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Inhibition of human parainfluenza virus type 1 sialidase by analogs of 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid

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Eleven novel analogs of 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu5Ac2en) modified at the C-4 and C-9 positions were designed and tested for their ability to inhibit sialidase of human parainfluenza virus type 1 (hPIV-1). The analogs modified by the cyanomethyl, amidinomethyl, and thiocarbamoylmethyl groups at the C-4 position exhibited potent inhibition against hPIV-1 sialidase compared with Neu5Ac2en. The most effective compound was thiocarbamoylmethyl analog (4-*O*-thiocarbamoylmethyl-Neu5Ac2en). The activity of 4-*O*-thiocarbamoylmethyl-Neu5Ac2en causing 50% enzyme inhibition at a concentration of approximately 1.0×10^{-5} M was 30-fold larger than Neu5Ac2en. While, the analogs of Neu5Ac2en modified by the azido and *N*-acetyl groups at the C-9 showed a decrease in inhibition of sialidase compared with the 9-hydroxy analogs. In addition, 4-*O*-thiocarbamoylmethyl-Neu5Ac2en strongly inhibited hPIV-1 infections of Lewis lung carcinoma-monkey kidney cells in comparison with Neu5Ac2en. The present findings would provide useful information for the development of anti-human parainfluenza virus compounds.

Keywords: parainfluenza virus, sialic acid, sialidase inhibitor, Neu5Ac2en

Introduction

Human parainfluenza virus type 1 (hPIV-1) which belongs to genus *Respirovirus,* family *Paramyxoviridae*, is an important human pathogen causing upper and lower respiratory disease and is known to be a cause of laryngotracheobronchitis (croup) in infants and young children [1,2]. However, there is no known potential inhibitor against hPIV-1 infection. hPIV-1 has two spike glycoproteins, the hemagglutinin-neuraminidase (HN) glycoprotein and the fusion (F) glycoprotein, embedded in the envelope [3,4]. Paramyxovirus infection is initiated by the attachment of the HN glycoprotein to sialic acid–containing glycolipids and glycoproteins of the target cells [5–7], while the sialidase is important for release of the virus from infected cells [8]. The HN glycoprotein is, therefore, one of the major targets for therapeutics agents. In an early study, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu5Ac2en) and *N*-acyl derivatives had been shown to inhibit Newcastle disease virus (NDV), simian virus 5, and Sendai virus sialidases [9]. It was also recently demonstrated that Neu5Ac2en inhibited not only sialidase activity of hPIV-3 but also cell fusion, and blocked the viral replication in the cell culture [10]. In addition, 2,3-didehydro-2,4-dideoxy-4-guanidino-*N*-acetylneuraminic acid (4-guanidino-Neu5Ac2en), which was designed on the basis of the crystal structure of influenza virus neuraminidase, very powerfully inhibited the sialidase activity and viral replication [11], was shown to inhibit hPIV-3 sialidase [12].

The crystal structures of NDV HN glycoprotein in complex with Neu5Ac2en were determined by X-ray diffraction [13]. The crystallographic information on the structure of NDV HN glycoprotein with Neu5Ac2en showed that the interactions around the carboxy group of Neu5Ac2en were very similar to those seen in other sialidase-Neu5Ac2en complexes and the amino acid residues interacting with C-6, C-7, and C-9 glycerol side chains and the C-5 acetamido group were largely conserved across seven paramyxoviruses including hPIV-1, although, the catalytic pocket had a large cavity in the direction of 4-hydroxy group of Neu5Ac2en compared with influenza virus and bacterial sialidases [13]. These findings indicated that analogs

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of Neu5Ac2en modified at the C-4 are useful for the development of anti-paramyxovirus agents. We recently designed novel Neu5Ac2en analogs modified at the C-4 and C-9 [14]. In the present study, we analyzed the effects of the analogs for hPIV-1 sialidase activity and the viral replication.

Materials and methods

Materials

Viruses

The hPIV-1 strain C35 (ATCC VR-94) was obtained from the American Type Culture Collection (Manassas, VA). The hPIV-1 clinical isolates Cl-5, Cl-11, and Cl-14 were generously donated by Dr. Portner (St. Jude Children's Research Hospital, Memphis, Tennesse). The hPIV-1 Cl-5, Cl-11, and Cl-14 were isolated from infected children in 1973, 1979, and 1983, respectively [15]. Lewis lung carcinoma–monkey kidney (LLC-MK2) cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum. Confluent monolayers of LLC-MK2 cells were infected with the hPIV-1 strain C35 or clinical isolates (approximately 10 PFU per cell) in serum-free MEM containing acetylated trypsin (1 μg/ml). Three days after infection, virions in the culture medium were collected. The virus was purified by sedimentation through 30%–50% sucrose gradients [16,17].

Anti-hPIV-1 antibody

Rabbit anti-hPIV-1 antibody was raised against whole hPIV-1 strain C35 by subcutaneous immunization of rabbits as described previously [18] and purified using the HiTrap Protein G column (Amersham Pharmacia Biotech, Uppsala, Sweden).

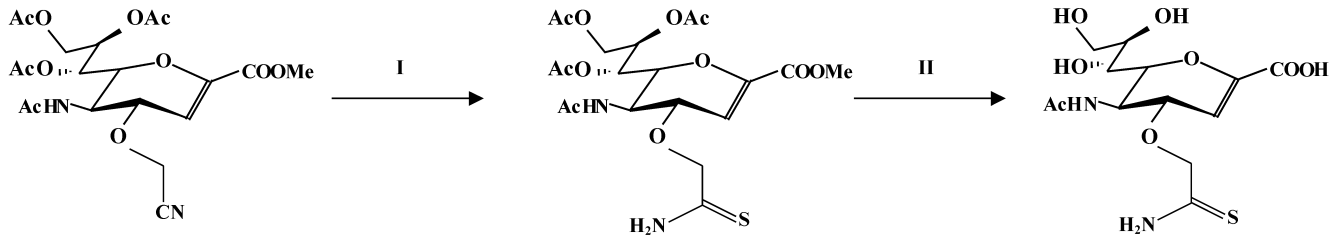
Analogs of Neu5Ac2en

The analogs, except 4-*O*-thiocarbamoylmethyl-Neu5Ac2en, were synthesized as described previously [14]. 5-acetamido-2,6-anhydro-4-*O*-thiocarbamoylmethyl-3,5-dideoxy-D-glycero-D-galacto-non-2-enoic acid (4-*O*-thiocarbamoylmethyl-Neu5Ac2en) was prepared from the derivative of 4-*O*-cyanomethyl-Neu5Ac2en [14] by transformation with AcSH-pyridine and by treatment with KOH-MeOH according to Scheme 1.

To a solution of methyl 5-acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-*O*-cyanomethyl-3,5-dideoxy-D-glycero-D-galacto-non-2-enonate (compound 1) (53 mg, 0.11 mmol) in pyridine (0.5 ml) and CH_2Cl_2 (0.5 ml) was added thioacetic acid (61 mg, 0.55 mmol) at room temperature under Ar, and the reaction mixture was stirred for 12 h. After removal of the solvent, the residue was chromatographed on silica gel using CH_2Cl_2-MeOH (10:1) to give methyl 5-acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-*O*-thiocarbamoylmethyl-3,5-dideoxy-D-glycero-D-galacto-non-2-enonate (compound 2) (52 mg, 94%). ^{1}H-NMR ($CDCl_3$) δ: 1.98–2.12 (m, 12H, AcO and AcN), 3.82 (s, 3H, MeO), 4.38, 4.57 (d, each 1H, *J*gem = 16 Hz, -OCH_2CSN$\underline{H_2}$), 6.12 (d, 1H, J = 3 Hz, H-3).

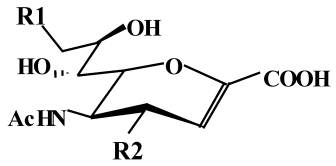
Compound 2 (52 mg) was dissolved in a solution of 0.1 M KOH in MeOH (1:1) (5 ml). The reaction mixture was stirred for 15 h at room temperature and neutralized with Amberlite 120 (H^+). The precipitates were filtered off through Celite 545 and the filtrate was desalted with ASAHI CHEMICAL Micro Acylizer G1 and the resulting aqueous solution was concentrated. The residue was chromatographed on silica gel using $CHCl_3$-MeOH-H_2O (60:60:10) to give 4-*O*-thiocarbamoylmethyl-Neu5Ac2en (compound 3) (19 mg, 52%) as a powder, after lyophilization. ^{1}H-NMR (D_2O):δ 2.11 (s, 3H, AcN), 3.65–3.72 (m, 2H, H-8, H-9a), 3.90–4.01 (m, 2H, H-7, H-9b), 4.47, 4.58 (d, each 1H, J_{gem} = 17 Hz, -OC$\underline{H_2}$CSNH_2), and 5.82 (d, 1H, J = 2 Hz, H-3). Positive f.a.b.-m.s. (NBA): $(M+Na)^+$ 387 m/z.

^{1}H NMR spectra were recorded with a Jeol EX 270 (Japan). Chemical shifts are expressed in ppm relative to Me4Si (δ = 0) in $CDCl_3$ and in D_2O referenced to HOD (4.85 ppm) as an internal standard. Fast atom bombardment (FAB) mass spectra were obtained with a Jeol SX-102 (Japan) mass



I) AcSH-pyridine
II) 0.1 M KOH-MeOH

Scheme 1.



Neu5Ac2en: R1=OH, R2=OH

4-guanidino-Neu5Ac2en: R1=OH, R2=NHC(=NH)NH_2

1a: R1=OH, R2=$OCH_2C(=NH)NH_2$

1b: R1=OH, R2= $OCH_2C(=O)NH_2$

1c: R1=OH, R2=$OCH_2C(=S)NH_2$

1d: R1=OH, R2=OCH_2CN

2a: R1=NHAc, R2=$OCH_2C(=NH)NH_2$

2b: R1=NHAc, R2=$OCH_2C(=O)NH_2$

2c: R1=NHAc, R2=$OCH_2C(=S)NH_2$

2d: R1=NHAc, R2=OCH_2CN

3a: R1=N_3, R2=$OCH_2C(=NH)NH_2$

3b: R1= N_3, R2=$OCH_2C(=O)NH_2$

3c: R1=N_3, R2=$OCH_2C(=NH)OCH_3$

Figure 1. Structures of the analogs of Neu5Ac2en. The analogs of Neu5Ac2en (1a–3b) were synthesized as described under Materials and methods. Neu5Ac2en, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid; 1a, 4-*O*-amidinomethyl-Neu5Ac2en; 1b, 4-*O*-carbamoylmethyl-Neu5Ac2en; 1c, 4-*O*-thiocarbamoylmethyl-Neu5Ac2en; 1d, 4-*O*-cyanomethyl-Neu5Ac2en; 2a, 9-acetamido-4-*O*-amidinomethyl-Neu5Ac2en; 2b, 9-acetamido-4-*O*-carbamoylmethyl-Neu5Ac2en; 2c, 9-acetamido-4-*O*-thiocarbamoylmethyl-Neu5Ac2en; 2d, 9-acetamido-4-*O*-cyanomethyl-Neu5Ac2en; 3a, 9-azido-4-*O*-amidinomethyl-Neu5Ac2en; 3b, 9-azido-4-*O*-carbamoylmethyl-Neu5Ac2en; 3c, 9-azido-4-*O*-methoxy-iminomethyl-Neu5Ac2en.

spectrometer in the positive ion mode using NBA matrix. Column chromatography was performed on Silica Gel Merck 60 (70–230 mesh). All reactions were monitored by TLC (Silica Gel 60-F_{254}, E. Merck, Germany) by charring after spraying with 5% H_2SO_4 in MeOH and then heating.

4-guanidino-Neu5Ac2en was supplied by Glaxo Wellcome Research and Development, Stevenage, UK. The structures of the analogs used in the present study are shown in Figure 1.

Methods

Sialidase inhibition assay

Sialidase activity was assayed by the fluorometric assay using 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (Sigma–Aldrich Japan, Japan). hPIV-1 was diluted with 0.9% NaCl solution. The analogs of Neu5Ac2en were diluted with 0.1 M acetate buffer (pH 4.6). Five microliters of the virus suspension (protein 2 μg/μl) was incubated with 5 μl of each Neu5Ac2en analog dilution (0.001–2.5 mM) for 30 min at 37°C. After addition of 10 μl of 4 mM 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (4MU-Neu5Ac) in 0.1 M acetate buffer (pH 4.6), the mixture was incubated for 30 min at 37°C. The reaction was stopped by adding 500 μl of carbonate buffer (pH 10.8). The fluorescence of the released 4-methylumbelliferone was measured using a fluorescence spectrophotometer (Hitachi F4010, Japan) with excitation at 355 nm and emission at 460 nm. As controls, Neu5Ac2en and 4-guanidino-Neu5Ac2en were used instead of the analogs. The concentration causing 50% inhibition of the sialidase activity was determined by plotting the percentage inhibition against the concentration of each analog. Assays were determined in duplicate.

Inhibition of hPIV-1 infection by 4-*O*-thiocarbamoylmethyl-Neu5Ac2en

Confluent monolayers of LLC-MK2 cells (1.9 cm^2) in 24-well plates (Corning Costar Corporation, Cambridge, MA) were inoculated with 200 μl of hPIV-1 (0.2 μg/ml) at room temperature in MEM with discontinuous swinging in the presence of increasing concentrations of 4-*O*-thiocarbamoylmethyl-Neu5Ac2en (0.2–20 mM). After 1 h, the monolayers were incubated for one day at 34°C in 0.5 ml of MEM containing 0.1% BSA and 5 μg acetylated trypsin/ml, without removal of the inoculum. The monolayers in each well were washed three times with PBS, fixed with 1 ml of methanol at room temperature for 5 min, and washed three more times with PBS. Anti-hPIV-1 rabbit antibody diluted 1:500 with 200 μl of PBS containing 0.5% BSA and 0.05% Tween 20 (blocking solution 3) were added to wells. After incubation at room temperature for 30 min, antibody solution was removed by suction. The wells were washed three times with PBS and incubated at room temperature for 30 min with horseradish peroxidase–conjugated Protein A diluted 1:3,000 with blocking solution 3. After the plates were washed three times with PBS, the viral antigen-positive cells in each well were detected by incubation with 0.5 ml of immunostaining reagent [18,19] containing *N,N*-diethylphenylene-diamine monohydrochloride and 4-chloro-1-naphthol. The wells were washed three times with PBS. Mock-infected LLC-MK2 cells were fixed and stained as negative controls. Infectious cells were defined as the mean of three counts of blue-stained cells within an area of 3.8 mm^2. For counting purposes, the cells were magnified × 200 using an inverted microscope (IMT-2; OLYMPUS, Tokyo, Japan). As a control, the inhibition activity of Neu5Ac2en was examined in the same manner.

Results and discussion

Inhibition of hPIV-1 sialidase by Neu5Ac2en analogs

The crystallographic information on the structure of NDV HN glycoprotein with Neu5Ac2en suggested that the catalytic pocket of hPIV-1 HN glycoprotein also has a large cavity in

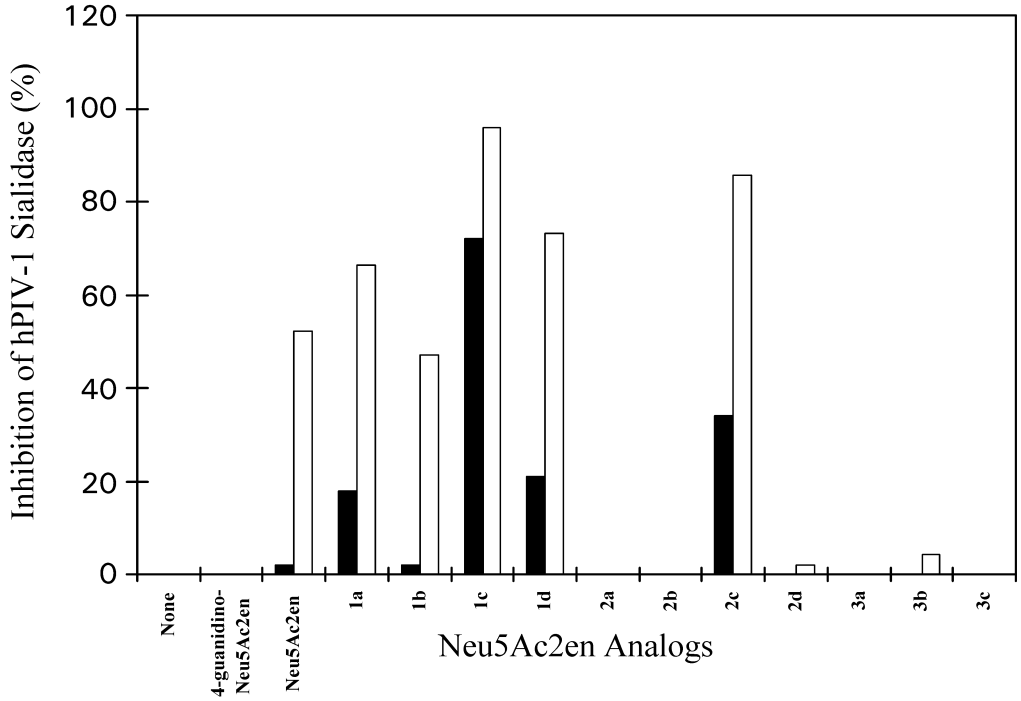


Figure 2. Inhibition of hPIV-1 sialidase activity by the analogs of Neu5Ac2en. hPIV-1 strain C35 suspension was incubated with 4MU-Neu5Ac in presence of each Neu5Ac2en analog dilution at the concentration of 25 μM (closed column) and 250 μM (open column) for 30 min at 37°C. After incubation, 4-methylumbelliferone released from 4MU-Neu5Ac was assayed by the fluorometric assay as described in Materials and methods. Values are expressed as a percentage inhibition of the enzyme activity. The columns are the means for duplicate experiments.

the direction of 4-hydroxy group of Neu5Ac2en [13]. We, therefore, preliminarily tested for eleven new analogs of Neu5Ac2en modified at the C-4 and C-9 positions (Figure 1) to inhibit sialidase of the hPIV-1 strain C35 at 25 and 250 μM by the fluorometric assay using 4MU-Neu5Ac (Figure 2). The amount of substrate hydrolyzed by the hPIV-1 sialidase was in direct proportion to the incubation time (0–60 min) under the conditions tested without the analogs (data not shown) and so inhibition of the sialidase activity by the analogs was determined 30 min after the addition of the substrate. The 9-hydroxy-Neu5Ac2en analogs modified by the amidinomethyl, thiocarbamoylmethyl, and cyanomethyl groups at the C-4 (Figure 1, **1a**, **1c**, and **1d**) and 9-acetamido-4-*O*-thiocarbamoylmethyl-Neu5Ac2en (Figure 1, **2c**) exhibited potent inhibition toward hPIV-1 sialidase compared with Neu5Ac2en. To estimate the order of magnitude of the activity for the analogs, the concentration causing 50% inhibition of the sialidase activity (IC_{50}) of the four substitutions were determined by plotting percentage inhibition against the concentration of each analog. The most effective compound against the sialidase was 4-*O*-thiocarbamoylmethyl-Neu5Ac2en (IC_{50} of 1.0×10^{-5} M) which was about 30-fold higher affinity compared with Neu5Ac2en (Table 1). While the analogs of Neu5Ac2en modified by the *N*-acetyl group at the C-9 showed a decrease in inhibition of the sialidase compared with the 9-hydroxy analogs (Figure 2).

In a previous study, the analogs of 4-nitrogen substitutive Neu5Ac2en containing 4-guanidino-Neu5Ac2en were designed and their inhibition for human parainfluenza virus type 2 (hPIV-2) was tested. 4-guanidino-Neu5Ac2en showed a less inhibitory effect than Neu5Ac2en [20]. In the present study, 4-guanidino-Neu5Ac2en had much lower inhibitory activity toward hPIV-1 sialidase in the conditions tested. The inhibition of 4-guanidino-Neu5Ac2en for hPIV-1 was also weaker than Neu5Ac2en in addition to hPIV-2.

Table 1. Inhibition (IC_{50}) against hPIV-1 sialidase for Neu5Ac2en, 9-acetamido-4-*O*-thiocarbamoylmethyl-Neu5Ac-2en, and the 9-hydroxy-Neu5Ac2en analogs modified by the amidinomethyl, thiocarbamoylmethyl, and cyanomethyl groups

Compound	IC_{50} (10^{-5} M)
Neu5Ac2en	30
9-acetamido-4-*O*-thiocarbamoylmethyl-Neu5Ac2en	4.2
4-*O*-amidinomethyl-Neu5Ac2en	10
4-*O*-thiocarbamoylmethyl-Neu5Ac2en	1.0
4-*O*-cyanomethyl-Neu5Ac2en	6.0

The cocrystal structure of the globular head region of NDV HN glycoprotein with Neu5Ac2en showed that the interactions around the carboxy group of Neu5Ac2en were very similar to those seen in other NA-Neu5Ac2en complexes and the amino acid residues interacting with the C-6, C-7, and C-9 glycerol side chains and the C-5 acetamido group were largely conserved across seven paramyxoviruses including hPIV-1, hPIV-2, and hPIV-3. However, the 4-hydroxy group of Neu5Ac2en made no interactions with the amino acid residues of the HN glycoprotein and fronted on a large cavity lined with several invariant residues that form a hydrogen bond network [13]. These structural findings indicated that modification at the C-4 position of Neu5Ac2en, to fit on the large cavity of the HN glycoprotein has important effects on the design of potent inhibitors against sialidases from human parainfluenza viruses. 4-*O*-thiocarbamoylmethyl-Neu5Ac2en may fit on the cavity of the hPIV-1 HN glycoprotein in comparison with the other analogs tested. The property of the carbamoylmethyl group accurately approximates the thiocarbamoylmethyl group, however, the modification of carbamoylmethyl group showed a minor effect in the degree of the inhibition in comparison to the thiocarbamoylmethyl group (Figure 2). The bulkiness of the group may effect the interaction with the cavity.

4-guanidino-Neu5Ac2en (IC_{50} of 2.5×10^{-4} M) for hPIV-3 sialidase exhibited stronger inhibition compared with Neu5Ac2en (2.1×10^{-3} M) [12]. While 4-guanidino-Neu5Ac2en showed lower inhibitory activity toward hPIV-1 sialidase. An earlier study of the kinetic parameters of hPIV-3 sialidase with fluorometric assay reported that there was little difference between the Michaelis-Menten constants and the catalytic rate of hPIV-3 sialidase for the substrate (4MU-Neu5Ac) compared with *C. perfringens* enzyme [21]. In addition, Neu5Ac2en had exhibited K_i values and IC_{50} values in the range from 10^{-6} to 10^{-5} M for bacterial enzymes from various sources including *C. perfringens* and hPIV-2 sialidase [9,20–23]. These reports suggest that the difference in the affinity of Neu5Ac2en for hPIV-2 and hPIV-3 sialidases is not due to the difference in kinetic parameters in the reactions of 4MU-Neu5Ac with each virus sialidase. We examined whether 4-*O*-thiocarbamoylmethyl-Neu5Ac2en is a potent inhibitor for hPIV-3 sialidase, however, the analog had no significant effect compared with that of 4-guanidino-Neu5Ac2en and Neu5Ac-
2en (data not shown). The present findings suggest that hPIV-3 has a different microsphere that interacts with the C-4 position of Neu5Ac2en in the cavity of the catalytic pocket in the HN glycoprotein. The inhibition of Neu5Ac2en for hPIV-1 sialidase was decreased by modification of the C-9 position. The structural findings of the NDV HN glycoprotein indicates that the interaction with all three hydroxy groups of the C-6, C-7, and C-9 glycerol side chains is a distinctive feature of paramyxovirus HN glycoproteins and an important determinant of sialic acid recognition. Therefore, the modification of hydroxy groups of the glycerol side chain appears to have decreased the affinity of analogs for hPIV-1.

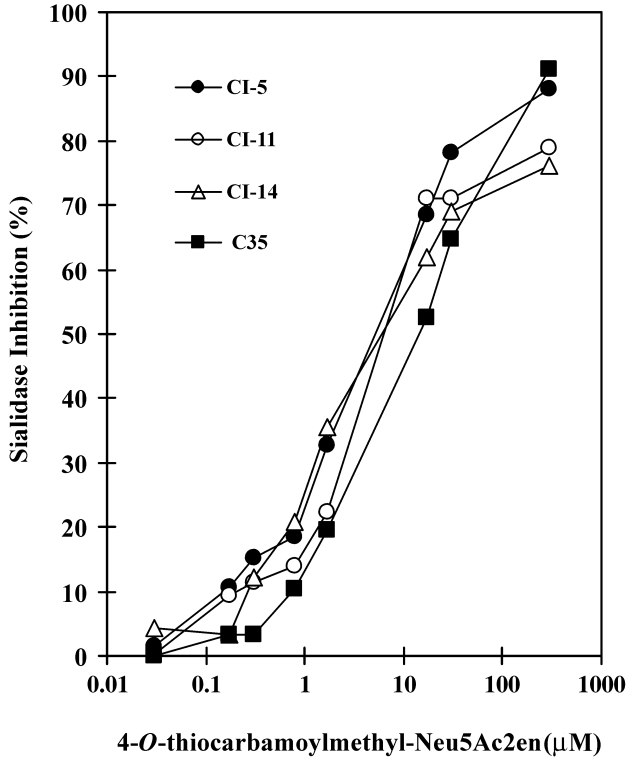


Figure 3. Inhibition of sialidases of hPIV-1 clinical isolates by 4-*O*-thiocarbamoylmethyl-Neu5Ac2en. Each hPIV-1 clinical isolate was incubated with 4-*O*-thiocarbamoylmethyl-Neu5Ac2en solution (0.001–2.5 mM) for 30 min at 37°C and their sialidase activities were assayed by the fluorometric assay. hPIV-1 strain C35 served as the control. Values are expressed as the percentage inhibition of the enzyme activity in duplicate.

Inhibition of sialidases of hPIV-1 clinical isolates by 4-*O*-thiocarbamoylmethyl-Neu5Ac2en

To clarify whether marked inhibition of 4-*O*-thiocarbamoylmethyl-Neu5Ac2en for the hPIV-1 strain C35 is a general property of hPIV-1 sialidase, we examined the inhibitory activity of 4-*O*-thiocarbamoylmethyl-Neu5Ac2en towards the hPIV-1 strains isolated from infected patients during different years. The analog showed a similar inhibition against all of the isolates as the C35 strain (Figure 3). This finding suggests that 4-*O*-thiocarbamoylmethyl-Neu5Ac2en is a universal inhibitor for hPIV-1 sialidase independent of the isolates.

Inhibition of hPIV-1 infection by 4-*O*-thiocarbamoylmethyl-Neu5Ac2en

We verified the ability of 4-*O*-thiocarbamoylmethyl-Neu5Ac2en to neutralize hPIV-1 infection. The hPIV-1 strain C35 was incubated with the analog in the infection to LLC-MK2 cells. The number of infected cells was scored as a percentage of viral antigen-positive cells without the analog (Figure 4). 4-*O*-thiocarbamoylmethyl-Neu5Ac2en inhibited hPIV-1 infection in a dose-dependent manner. The activity of 4-*O*-thiocarbamoylmethyl-Neu5Ac2en causing 50% inhibition of

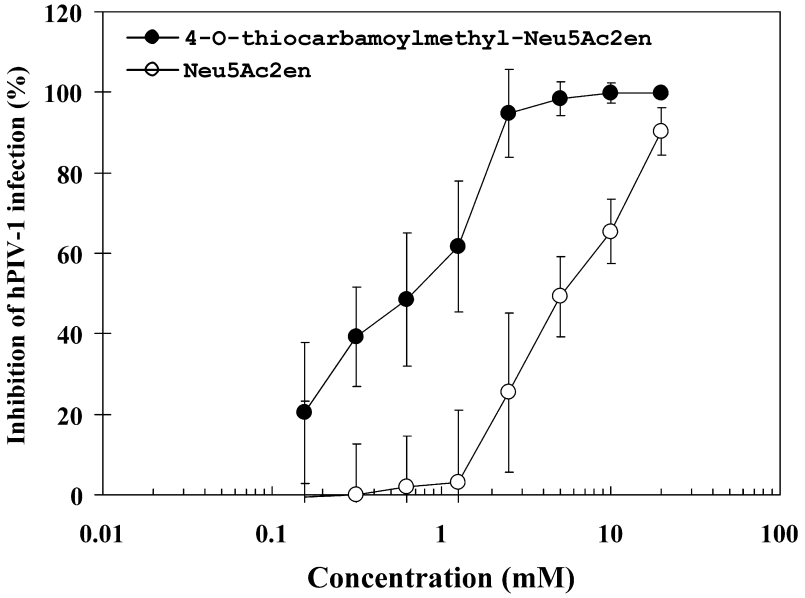


Figure 4. Inhibition of hPIV-1 infection to LLC-MK2 cells by 4-*O*-thiocarbamoylmethyl-Neu5Ac2en. LLC-MK2 cells were infected with hPIV-1 strain C35 in the presence of 4-*O*-thiocarbamoylmethyl-Neu5Ac2en (0.2–20 mM) as described in Materials and methods. The inhibition of hPIV-1 infection is expressed as a percentage relative to the total number of the viral antigen-positive cells without the analog. The values are the mean ± SD of three measurements. Neu5Ac2en served as the control.

virus-infected cells at a concentration of approximately 0.5×10^{-4} M was 10-fold larger than Neu5Ac2en.

The study of the high and low pH crystal structures of NDV HN glycoprotein indicated that the catalytic site may provide both sialic acid binding and hydrolysis activity by a conformational switch [13]. Our recent studies showed that hPIV-1 preferentially binds to neolacto-series gangliosides containing branched *N*-acetyllactosaminoglycans with terminal Neu5Acα2-3Gal but no other ganglio-series gangliosides are bound by the virus [24]. We therefore examined whether 4-*O*-thiocarbamoylmethyl-Neu5Ac2en inhibits hemagglutination by hPIV-1, however a concentration of 20 mM 4-*O*-thiocarbamoylmethyl-Neu5Ac2en, which apparently inhibited hPIV-1 infection, did not prevent agglutination of guinea pig erythrocytes by hPIV-1 (data not shown). The results suggest that the primary mechanism of the antiviral activity by 4-*O*-thiocarbamoylmethyl-Neu5Ac2en is inhibition of the sialidase activity. The present findings would provide useful information for the development of anti-human parainfluenza virus compounds.

Acknowledgments

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