasmon resonance; ST, sialyltransferase.

**References**


