Published in final edited form as:
Virology. 2011 June 20; 415(1): 12–19. doi:10.1016/j.virol.2011.03.024.

High-throughput neuraminidase substrate specificity study of human and avian influenza A viruses

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Abstract

Despite the importance of neuraminidase (NA) activity in effective infection by influenza A viruses, limited information exists about the differences of substrate preferences of viral neuraminidases from different hosts or from different strains. Using a high-throughput screening format and a library of twenty α2–3- or α2–6-linked *para*-nitrophenol-tagged sialylgalactosides, substrate specificity of NAs on thirty-seven strains of human and avian influenza A viruses was studied using intact viral particles. Neuraminidases of all viruses tested cleaved both α2–3- and α2–6-linked sialosides but preferred α2–3-linked ones and the activity was dependent on the terminal sialic acid structure. In contrast to NAs of other subtypes of influenza A viruses which did not cleave 2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid (Kdn) or 5-deoxy Kdn (5d–Kdn), NAs of all N7 subtype viruses tested had noticeable hydrolytic activities on α2–3-linked sialosides containing Kdn or 5d–Kdn. Additionally, group 1 NAs showed efficient activity in cleaving *N*-azidoacetylneuraminic acid from α2 –3-linked sialoside.

Keywords

Carbohydrate; Influenza A virus; Inhibitors; Neuraminidase; Sialic acid; Sialosides; Substrate specificity studies

Introduction

Human influenza A virus is an important pathogen that causes an acute viral disease of the respiratory tract in millions of people each year. Avian influenza A virus outbreaks have caused major losses for the poultry industry (Spicuzza et al., 2007), but can also cause

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deadly diseases (e.g. H5N1) as well as less severe infections in humans (Swayne and Halvorson, 2008). The 2009 pandemic by an influenza A virus strain of mixed avian and swine origins have stimulated renewed interest into emerging influenza virus strains.

Influenza A virions contain two types of surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), both recognizing sialic acid-containing receptors on the host cell surface. HAs bind to cell surface sialyloligosaccharides and mediate the entry of the virus into the cell (Wiley and Skehel, 1987). NAs are believed to prevent the aggregation of progeny virions by removing sialic acid from the carbohydrate moieties of newly synthesized HA and NA glycopolypeptides (Palese et al., 1974) and facilitate the spreading of progeny virions by removal of sialic acid from the glycoconjugates on the surface of infected host cells (von Itzstein, 2007). Recent studies found that in addition to its previously recognized functions, NA plays a direct and early role in influenza A virus entry into host target cells. The presence of NA significantly enhanced both pseudotype infectivity and cell-cell fusion (Su et al., 2009). Therefore, understanding the substrate specificity of influenza A virus NAs is critical for preventing direct transmission of virus from avian or other species to human and for developing new potent NA inhibitors.

Neuraminidases (EC 3.2.1.18) are sialoside hydrolyzing enzymes which cleave the glycosidic linkages between the terminal sialic acid and an adjacent sugar residue. To date, studies on the substrate specificity of influenza A virus neuraminidases are limited to a small number of influenza strains and a restricted set of sialosides. The NAs of N2 influenza A viruses isolated from different hosts were shown to differ in their ability to distinguish α2–3-linked and α2–6-linked sialyl lactose (Baum and Paulson, 1991; Couceiro and Baum, 1994; Franca de Barros et al., 2003; Kobasa et al., 1999). The recognition of underlying glycan structures of sialosides by human influenza A viruses has been observed (Katinger et al., 2004). More recently, the substrate specificity of the neuraminidases of H1N1 influenza A virus and avian-human reassortant influenza A viruses were studied using six sialosides, including BODIPY-labeled α2–3-linked sialyl lactose, α2–3-linked sialyl *N*-acetyllactosamine, sialyl Lewis c, sialyl Lewis a, α2–6-linked sialyl lactose, and α2–6-linked sialyl *N*-acetyllactosamine (Mochalova et al., 2007; Shtyrya et al., 2009). Most NAs used for these substrate specificity studies were either recombinant enzymes or expressed by purified viruses.

Here, we report on a substrate specificity study of NAs on thirty-seven human and avian influenza A viruses using gradient purified and/or non-purified viral particles conducted by employing a high-throughput screening format using a library of twenty α2–3- or α2–6-linked *para*-nitrophenol-tagged sialyl galactosides Siaα2–3/6Galβ*p*NP synthesized by a highly efficient one-pot three-enzyme system (Cao et al., 2009).This proof-of-principle study demonstrates that the obtained information can be used to design novel neuraminidase inhibitors that target a select group of NAs on influenza A viruses.

Results

Viruses analysis

As shown in Table 1, thirty-seven viral strains were used in this study. Among these viral strains, twenty-two were avian-origin influenza A viruses isolated from free-flying wild birds or domestic captive birds in different locations of California and at various times (1996, 1998, 2006, 2005, 2007, 2008) with different hemagglutinin (belonging to group 1 hemagglutinin H1, H5, H6, H12 or group 2 hemagglutinin H3, H4, H10) and neuraminidase subtypes (belonging to group 1 neuraminidase N1, N5, N8 or group 2 neuraminidase N2, N6, N7, and N9); ten were avian H9N2 subtype influenza A viruses either isolated from domestic birds in Hong Kong in different years (1978, 1979, 1988, 1993, 1997, 1998, 1999)

or isolated from duck in Hong Kong in 1979 and adapted to quail or chicken; one was a human H9N2 influenza A virus HK/2108/03 isolated in 2003; and four were human influenza A viruses belonging to H1N1, H3N1 and H3N2. Among human influenza A viruses, A/Memphis71 is a reassortant carrying the hemagglutinin of A/Memphis/1/71 (H3) and the neuraminidase of A/Bellamy/42 (N1). H9N2 avian influenza A viruses from birds with demonstrated adaptation to mammals (Wan et al., 2008; Xing et al., 2011) were classified into a separate group in order to test the effect of the adaptation and interspecies transmission of viruses from avian to mammalian species on the substrate specificity of neuraminidase.

Substrate specificity of neuraminidases from avian and H9N2 influenza viruses

Using twenty α2–3- and α2–6-linked sialyl galactosides including Siaα2–3/6Galβ*p*NP containing *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), 2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid (Kdn), or their C5-derivatives (Table 2) (Cao et al., 2009), a substrate specificity study of neuraminidases from different strains of viruses all normalized to the same HA-titer shows important similarities and differences (Table 3 and Fig. S1)

All influenza A viruses tested have relatively good sialic acid cleavage activities towards α2– 3-linked sialosides Neu5Acα2–3Galβ*p*NP (compound **1a**), Neu5AcFα2–3Galβ*p*NP (compound **2a**), and Neu5Gcα2–3Galβ*p*NP (compound **5a**) although some viruses [A/mallard/California/8035/2008 (H5N2), A/green winged teal/California/8204/2008 (H5N9), A/Quail/Hong Kong/A28945/1988 (H9N2), A/Duck/Hong Kong/Y280/1997 (H9N2), A/Chicken/Hong Kong/G9/1997 (H9N2)] have weaker neuraminidase activities than others as shown by different absorption maxima indicated in the graphs (Fig. S1). All viruses tested have higher activity towards α2–3-linked sialosides (compounds **1a–10a**) than α2–6-linked sialosides (compounds **1b**-**10b**). Neu5Acα2–3Galβ*p*NP (compound **1a**) is the best substrate for all viruses tested. In contrast, α2–3- or α2–6-linked sialosides containing C5-OMe modified Neu5Ac (compounds **3a** and **3b**) and C5-F, -OMe, or $-N_3$ modified Kdn (compounds **7a–9a, 7b–9b**) are not effectively cleaved by any of the viral neuraminidases tested here. Among α2–6-linked sialosides tested (compounds **1b–10b**), only the ones containing Neu5Ac or Neu5AcF (compound **1b** and **2b**) are suitable but weak substrates for the viral neuraminidases (Fig.S1).

Despite the similarities described above, the viruses showed different neuraminidase activities to the modifications on the C-5 hydroxyl group of sialic acid in the sialoside substrates. For example, α2–3-linked sialoside containing Neu5AcN_3 (compound **4a**), an azido-modified Neu5Ac, is a good substrate for some viral neuraminidases tested (N1, N5, most N7, and some N2) but not others (most N2, some N7, and N9). Many N7-expressing influenza A viruses cleaved α2–3-linked sialosides containing either Kdn (compound **10a**) or its 5-deoxy derivative (5d–Kdn) (compound **6a**), a unique substrate specificity not shared by the other strains tested.

H9N2 avian influenza A viruses continue to circulate worldwide. In Asia, H9N2 viruses have caused disease outbreaks and established lineages in land-based poultry (Aamir et al., 2007; Alexander, 2007; Guo et al., 1999; Kim et al., 2006; Naeem et al., 1999; Nili and Asasi, 2003). Importantly, H9N2 viruses have occasionally transmitted from land-based poultry to mammals, including humans. Mild respiratory diseases of humans were reported in Hong Kong and mainland China in 1999 and again in Hong Kong in 2003 (Butt et al., 2005; Lin et al., 2000; Peiris et al., 2001). We analyzed a series of H9N2 influenza A viruses isolated from ducks, quail, chicken, pheasant, and humans in Hong Kong over a period of 25 years (1978–2003) and two laboratory-adapted viruses (QA23 and Qa23CkA10) (Table 1). This provided an excellent opportunity to correlate possible changes of neuraminidase

substrate specificity with interspecies transmission and avian to human adaptation (Wan et al., 2008). Similar to other N2 influenza A viruses (Fig. S1B), α2–3- and α2–6-linked sialosides containing Neu5Ac or Neu5AcF (compounds **1a, 1b, 2a**, and **2b**), and α2–3-linked sialoside containing Neu5Gc (compound **5a**) were proven to be good substrates. However, the α2–3-linked sialoside containing $Neu5AcN_3$ (compound **4a**) was a weak substrate for some N2 strains such as dk/702/79 and Q/29209-1/93 (Fig. S1F) but not a substrate for others. No other compounds were substrates for these viruses. Significantly, there were noticeable differences between the overall sialidase activity and the relative ratio of α2–6-sialidase versus α2–3-sialidase activities among H9N2 strains isolated from different species and different times. The laboratory adapted H9N2 viruses showed lower relative α2–6-sialidase activity while the human H9N2 virus showed higher relative α2–6-sialidase activity (Fig. S1F).

Substrate specificities of neuraminidases from human influenza A viruses

A similar pattern of substrate specificity was observed for both sucrose-gradient-purified (Fig. S2A) and non-purified human influenza A viruses (Fig. S2B) (Table 3). The latter were used in form of virus-containing allantoic fluid. The purification process did, however, seem to decrease the neuraminidase activity of the purified viruses, requiring the use of higher viral amounts in the assays. Similar to the avian influenza A viruses tested, the neuraminidases from human viruses cleaved α2–3-linked sialosides with higher efficiency than α2–6-linked sialosides. All four human viruses cleaved Neu5Ac and Neu5AcF efficiently from α2–3- and α2–6-linked sialosides (compounds **1a, 1b, 2a**, and **2b**). α2–3-Linked sialoside containing Neu5Gc (compound **5a**) is also a suitable substrate for these human virus strains. In contrast, α2–3-linked sialoside containing $Neu5AcN_3$ ($Neu5AcN_3$α2–3Galβ*p*NP, compounds **4a**) is a suitable substrate for A/PR8 and A/Mem71 (N1 type), but not for either A/Udorn72 or A/Philippines (N2 type). This result was consist with the NA activity of avian influenza A viruses described above. The human influenza A viruses tested were unable to cleave α2–3/6-linked sialosides containing Neu5AcOMe, Kdn, or Kdn derivatives (compounds **3a, 3b, 6a–10a**, and **6b–10b**). Overall, the sialic acid hydrolysis activities of human influenza A viruses were lower than those of avian influenza strains, as higher titers of human viruses (500–10,000 HAU mL^{-1}) had to be used to achieve similar hydrolysis efficiency to avian viruses (12.8 HAU mL^{-1}).

Inhibition studies of viral neuraminidases

A sialidase transition state analog, Neu5Ac2en, is a nonselective inhibitor against sialidases, including influenza A virus NAs. To test the inhibitory activity of the non-selective inhibitor Neu5Ac2en, we synthesized it and its C5-derivatives Neu5Ac2en derivatives 2-deoxy-2,3-dehydro-*N*-glycolylneuraminic acid (Neu5Gc2en), 2-deoxy-2,3-dehydro-*N*-azidoacetylneuraminic acid ($Neu5AcN_3$2en), and 2-deoxy-2,3-dehydro-Kdn (Kdn2en) (Li et al., 2011). These compounds were tested against NAs from different viruses, using a microtiter-plate based inhibition assay (Li et al., 2011) and their activities were compared to that of a commercially available influenza A virus NA inhibitor Zanamivir (a C4-guanadino derivative of Neu5Ac2en). As shown in Table 4, when Neu5Acα2–3Galβ*p*NP (compound **1a**) was used as the neuraminidase substrate for the inhibition assays, the IC_{50}s of Neu5Ac2en were in the range of 2.6–48.3 μM for nine avian influenza A viruses with four NA subtypes and two human influenza A viruses with different NA subtypes. The inhibitory activity of Neu5Ac2en was the least efficient for avian influenza A viruses Q/29209-1/93 (IC_{50} = 48.3±5.8 μM) and mal/6957/08 (IC_{50} = 23.2±1.5 μM) and most efficient for avian influenza A viruses mal/8212/08 (IC_{50} = 3.8±0.4 μM) and wi/HKWF541/07 (IC_{50} = 1.8±0.5 μM), as well as human A/PR8 (IC_{50} = 2.6±0.5 μM). In comparison, commercial influenza virus specific inhibitor Zanamivir has IC_{50}s in a range of 3.3–94.4 nM, indicating that Zanamivir is about three orders of magnitude more efficient than Neu5Ac2en. The Neu5Ac

cleavage activities of Neu5Acα2–3Galβ*p*NP by neuraminidases of human virus A/PR8 (IC_{50} = 3.3±0.2 nM) and avian viruses mal/8212/08 (IC_{50} = 6.5±0.3 nM) and Qa23CkA10 (IC_{50} = 9.5±1.6 nM) were more susceptible to Zanamivir inhibition than other viruses tested, especially avian viruses Q/29209-1/93 (IC_{50} = 59.9±3.6 nM) and mal/6957/08 (IC50 = 49.9±3.1 nM) as well as human virus A/Udorn72 (IC_{50} = 94.4±6.2 nM). When Neu5Acα2–6Galβ*p*NP (compound **1b**) was used as the neuraminidase substrate for some viruses which have significant α2–6-sialidase activity, the IC_{50}s of Neu5Ac2en were in sub micromolar range (1.8–11.3 μM) and those of Zanamivir were in the range of 3.2–78.8 nM.

Because of the differences of virus NAs in their effectiveness of cleaving sialosides containing different C5-modified sialic acids, we hypothesized that C5-derivatives of the nonselective sialidase inhibitor Neu5Ac2en might act as selective inhibitors against NAs of certain influenza A strains. To test this hypothesis, the inhibitory activities of three C5-analogs of Neu5Ac2en: Neu5Gc2en, $Neu5AcN_3$2en, and Kdn2en, were tested against nine non-purified avian influenza A viruses and the two purified human influenza A viruses. As shown in Table 5, these three compounds were less potent inhibitors compared to Neu5Ac2en. Except for A/PR8 whose IC_{50} of Neu5Gc2en was around 100 μM, the IC_{50}s of Neu5Gc2en for the other virus NAs were >100 μM. Furthermore, for all virus NAs tested, the IC_{50}s of $Neu5AcN_3$2en were > 1 mM and those of Kdn2en were > 10 mM. The higher IC_{50} values obtained for Neu5Gc2en (IC_{50} > 100 μM), $Neu5AcN_3$2en (IC_{50} > 1 mM), and especially Kdn2en (IC_{50} > 10 mM) agreed well with the observation that sialosides containing Neu5Gc, $Neu5AcN_3$, and Kdn were less efficient substrates than Neu5Ac-containing sialosides for influenza A viruses tested here (Fig. S1 and Fig. S2) .

Discussion

Avian influenza A virus NAs have relatively high activity and exhibit substrate preference for α2–3-sialylated glycan substrates, while human virus NAs have lower activity and cleave both α2–3- and α2–6-sialylated glycans. Preferences between avian and human NAs differ also for the internal glycan structures of the sialoside substrates (Shtyrya et al., 2009). The binding of HAs (i.e. ligand specificity) to host cells needs to match the activity of NAs (i.e. substrate specificity) to achieve efficient viral infection and replication (Mitnaul et al., 2000; Shtyrya et al., 2009; Wagner et al., 2000). Despite the existence of more than 50 different natural sialic acid forms (Angata and Varki, 2002; Chen and Varki, 2010; Schauer, 2000), little information exists about the ligand preferences of HAs, or the substrate specificities of NAs on human and avian influenza A viruses with regard to the terminal sialic acid structures of sialosides.

Some similarities were identified among the NAs of all tested avian and human influenza A viruses. First, α2–3-linked sialosides were better substrates than α2–6-linked sialosides. Second, among 20 sialyl galactosides tested, Neu5Acα2–3Galβ*p*NP (compound **1a**) containing the most abundant sialic acid form, Neu5Ac, was the best substrate for all virus NAs. Third, substitution of one of the three hydrogen atoms by a fluorine atom on the *N*-acetyl group of Neu5Ac-terminated sialosides did not significantly decrease the activities of virus NAs, indicating the similarity between a hydrogen and fluorine. This property is very similar to that observed for human and bacterial sialidases (Cao et al., 2009; Li et al., 2011). Fourth, α2–3-linked sialoside containing a non-human sialic acid form, Neu5Gc, was a suitable substrate for all human and avian influenza A virus NAs tested, despite the lack of such monosaccharide at the site of host infection.

Despite these similarities, obvious differences existed between the degrees of substrate preference between the tested viral NAs for α2–6-linked sialosides versus α2–3-linked sialosides. Furthermore, N7 subtype NAs had distinct subtype specificities. They were the

only viral NAs that used α2–3-linked sialoside substrates containing Kdn (compound **6a**) or 5-deoxy-Kdn (5d–Kdn, compound **10a**).

Our detailed analysis of the substrate specificity of 27 different influenza A virus NAs revealed that their relative preference for α2–3-linked sialoside substrates differed between host origin and time of isolation (Table S1). Although the ratios of relative neuraminidase activities on Neu5Acα2–6Galβ*p*NP (compound **1b**) versus Neu5Acα2–3Galβ*p*NP (compound **1a**) substrates were all below 1, they varied from low, such as for avian influenza A viruses sh/HKWF216/07 (H6N1, 0.09), mal/8322/08 (H6N1, 0.09), and dk/K90/05 (H6N8, 0.06), to high, as for human influenza A virus A/Udorn72 (H3N2, 0.64) and three other human viruses (**1b**/**1a** ratio > 0.4). In contrast to human influenza viral NAs, the ratios of activities on Neu5Acα2–6Galβ*p*NP (compound **1b**) versus Neu5Acα2–3Galβ*p*NP (compound **1a**) substrates were relatively low for most NAs of avian influenza A viruses except for gos/HKWF446/07 (H10N7, 0.57), dk/702/79 (H9N2, 0.43), Q/29209–1/93 (H9N2, 0.49), ck/G9/97 (H9N2, 0.48). These results were consistent with previous studies demonstrating that Neu5Acα2–3Gal was a preferred substrate over Neu5Acα2–6Gal for avian, swine, and N2 human influenza A viruses (Kobasa et al., 1999).

Based on their three-dimensional structures, neuraminidases from nine NA subtypes can be separated into two groups: Group 1 NA viral strains containing N1, N4, N5, and N8 subtypes and group 2 NA strains containing N2, N3, N6, N7, and N9 subtypes (Russell et al., 2006a). The major structural difference between group 1 and group 2 is a cavity observed in the active site close to ligand binding site of group 1 NAs, which is absent in the group 2 NAs (Russell et al., 2006a). Therefore, substrates for group 1 NAs might not be hydrolyzed by group 2 NAs. Based on their structural similarities, NAs in group 1 might have similar substrate specificities, and differ from those shared by group 2 NAs. Indeed, the substrate specificities of group 1 and 2 NAs revealed distinct patterns. As shown in Table 3, group 1 NAs have good cleavage activity against α2–3-linked sialosides containing Neu5AcF (compound **2a**), $Neu5AcN_3$ (compound **4a**), and Neu5Gc (compound **5a**), as well as Neu5Acα2–3Galβ*p*NP (compound **1a**). In contrast, group 2 NAs have good activity towards α2 –3-linked sialosides containing Neu5AcF (compound **2a**), and Neu5Gc (compound **5a**) as well as Neu5Ac α2–3Galβ*p*NP (compound **1a**). However, $Neu5AcN_3$α2–3Galβ*p*NP (compound **4a**) is not a good substrate for most group 2 NAs, except for most N7 subtype strains and some belonging to the H9N2 subtype.

We demonstrate here that using a high-throughput approach to screen a library of *para*-nitrophenol-tagged α2–3- and α2–6-linked sialosides Siaα2–3/6GalβpNP is a highly effective means to conduct substrate specificity studies of sialidases from many influenza viruses. The neuraminidases of non-purified influenza A viruses share the same substrate specificity pattern as those from purified viral particles. Therefore, non-purified influenza A viruses can be directly used for substrate specificity assays, saving on labor and time-consuming viral isolation procedures and preventing the activity loss of NAs observed during the viral particle purification process. In addition, the inhibitory activity of two know NA inhibitors, Zanamivir and Neu5Ac2en, and three new candidate inhibitors, Neu5Gc2en, $Neu5AcN_3$2en and Kdn2en, were efficiently characterized using Neu5Acα2–3/6Galβ*p*NP as substrate for viral NAs. These high-throughput substrate specificity and inhibition assays are much more efficient than the reported assays using peroxidase-linked lectin or fluorescence detection coupled with separation by an anion-exchange microcartridge (Mochalova et al., 2005) or a DEAE filter (Mochalova et al., 2007). The knowledge gained about the substrate specificity of individual influenza strains utilizing these rapid assays allows for the design of inhibitors that selectively target a distinct group of influenza A virus NAs.

Material and methods

Viruses

Thirty-seven avian and human influenza A virus strains were used in this study (Table 1). Unless indicated otherwise, the avian viruses were provided by the Cardona group and were collected through surveillance of wild birds done as part of Centers of Excellence in Influenza Research and Surveillance or Avian Influenza Coordinated Agricultural projects (A/northern shoveler/California/JN950/2006). A/emu/California/2092/1996 and A/pheasant/California/2373/1998 were from diagnostic cases submitted to the California Animal Health and Food Safety Laboratory. A human H9N2 virus preparation (A/Hong Kong/2108/2003) was provided by Dr. Yi Guan at the University of Hong Kong. All avian H9N2 subtype viruses excluding A/pheasand/California/2373/1998 were from Dr. Daniel Perez. Among H9N2 avian influenza A viruses, QA23 and Qa23CkA10 viruses are laboratory-adapted viruses. QA23 virus was developed by adapting a wild type duck H9N2 viral isolate, influenza dk/702/79 virus, in quail through 23 serial lung passages, and the chicken-adapted QA23CkA10 virus was obtained from the 10^{th} chicken lung passage of the QA23 virus (Hossain et al., 2008). Allantoic-fluid containing the virus strains were used for the assays.

Among the strains used, four were human influenza A viruses (A/Puerto Rico/34/8, H1N1; A/Memphis71, H3N1; A/Udorn/307/72, H3N2; and A/Philippines/2/82/X-79, H3N2). A/Memphis71 is a reassortant influenza A virus strain carrying the hemagglutinin of A/Memphis/1/71 (H3) and the neuraminidase of A/Bellamy/42 (N1). All four human influenza A viruses used were propagated from frozen stocks originally obtained from Lorena Brown (University of Melbourne, Australia) and Charles Stephensen (UC Davis). Both sucrose-gradient purified virus particles and allantoic-fluid containing the human virus strains were used for the assays to test the effect of potential contaminating proteins in the allantoic fluid and/or the effects of the virus purification procedures on the activity and substrate specificity of viruses.

Virus propagation

Avian virus isolates were passaged by inoculation into 9–11 day specific-pathogen-free (SPF) embryonating chicken eggs (SPAFAS, Charles River) following standard methods (WHO, 2002). Allantoic fluid samples were tested for hemagglutination using 0.5% chicken blood (Colorado Serum Company, Denver, CO) as described previously (Li et al., 2008; WHO, 2002).

For human virus, fertilized hen eggs were incubated for 10 days with constant rotation at 37°C and 65% humidity. Eggs containing live embryos were infected with predetermined optimal concentrations of influenza virus strains by inoculation into the allantoic cavity and incubated for 2 days at 35°C followed by overnight incubation at 4°C. Allantoic fluid was harvested, batched, and centrifuged (8000 × g, 15 min at 4°C). Supernatants were aliquoted, snap-frozen on dry ice and stored at −80°C until used.

Purification of human influenza A virus

For virus purification, batches of allantoic fluid from infected eggs were centrifuged. Virus particles were precipitated from supernatants by overnight incubation at 4°C with 8% polyethylene glycol 6000 solution (Sigma- Aldrich, Dallas, TX) and pelleted by centrifugation (12,000 × g, 30 min at 4°C). To release the virus, the pellet was resuspended in phosphate buffered saline (PBS), sonicated, and centrifuged for 5 min at 3000 × g. The supernatant was collected and centrifuged (24,000 × g, 2 h at 4°C). The virus pellets were resuspended in a small volume of PBS and separated by sucrose gradient centrifugation (linear gradient 70–25%, 24,000 × g, 2 h at 4°C). The virus band was harvested,

resuspended in PBS and centrifuged (24,000 × g, 2 h at 4°C). The pellet containing the virus was stored in a small volume of PBS at 4°C. The virus concentration (HAU/mL) was determined by standard hemagglutination assays using chicken red blood cells (Li et al., 2008; WHO, 2002).

Sialosides and neuraminidase inhibitors

The sialosides used in this study were synthesized using a one-pot three-enzyme approach as described previously (Cao et al., 2009). Zanamivir was from TCI America Ltd. (Portland, OR). Neuraminidase inhibitors 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en), 2-deoxy-2,3-dehydro-*N*-glycolylneuraminic acid (Neu5Gc2en), 2-deoxy-2,3-dehydro-*N*-azidoacetylneuraminic acid ($Neu5AcN_3$2en), and 2-deoxy-2,3-dehydro-Kdn (Kdn2en) were synthesized using a chemoenzymatic method as reported (Li et al., 2010).

Sialidase substrate specificity assays

Twenty sialyl galactosides including Siaα2–3/6Galβ*p*NP containing *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), 2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid (Kdn), or their C5-derivatives (Table 2) (Cao et al., 2009) were used to study the substrate specificity of neuraminidases on thirty-seven influenza A viruses using a microtiter plate-based high-throughput colorimetric assay method (Cao et al., 2009; Chokhawala et al., 2007). All sialidase assays were carried out at 37°C in duplicate in 384-well plates (Fisher Scientific, Chicago, IL) in a final volume of 20 μL each containing MES buffer (100 mM, pH 5.0), a certain titer of allantoic fluid containing virus or sucrose-purified viral particles, a sialoside substrate (0.3 mM), and an *Aspergillus oryzae* β-galactosidase (12 μg, 126 mU) from Sigma (St. Louis, MO). The amount of the β-galactosidase required to completely hydrolyze the Galβ*p*NP within the time frame of the assay was pre-determined and confirmed by control assays with Galβ*p*NP (0.3 mM). The reactions were carried out for 60 min and were stopped by adding pre-chilled CAPS buffer (*N*-cyclohexyl-3-aminopropane sulfonic acid, 40 μL, 0.5 M, pH 10.5). The amount of the *para*-nitrophenolate formed was determined by measuring the $A_{405\ nm}$ of the reaction mixtures using a microtiter plate reader. As described before (Cao et al., 2009; Chokhawala et al. 2007), if a sialoside is the substrate of the neuraminidase of the virus tested, the sialic acid on the sialoside will be cleaved off by the neuraminidase to provide Galβ*p*NP, which is quickly hydrolyzed by the excess amount of β-galactosidase in the reaction mixture to give *para*-nitrophenol. Addition of the pre-chilled CAPS buffer at the end of the incubation converts most of the *para*-nitrophenol to *para*-nitrophenolate whose concentration can be determined by measuring its absorption at 405 nm. The $A_{405\ nm}$ reading is proportional to the amount of the sialic acid released from the sialoside by the neuraminidase of the virus and can be used to compare the substrate specificity of different neuraminidases on the surface of different viral strains.

Inhibition assay of viral neuraminidases

Inhibition assays were carried out in duplicate in 384-well plates in a total volume of 20 μL each in MES buffer (100 mM, pH 5.0) similar to that described above for the sialidase substrate specificity assays except that a fixed concentration of Neu5Acα2–3Galβ*p*NP or Neu5Acα2– 6Galβ*p*NP (1 mM) was used as the substrate and different concentrations of sialidase inhibitors were used. To determine IC_{50} values for Zanamivir and Neu5Ac2en, at least 11 different concentrations varying from 0 to 10.0 mM were used. IC_{50} values were obtained using Grafit 5.0 for logarithm concentration-response plots. When Neu5Gc2en (100 μM, 500 μM, and 1 mM), $Neu5AcN_3$2en (1 mM, 5 mM, and 10 mM), or Kdn2en (10 mM) was used as an inhibitor, only certain concentrations were chosen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge the provision of influenza A viruses for this work from Drs. Yi Guan, Daniel Perez and Peter Woolcock. This work was supported by NIH grant R01GM076360–04S1. X.C. is a Camille Dreyfus Teacher-Scholar and a UC-Davis Chancellor's Fellow.

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Table 1

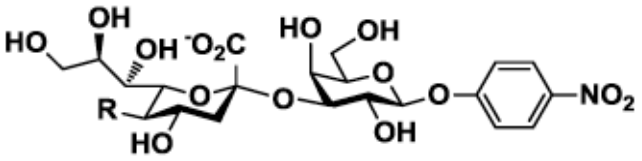
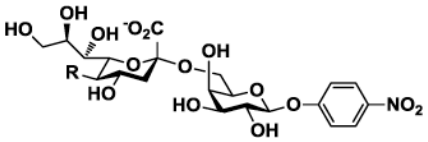
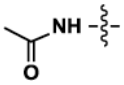
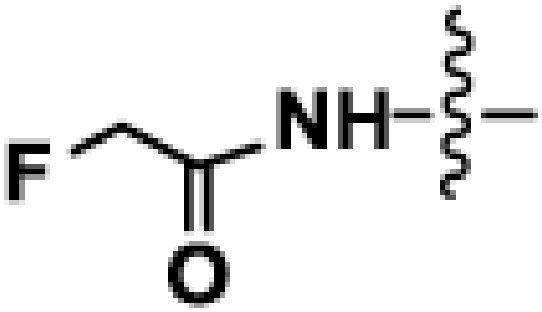
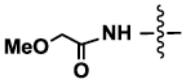
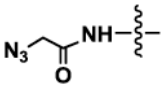
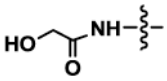
Influenza A viruses used in this study.

	Viral strain	Type	Abbreviation
Avian influenza A viruses			
N1	A/northern shoveler/California/HKWF569/2007	H3N1	sh/HKWF569/07
	A/northern shoveler/California/HKWF216/2007	H6N1	sh/HKWF216/07
	A/mallard/California/8212/2008	H6N1	mal/8212/08
	A/mallard/ California/8322/2008	H6N1	mal/8322/08
	A/green winged teal/California/8326/2008	H1N1	tel8326/08
N2	A/northern shoveler/California/HKWF268/2007	H6N2	sh/HKWF268/07
	A/green winged teal/California/7972/2008	H6N2	tel/7972/08
	A/mallard/California/8035/2008	H5N2	mal/8035/08
N5	A/american wigeon/California/HKWF295/2007	H6N5	wi/HKWF295/07
	A/american wigeon/California/HKWF541/2007	H6N5	wi/HKWF541/07
	A/american wigeon/California/8352/2008	H12N5	wi/8352/08
N6	A/env/California/7896/2008	H4N6	dk/7896/08
N7	A/emu/California/2092/1996	H10N7	emu/2092/96
	A/northern shoveler/California/JN950/2006	H10N7	sh/JN950/06
	A/greater white-fronted goose/California/HKWF446/2007	H10N7	gos/HKWF446/07
	A/northern shoveler/California/HKWF848/2007	H3N7	sh/HKWF848/07
	A/northern shoveler/California/HKWF1021/2007	H3N7	sh/HKWF1021/07
	A/northern shoveler/California/HKWF1128/2007	H2N7	sh/HKWF1128/07
	A/cinnamon teal/California/HKWF1111/2007	H5N7	tel/HKWF1111/07
	A/mallard/California/6957/2008	H10N7	mal/6957/08
N8	A/ring-necked duck/California/K90/2005	H6N8	dk/K90/05
N9	A/green winged teal/California/8204/2008	H5N9	tel/8204/08
H9N2 avian	A/duck//Hong Kong/448/1978	H9N2	dk/448/78
	A/duck/Hong Kong/702/1979	H9N2	dk/702/79
	A/quail/Hong Kong/A28945/1988	H9N2	Q/A28945/88
	A/quail/Arkansas/29209-1/1993	H9N2	Q/29209-1/93
	A/duck/Hong Kong/Y280/1997	H9N2	dk/Y280/97
	A/chicken/Hong Kong/G9/1997	H9N2	ck/G9/97
	A/pheasant/California/2373/1998		pt/2373/98
	A/chicken/Hong Kong/SF3/1999	H9N2	ck/SF3/99
	A/duck/Hong Kong/702/1979-quail adapted23	H9N2	QA23
	A/duck/Hong Kong/702/1979-chicken adaptedA10	H9N2	Qa23CkA10
H9N2 human[a]	*A/Hong Kong/2108/2003 (H9N2)*	*H9N2*	*HK/2108/03*
Human influenza A viruses[a]			
N1	*A/Puerto Rico/34/8*	*H1N1*	*A/PR8*
	A/Memphis/71	*H3N1*	*A/Mem71*
N2	*A/Udorn/307/72*	*H3N2*	*A/Udorn72*
	A/Philippines/2/82/X-79	*H3N2*	*A/Philips*

[a] Human influenza A viruses are shown in italics.

Table 2

Structures of *para*-nitrophenol-tagged α2–3- and α2–6-linked sialyl galactosides Siaα2–3/6Galβ*p*NP containing different sialic acid forms (Cao et al., 2009) used for substrate specificity studies of neuraminidases on the surface of viral particles.

Number	Compounds	Structure
1a–10a	Siaα2–3Galβ*p*NP	
1b–10b	Siaα2–6Galβ*p*NP	
	Sialic acid name	**Sialic acid structure**
1a or 1b	**Neu5Ac** (R= NHAc)	
2a or 2b	**Neu5AcF** (R= $NHCOCH_2F$)	
3a or 3b	**Neu5AcOMe** (R= $NHCOCH_2OMe$)	
4a or 4b	**Neu5AcN3** (R= $NHCOCH_2N_3$)	
5a or 5b	**Neu5Gc** (R= $NHCOCH_2OH$)	

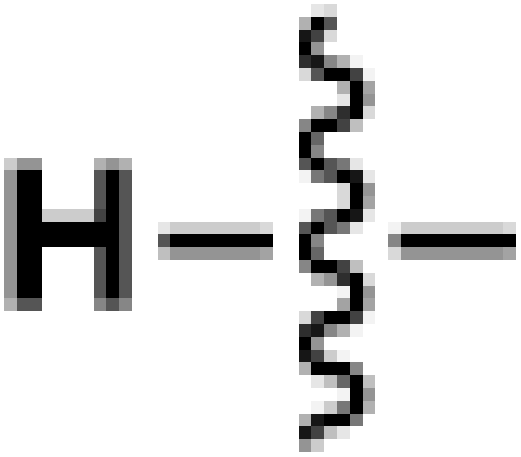
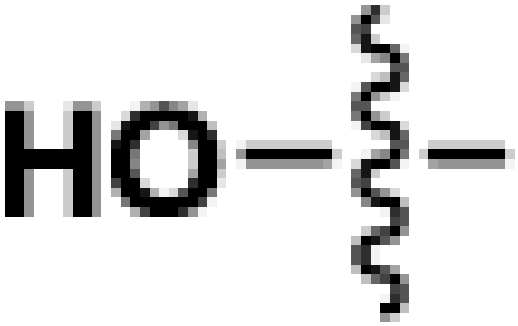
Number	Compounds	Structure
6a or 6b	**5d–Kdn** (R= H)	
7a or 7b	**5F–Kdn** (R= F)	
8a or 8b	**5Me-Kdn** (R= OMe)	
9a or 9b	**5N_3-Kdn** (R= N_3)	
10a or 10b	**Kdn** (R= OH)	

Table 3

Substrate specificities of neuraminidases on avian and human influenza viruses.

Type	Viral strain	Substrate Specificity[b]							
		Comp. a						Comp.b	
		a_{1a}	2a	4a	5a	6a	10a	1b	2b
N1	sh/HKWF569/07	72	++++	++++	++++	−	−	++	+
	sh/HKWF216/07	64	++++	+++	++++	−	−	−	−
	mal/8212/08	60	++++	+++	++++	−	−	+	−
	mal/8322/08	52	++++	++	+++	−	−	−	−
	tel8326/08	88	++++	+	++++	−	−	+++	++
N2	sh/HKWF268/07	59	++++	+	+++	−	−	++	++
	tel/7972/08	40	+++	−	+++	−	−	++	+
N5	wi/HKWF295/07	41	++++	++	+++	−	−	+	−
	wi/HKWF541/07	71	++++	+++	++++	−	+	++	+
	wi/8352/08	34	++++	+++	+++	−	−	+	−
N6	dk/7896/08	28	++++	+	++	−	−	++	+
N7	emu/2092/96	32	++++	−	+++	+	+	++	+
	sh/JN950/06	57	++++	+	+++	+	+	++	+
	gos/HKWF446/07	88	++++	+++	++++	++	+++	+++	+++
	sh/HKWF848/07	88	++++	+++	++++	++	++	+++	++
	sh/HKWF1021/07	78	++++	+++	++++	+	++	++	++
	sh/HKWF1128/07	46	++++	++	+++	+	+	++	+
	tel/HKWF1111/07	42	++++	+	++	−	+	+	+
	mal/6957/08	81	++++	+	++++	+	+	++	++
N8	dk/K90/05	72	++++	+	+++	−	−	−	−
H9N2 avian	dk/448/78	78	++++	+	+++	−	−	++	++
	dk/702/79	76	++++	++	++++	−	+	+++	++
	Q/29209-1/93	84	++++	++	++++	−	−	+++	++
	pt/2373/98	73	++++	−	+++	−	−	++	+
	ck/SF3/99	69	++++	−	+++	−	−	++	++
	QA23	37	++++	−	++	−	−	+	+

Type	Viral strain	Substrate Specificity[b]							
		Comp. a						Comp.b	
		[a]1a	2a	4a	5a	6a	10a	1b	2b
	Qa23CkA10	37	++++	−	++	−	−	+	+
H9N2 Human[c]	*HK/2108/03*	32	++++	−	+++	−	−	+++	++
Human unpurified[c]									
N1	*A/PR8*	44	++++	++	+++	−	−	+++	++
	A/Mem71	35	++++	++	+++	−	−	+++	++
N2	*A/Udorn72*	22	++++	−	+++	−	−	++++	+++
	A/Philips	34	++++	−	++	−	−	+++	+++
Human purified N1[c]	*A/PR8 (purified)*	84	++++	++	++++	−	−	++++	+++
	A/Mem71(purified)	82	++++	+++	++++	−	−	+++	+++

[a]Absolute values for the percentage hydrolysis of Neu5Aca2–3Gal*p*pNP(compound **1a**) and relative value for all other compounds. Relative values: Virus neuraminidase activity using Neu5Acα2–3Galβ*p*NP (compound **1a**) as the substrate is considered as 100%. "++++" represents activity ≥ 60%; "+++" represents activity ≥ 40% but < 60%; "++" represents activity ≥20% but <40%; "+" represents activity ≥10% but <20%; "-" represents activity < 10%.

[b]The results for viruses with low activity [A_{405max} is below 0.2 when Neu5Acα2–3Galβ*p*NP (**1a**) is used as the substrate or the relative activity is below 10%]are not listed. These include viruses mal/8035/08 (H5N2), tel/8204/08 (H5N9), Q/A28945/88 (H9N2), dk/Y280/97 (H9N2), and ck/G9/97 (H9N2).

[c]Human influenza A viruses are shown in italics. Underlined are group 1 NA viral strains. Others are group 2 NA viral strains.

Table 4

IC_{50}s of Zanamivir and NeuAc2en for activities of human and influenza A virus neuraminidases[a]

Viral Strain	Type	IC_{50}			
		Neu5Acα2–3GalβpNP		Neu5Acα2–6GalβpNP	
		Zanamivir (nM)	Neu5Ac2en (uM)	Zanamivir (nM)	Neu5Ac2en (μM)
sh/HKWF569/07	H3N1	14.3±1.6	10.7±1.4	ND	ND
mal/8212/08	H6N1	6.5±0.3	3.8±0.4	ND	ND
wi/HKWF541/07	H6N5	17.2±2.8	1.8±0.5	ND	ND
sh/JN950/06	H10N7	10.4±1.4	5.0±1.3	ND	ND
gos/HKWF446/07	H10N7	23.6±2.7	7.9±1.4	ND	ND
mal/6957/08	H10N7	49.9±3.1	23.2±1.5	ND	ND
Q/29209-1/93	H9N2	59.9±3.6	48.3±5.8	12.8±1.0	7.2±1.3
ck/SF3/99	H9N2	20.3±1.3	6.7±0.8	ND	ND
Qa23CkA10	H9N2	9.5±1.6	6.4±1.2	ND	ND
A/PR8[*]	*H1N1*	*3.3±0.2*	*2.6±0.5*	*3.2±0.5*	*1.8±0.4*
A/Udorn72[*]	*H3N2*	*94.4±6.2*	*17.8±4.7*	*78.8±6.8*	*11.3±2.7*

[a] Unpurified avian influenza A viruses (12.8 HAU mL^{-1}) and purified human viruses (A/PR8, 1×10^4 HAU mL^{-1}; A/Udon, 5×10^3 HAU mL^{-1}) were used. The reactions were carried out at 37 °C for 60 min with 1 mM of substrate.

[*] Purified human influenza A virus; ND, not determined.

Table 5

Percentage inhibition of Neu5Gc2en, Neu5AcN$_3$2en, and Kdn2en against influenza A virus neuraminidases using Neu5Acα2–3Galβ*p*NP or Neu5Acα2–6Galβ*p*NP as the substrate.[a]

Viral Strain	Type	% inhibition with Neu5Acα2–3Galβ*p*NP as the substrate						
		Neu5Gc2en			Neu5AcN$_3$2en			Kdn2en[b]
		100 μM	500 |μM	1 mM	1 mM	5 mM	10 mM	10 mM
sh/HKWF569/07	H3N1	24.1±5.6	56.3±0.7	72.9±0.3	27.6±0.3	51.9±0.1	61.6±1.3	7.5±0.4
mal/8212/08	H6N1	40.2±1.9	78.4±1.0	87.3±0.6	39.1±1.2	72.9±1.0	80.5±0.2	22.9±1.7
wi/HKWF541/07	H6N5	14.9±2.1	53.9±1.5	71.7±0.1	29.3±1.9	66.3±1.7	77.1±0.3	10.7±2.5
sh/JN950/06	H10N7	42.2±0.5	81.0±1.2	90.9±0.4	29.9±0.6	65.7±0.7	73.5±1.2	45.9±0.4
gos/HKWF446/07	H10N7	29.1±0.7	66.3±4.1	82.7±1.8	26.8±0.6	53.2±0.8	65.4±0.2	40.5±1.3
mal/6957/08	H10N7	14.3±2.0	51.6±1.5	71.9±0.2	8.6±0.4	34.2±0.4	52.2±2.0	18.8±0.9
Q/29209-1/93	H9N2	6.1±0.2	29.5±4.6	53.9±0.4	6.7±0.1	23.2±0.7	36.0±1.7	–
ck/SF3/99	H9N2	36.1±0.9	77.4±0.3	87.7±0.2	30.1±0.6	67.5±1.1	79.0±0.2	13.1±0.1
Qa23CkA10	H9N2	37.6±0.6	76.4±0.9	85.9±0.3	23.3±0.9	63.0±0.1	72.1±1.6	–
A/PR8[*]	*H1N1*	*48.5±2.2*	*84.6±0.2*	*90.4±0.2*	*66.7±0.5*	*88.9±0.9*	*90.1±0.4*	*42.2±2.4*
A/Udorn72[*]	*H3N2*	*18.1±0.2*	*42.3±0.3*	*57.6±2.0*	–	–	*10.0±0.6*	–
		% inhibition with Neu5Acoα2–6Galβ *p* NP as the substrate						
Q/29209-1/93	H9N2	39.4±0.5	80.2±1.27	90.5±0.4	23.8±2.2	57.3±0.3	64.2±1.4	–
A/PR8[*]	*H1N1*	*54.5±1.3*	*80.9±0.8*	*86.6±0.5*	*66.9±3.0*	*82.2±1.1*	*82.0±0.3*	*43.5±0.5*
A/Udorn72[*]	*H3N2*	*24.4±0.3*	*61.7±0.3*	*73.6±0.3*	–	*24.4±0.8*	*43.5±2.4*	–

[a] Unpurified avian influenza A viruses (12.8 HAU mL^{-1}) and purified human viruses (A/PR8, 1 × 10^4 HAU mL^{-1}; A/Udorn72, 5 × 10^3 HAU mL^{-1}; A/Mem71, 5 × 10^3 HAU mL^{-1}; A/Philips: 2 × 10^4 HAU mL^{-1}) were used. The reactions were carried out at 37°C for 60 min with 1 mM of substrate.

[b] Kdn2en did not show any inhibition against the neuraminidase activity of A/Mem71 and A/Philips.

[*] Purified human influenza A virus.

"–" no inhibition