Human neuraminidase isoenzymes show variable activities for 9-*O*-acetyl-sialoside substrates

Carmanah D. Hunter, Neha Khanna, Michele R. Richards, Reza Rezaei Darestani, Chunxia Zou, John S. Klassen, and Christopher W. Cairo*

Alberta Glycomics Centre, Department of Chemistry, University of Alberta, Edmonton Alberta, T6G 2G2, Canada

*To whom correspondence should be addressed. Tel.: 780 492 0377; fax: 780 492 8231; e-mail: ccairo@ualberta.ca

ABSTRACT

Recognition of terminal sialic acids is central to many cellular processes, and structural modification of sialic acid can disrupt these interactions. A prominent, naturally occuring, modification of sialic acid is 9-*O*-acetylation (9-*O*-Ac). Study of this modification through generation and analysis of 9-*O*-Ac sialosides is challenging due to the lability of the acetate group. Fundamental questions regarding the role of 9-*O*-Ac sialic acids remain unanswered, including what effect it may have on recognition and hydrolysis by the human neuraminidase enzymes (hNEU). To investigate the substrate activity of 9-*O*-acetylated sialic acids (Neu5,9Ac$_2$) we synthesized an acetylated fluorogenic hNEU substrate 2′-(4-methylumbelliferyl)-9-*O*-acetyl-α-D-*N*-acetylneuraminic acid. Additionally, we generated a panel of octyl sialyllactosides containing modified sialic acids including variation in linkage, 9-*O*-acetylation, and C-5 group (Neu5Gc). Relative rates of substrate cleavage by hNEU were determined using fluorescence spectroscopy and electrospray ionization mass spectrometry. We report that 9-*O*-acetylation had a significant, and differential, impact on sialic acid hydrolysis by hNEU with general substrate tolerance following the trend of Neu5Ac > Neu5Gc >> Neu5,9Ac$_2$ for NEU2, NEU3, and NEU4. Both NEU2 and NEU3 had remarkably reduced activity for Neu5,9Ac$_2$ containing substrates. Other isoenzymes appeared to be more tolerant, with NEU4 even showing increased activity on Neu5,9Ac$_2$ substrates with an aryl aglycone. The impact of these minor structural changes to sialic acid on hNEU activity was unexpected, and these results provide evidence of the substantial influence of 9-*O*-Ac modifications on hNEU enzyme substrate specificity. Furthermore, these findings may implicate hNEU in processes governed by 9-*O*-acetyltransferases and -esterases.

INTRODUCTION

Sialic acids are a structurally diverse family of carbohydrates; however, the influence of subtle structural changes on their biological function is not well understood. These 9-carbon, α-keto acids are often the terminal (non-reducing) carbohydrate residue of human glycans.[1] Their location at the periphery of the glycan allows sialic acids to play roles in development, immune response, host-pathogen interactions, and tumor metastasis.[2, 3] The most common sialic acid in humans is the 5-*N*-acetylneuraminic acid (Neu5Ac), and it is considered to be the precursor for most sialic acids. Common modifications of Neu5Ac include a glycolyl group at N-5 (Neu5Gc), and *O*-acetate, -sulfate, -lactate, or -phosphate ester modifications of hydroxyl groups.[1] The 9-*O*-acetylated form of sialic acid (Neu5,9Ac_2) has been implicated in blocking lectin binding,[4] yet also enhances the affinity of influenza C hemagglutinin.[5] Ligands for immune cell lectins, such as Siglecs, can be masked by 9-*O*-acetylation of sialic acid.[6] The presence of Neu5,9Ac_2 has been associated with cancer cell survival through prevention of GD3-mediated apoptosis,[7] and protection of sialoside substrates from bacterial and viral neuraminidases.[8] Despite its recognized importance, the specific roles of Neu5,9Ac_2 residues have remained unclear. Routine study of Neu5,9Ac_2 has been hampered by the lability of the *O*-acetate, particularly under basic conditions;[9-11] limiting detection strategies to the use of antibodies, lectins, or influenza C hemagglutinin-esterase.[12] This fact prompted the recent development of a hydrolytically stable analog of Neu5,9Ac_2 (9-acetamido-9-deoxy-*N*-acetylneuraminic acid) for glycan microarrays.[11]

The 9-*O*-acetylation of sialosides is regulated *in vivo* by the action of two opposing enzyme activities: sialate-*O*-acetyltransferases (SOAT) and sialate-9-*O*-acetylesterases (SIAE). Human SIAE have been identified[13, 14] and are implicated in several diseases including rheumatoid arthritis and type I diabetes.[15] In childhood acute lymphoblastic leukemia, there is increased sialic acid *O*-

acetylation as a consequence of both decreased SIAE activity and increased SOAT activity.[16] Furthermore, increased 9-*O*-acetylation has been observed in melanoma, small cell lung carcinoma, glioblastoma, and breast carcinomas.[17] The removal of the 9-*O*-acetyl group by treatment with SIAE induced apoptosis in both leukemia[18] and glioblastoma cells.[7] While human SIAEs have been known for decades,[13, 14] human SOATs have been more challenging to isolate, due to the sensitivity of AcT activity to membrane solubilization.[19] The protein CASD1 has recently been found to be essential for sialic acid 9-*O*-acetylation in humans;[20] however, this enzyme may not be responsible for SOAT activity in gangliosides.[17]

While both acetylesterases and acetyltransferases are critical to the prevalence of Neu5,9Ac_2, the ability of these residues to modulate activity of sialic acid modifying enzymes is not well understood. Neuraminidase (also called sialidase) enzymes are glycosyl hydrolases which cleave the glycosidic linkage of sialiosides (EC 3.2.1.18). Four distinct human neuraminidase (hNEU) isoenzymes have been identified (NEU1, NEU2, NEU3, and NEU4). The hNEU isoenzymes differ in subcellular localization, tissue expression, and substrate preference.[21-25] Seyrantepe et al. demonstrated that both the sialic acid aglycone and reducing-end sugar have a large impact on the relative activity of hNEU.[23] While NEU2 and NEU4 can cleave glycoproteins, glycolipids, and oligosaccharides;[23, 26] NEU1 cleaved only glycoproteins and oligosaccharides, [23, 27] and NEU3 demonstrated a strong preference for glycolipid substrates.[23, 28] Sialic acid diversity also impacts hNEU activity. The hNEU are reported to have different activity for Neu5Gc substrates compared to Neu5Ac substrates.[29,30] Sialic acid oligomers containing Neu5Gc residues have reduced substrate activity with NEU1, NEU2, and NEU4.[31] The Neu5,9Ac_2 residue is known to impede the activity of bacterial and viral neuraminidases.[32] Reports differ on whether Neu5,9Ac_2 residues are substrates for mammalian NEU.[33-35]

To the best of our knowledge, a comprehensive study probing the influence of sialic acid 9-*O*-acetylation on hNEU modulation has not been carried out. While recent work indicated that Neu5,9Ac$_2$, is a poor substrate for NEU2,[35] previous work with sialosides containing unnatural modifications at C-9 have suggested that variation among isoenzymes could result in disparate activity for Neu5,9Ac$_2$ substrates. Substitutions of the 9-OH of Neu5Ac with fluoride, methoxy, hydrogen, and azide groups almost completely inhibited NEU2 activity.[36] Sandbhor et al. reported that 9-azido, -amino, or -aryl groups reduced substrate activity of sialosides for NEU3.[37] Futhermore, the most selective inhibitors known for NEU1[38] and NEU4[39] involve modifications of 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (DANA) at the C-9 position.[25] These examples suggest that the C-9 pocket of hNEU enzymes is critical for recognition, and could result in differential activity for Neu5,9Ac$_2$ substrates among the isoenzymes.

Generation of defined sialosides containing Neu5,9Ac$_2$ is necessary for elucidation of enzyme specificity. While the generation of 9-*O*-acetyl sialiosides is challenging, there are examples using selective trimethyl orthoacetate chemistry.[40-46] Enzymatic strategies using O-acetyltransferases have been reported.[47] In order to test for effects of 9-O-acetylation on hydrolysis by hNEU, we generated a fluorogenic substrate containing Neu5,9Ac$_2$: 2′-(4-methylumbelliferyl)-α-D-9-*O*-acetyl-5-*N*-acetylneuraminic acid (4MU-Neu5,9Ac$_2$ **2**, **Figure 1a**). A similar strategy has been explored for testing the role of N-5 modifications of Neu5Ac on hNEU activity in cell lysates.[48] Using fluorogenic substrates in combination with purified isoenzymes, we observed that substitution of Neu5,9Ac$_2$ for Neu5Ac significantly inhibited cleavage by multiple isoenzymes of hNEU. Furthermore, we generated a panel of octyl sialyllactosides (**4**–**9**, **Figure 1c**) containing Neu5Gc and Neu5,9Ac$_2$ residues with variation of glycosidic linkages to Gal (either α(2→3) or α(2→6)). We observed that trisaccharides containing Neu5,9Ac$_2$ were generally poor substrates

for NEU2, NEU3, and NEU4 isoenzymes, with NEU2 having the greatest sensitivity to this modified residue. These findings support a role for enzymes that regulate 9-*O*-acetylation of Neu5Ac (i.e. SIAE, SOAT) in altering hNEU activity on cellular sialosides.

RESULTS AND DISCUSSION

To investigate the effect of 9-*O*-acetylation of sialic acid on hydrolysis by hNEU, we acetylated **1** at *O*-9 using the method of Furuhata and Ogura to generate 4MU-Neu5,9Ac$_2$ **2**.[46] Incubation of **1** or the 9-*O*-acetylated derivative, **2**, with hNEU allowed us to monitor the relative rates of hydrolysis by fluorescence spectroscopy. The four hNEU isoenzymes exhibited dramatic differences in their ability to hydrolyze Neu5,9Ac$_2$ (**Figure 1b**). The hNEU isoenzymes discriminated between the unmodified and 9-*O*-modified substrates to different extents. NEU4 was the only isoenzyme to show a significant (2-fold) preference for the Neu5,9Ac$_2$ substrate. Of the other isoenzymes, NEU3 had a 5-fold preference for the Neu5Ac substrate. Remarkably, NEU2 activity was almost completely inhibited by acetylation of the *O*-9 position.[35] Overall, these data clearly demonstrated that the 9-*O*-Ac modification could have a substantial, isoenzyme-specific, influence on hNEU activity. However, we considered that the 4MU-based substrates may be poor mimics of the physiological substrates of the enzymes, and proceeded to investigate differences in hNEU activity in the context of trisaccharide substrates.

Synthesis of octyl sialyllactosides. Human neuraminidase substrate preference depends not only on sialic acid but also on the reducing end sugars and aglycone.[37] For instance, NEU1 is known to prefer 4MU-Neu5Ac **1** over 3'-sialyllactose (the carbohydrate moiety of **3**), which in turn was cleaved more efficiently than gangliosides. NEU3 demonstrated the opposite preferences, with gangliosides being preferred over 4MU-Neu5Ac (**1**).[23] To generate substrates for hNEU similar to physiological targets, we prepared analogs of the ganglioside GM3 containing modified sialic acid residues (Neu5Ac, Neu5Gc, and Neu5,9Ac_2) using a chemoenzymatic strategy (**Figure 1c**). Previous work in our group had shown that an octyl chain is a sufficient mimic of the native ceramide in GM3 to maintain NEU3 activity.[37] This substitution simplified both the synthesis and purification of glycolipid substrates. Smutova et al. observed that the glycosidic linkage between sialic acid and galactose had a significant impact on hNEU activity; however, LacNAc derivatives did not show large differences from the corresponding Lac derivatives.[49] With these data in mind, we designed a panel of octyl-sialyllactoside analogues with variable sialic acid residues to investigate substrate activity of hNEU. We varied the anomeric linkage of the sialic acid to the galactose residue, preparing both α(2→3) (**4**, **6**, **8**) and α(2→6) (**5**, **7**, **9**; **Figure 1c**) linkages that could act as mimics of glycolipid and glycoprotein linkages, respectively. We also synthesized substrates containing Neu5Gc, as the N-glycolyl group has been proposed to influence NEU2 activity[36, 48] and this modified residue can be incorporated into human glycans from dietary sources.[50-52]

The most facile route to modified sialic acid-containing substrates is through the one-pot, chemoenzymatic method developed by Chen and coworkers.[42, 53, 54] This approach circumvents the challenges faced by chemical methods.[55, 56] The acceptor for the chemoenzymatic sialylation was β-octyl lactoside, obtained in 5 steps from lactose.[37] The Neu5Ac residue was converted to

Neu5,9Ac_2 in one step.[40] Efforts to convert Neu5Ac to Neu5Gc directly were less efficient in our hands.[57] We found that synthesis of the *N*-glycolyl-D-mannosamine (ManNGc), which was then converted to Neu5Gc chemoenzymatically, provided better yields of the product.[58] The starting materials Neu5Ac, Neu5,9Ac_2, and the acceptor were subjected to a one-pot, two-enzyme reaction (CSS and SiaT) to yield compounds **4**, **5**, **8**, and **9** (Figure S2). The Neu5Gc targets **6** and **7** were generated with a one-pot, three-enzyme (aldolase, CSS, SiaT) chemoenzymatic method starting from ManNGc, pyruvate, and β-octyl lactoside (see Figure S3).[42, 53, 54]

The Neu5,9Ac_2 sialyllactoside targets **8** and **9** presented unique challenges for chemoenzymatic synthesis. Optimal sialyltransferase conditions are alkaline (pH 8.8), which rapidly hydrolyzes the Neu5,9Ac_2 ester. Following Chen and coworkers protocol for chemoenzymatic synthesis of *O*-acetylated sialic acids, we lowered the pH of the reaction buffer to 7.2.[59] We observed partial hydrolysis at long reaction times, while shorter times (3 h) provided the best yields for Neu5,9Ac_2 α(2→6) product **9** (33%) after purification by C18 chromatography. Synthesis of the Neu5,9Ac_2 α(2→3) product **8** required purification by HPLC, and gave a low yield of the desired product (10%).

Solution phase kinetics of hNEU activity with octyl sialyllactosides. With octyl sialyllactosides **4**–**9** in hand as improved mimics of ganglioside targets, we sought to confirm changes to hNEU kinetics with these substrates. Substrates **4**–**9** lack any sensitive chromophore for detection of cleavage by fluorescence or UV spectroscopy. To monitor the reactions of **4**–**9** with hNEU we adapted a known assay for the detection of free sialic acid, in which malononitrile reacts with the ketone of open chain sialic acid under basic conditions to generate a fluorescent product with a limit of quantitation (LOQ) of 2 μM.[60] This fluorescent assay does not require modification of the substrates with a chromophore, and can therefore be used to enable the study of native substrates.[30, 49, 61] However, the low sensitivity of the assay in combination with the slow kinetics of NEU3 and NEU4 limited our analysis to the determination of relative rates (k_{rel}). In testing the activity of NEU1 with **4**, we found that the rate of cleavage was too slow for detection by this assay; therefore, we did not attempt to quantify the kinetics of **4**–**9** against NEU1 with the malonitrile assay (Figure S4). This observation is consistent with previous reports that gangliosides are poor substrates for NEU1.[23]

Among the remaining hNEU isoenzymes, we found that NEU2 was the least tolerant of modifications to the Neu5Ac residue or its glycosidic linkage. Due to this specificity, we had to alter the timescale of the experiments to obtain a full data set for NEU2. For the Neu5Ac and Neu5Gc α(2→3) substrates **4** and **6**, data was collected every 1 min over 4 min, while for substrates **5**, **7**, **8**, and **9** data was collected every 10 min over 40 min. Substrate **5**, which differs from **4** only in the α(2→6) glycosidic linkage, had more than a 30-fold reduction in activity. The Neu5Gc residue with an α(2→3)linkage **6**, showed a 2-fold loss of activity as compared to **4**; while the α(2→6)-linked Neu5Gc **7**, had very low activity (**Figure 1**). The observed preference of NEU2 for Neu5Ac over Neu5Gc contrasts previous reports using disaccharides containing a *p*-

nitrophenyl-galactoside.[36] These structural differences in the substrates are likely responsible for disagreement as hNEU substrate preference is influenced by the aglycone. Furthermore, the glucose residue of 3'-sialyllactose participates in two hydrogen bonds with NEU2 outside of the active site.[62] Both Neu5,9Ac_2 substrates **8** and **9** had remarkably low rates of cleavage. Substrates **8** and **9** were hydrolyzed at least 100-fold slower than **4**, consistent with the data collected for the 4MU-NANA substrates with NEU2 (**Figure 1**, Table S1). Our results are consistent with a recent observation that Neu5,9Ac_2 sialosides resulted in nearly complete loss of NEU2 hydrolysis activity.[35, 36]

We observed that NEU3 was more tolerant of substrate modifications than NEU2. NEU3 had a preference for α(2→3) over α(2→6) linkages (**Figure 1**, Table S1) which was only significant with modified sialosides (Neu5Gc and Neu5,9Ac_2).[49] The incorporation of Neu5Gc for substrates **6** and **7** both showed reduction in activity to approximately half of that for Neu5Ac substrate **4**. NEU3 activity showed a similar reduction for Neu5,9Ac_2 (**8**, **9**) substrates relative to **4**. Interestingly, while the C-9 pocket of NEU3 has been exploited for the design of inhibitors of NEU3,[61] it did not accommodate the acetate group, nor a C-9 methylamide.[38] We concluded that NEU3 had a modest preference for sialosides with an α(2→3) linkage and only minor discrimination between that Neu5Gc and Neu5,9Ac_2 substrates (~2-fold reduction). The NEU3 kinetic data suffered from large standard errors which we attributed to slow kinetics for this enzyme combined with the high LOQ of the malonitrile assay and limited quantities of the substrates. Therefore, we pursued alternative assays to confirm this finding (*vide infra*).

We found that NEU4 was also more tolerant than NEU2 with modified substrates. The NEU4 isoenzyme exhibited only a moderate preference for α(2→3) over α(2→6) linkages.[49] We observed NEU4 to have a preference for Neu5Ac over Neu5Gc residues. The crystal structure of

NEU2 and homology models developed for NEU3 and NEU4 suggest that these three isoenzymes use similar active site residues (N86, Y178, E218 of NEU2; N88, Y179, E225 of NEU3; N86, Y177, E222 of NEU4) to recognize the N5 group.[63] Surprisingly, NEU4 exhibited a significant discrimination against Neu5,9Ac$_2$ substrates (**8**, **9**) compared to the Neu5Ac substrate **4** (**Figure 1**, Table S1). This result is the opposite of the substrate preference we observed with our 4MU-NANA substrates (**1**, **2**). The NEU4 isoenzyme has previously been shown to have a significant preference for 4-MU substrates over gangliosides, and the aglycone may have an influence on the observed activity.[23] This hypothesis is consistent with the observed differences between 4MU-Neu5Ac and GM3 interacting with the NEU2 active site.[62] This result emphasized the influence of the aglycone on substrate recognition by hNEU, and highlighted the limitations of 4MU substrates for the study of hNEU substrate specificity. Together, our results suggested a currently unrecognized role for 9-*O*-acetyl modifications in the regulation of sialic acid catabolism.

ESI-MS kinetics. Our kinetic studies of trisaccharide substrates for hNEU highlighted a need for more sensitive assays. In particular, while the 4MU-based substrates clearly indicated a NEU3 preference for Neu5Ac, the nature of the trisaccharide assays could not clearly resolve the effect of Neu5,9Ac_2 substrates on NEU3 hydrolysis. To address this issue, we designed an electrospray ionization mass spectrometry (ESI-MS) assay to monitor the hydrolysis of octyl sialyllactosides by hNEU. Electrospray ionization is a sufficiently mild ionization technique to preserve the *O*-acetyl ester of **8** and **9** and is more sensitive than the malononitrile fluorescence assay. Additionally, the assay can simultaneously measure both substrate depletion and product formation by monitoring changes in substrate and product ion abundances, relative to an internal standard (leucine encephalin, and *N*-acetyl-9-azido-9-deoxy-neuraminic acid). The ESI-MS assay was implemented to monitor the kinetics of NEU3 with the α(2→3) substrates (**4**, **6**, and **8**; Table 1). Control experiments were performed in the absence of enzyme to confirm that changes in abundance of the substrate and product were not due to in-source (gas phase) fragmentation of the substrate ions. With this assay, we confirmed the trends observed in the malononitrile assay. We ascertained a two-fold decrease in activity for Neu5Gc substrate **6** relative to **4**. We observed a 7-fold reduction in activity for Neu5,9Ac_2 substrate **8** relative to **4** – consistent with the 4MU assay for substrates **1** and **2**.

ESI-MS can differentiate between modified sialic acids based on the differences in molecular weights. This feature allowed us to test for competition between different sialic acid-containing substrates for the hNEU active site, which is likely to occur in physiological settings. Relative rates were determined for substrates **4**, **6**, and **8** measured both in isolation and in a mixture of all three substrates (**Table 1**, **Figure 2**). The differences in relative rates for substrates measured individually and in a mixture were not significant by one-way ANOVA. Therefore, we

concluded that competition between substrates did not have a significant impact on sialic acid hydrolysis. These data confirmed the results of the malononitrile assay that NEU3 had substantially reduced activity Neu5Gc and Neu5,9Ac_2 substrates.

Molecular modeling of hNEU active sites with modified sialic acids. To provide insight into the surprising discrimination of hNEU for modified sialoside substrates, we performed molecular dynamics (MD) simulations of enzyme-substrate complexes. NEU2 is the only hNEU enzyme with an atomic resolution crystal structure.[64] Homology models for the other hNEU enzymes have been proposed based on the structure of NEU2.[63, 65] We used the crystal structure of NEU2 and our homology model of NEU3[65] to conduct MD simulations of NEU2 and NEU3 bound to the methyl sialyllactoside analogues of **4**, **6**, and **8**. The MD simulations provided structural insight into the observed relative rates of hydrolysis.

In NEU2, the preference for α(2→3)Neu5Ac over α(2→3)Neu5Gc substrates was supported by changes in the hydrogen bonding networks seen during MD simulations, particularly with the amino acid residues responsible for catalysis, the nucleophilic pair Tyr334–Glu218.[65] We found the H-bond between the carboxylate of Glu218 and H5N of **4** was occupied 24% of the time, but that same H-bond increased occupancy to 82% when **8** was in the active site (**Figure 3**). The increased occupancy of this Glu218 H-bond with H5N would make a key catalytic residue less available to deprotonate the nucleophile (Tyr334), reducing the rate of cleavage.

The C-9 pocket of NEU2 cannot accommodate large modifications of the glycerol side chain. In our MD simulations of **8** bound to NEU2, the 9-*O*-acetyl group forced the sialic acid ring into a boat conformation throughout the simulation (**Figure 3**, Figure S8 - S10, Table S2). This modification also led to further changes in the hydrogen bonding network in the NEU2 active site. When **4** was bound to NEU2, the O-7H of Neu5Ac formed a hydrogen bond to Glu111 96% of the

time. This hydrogen bond decreased occupancy to 81% for Neu5Gc in **6**, and only 2% for Neu5,9Ac$_2$ in **8**, with a concomitant increase on a H-bond between O-4H in Gal and Glu111 (68% of the simulation time). Residue Glu111 has been implicated as necessary for correct positioning of the substrate in the NEU2 active site.[64] Thus, we propose that the 9-*O*-Ac modification cannot be sterically accommodated by the NEU2 active site.

Our MD simulations also supported that the C-9 pocket in NEU3 was larger than that of NEU2; allowing the sialoside rings of **4**, **6**, and **8** to remain in chair conformations throughout the simulations (**Figure 3**, Figure S11, Table S3). Analysis of the hydrogen bonding networks between **4**, **6**, and **8** with NEU3 active site residues responsible for catalysis (Tyr370, Glu225)[65] showed differences for modified sialosides (**Figure 3**). With **4**, a hydrogen bond between phenolic hydrogen of Tyr370 and the C-1 carboxylate of Neu5Ac was occupied for only 5% of the MD simulation. The same H-bond had higher occupancy when **6** or **8** was in the active site of NEU3 – for **6**, it was occupied for 64% of the simulation, and for **8**, it was occupied for 41% of the simulation. Increased H-bonding should reduce the nucleophilicity of Tyr370, and decrease the relative rate of catalysis for **6** and **8** when compared with **4**. The acetate group at C-9 also caused a shift of the trisaccharide in the active site for **8** relative to **4** (**Figure 3**). This shift was accompanied by changes in hydrogen bonding to Asp50, one of the catalytic residues for NEU3.[65] For **4** and **6**, Asp50 formed two key H-bonds to the glycan – one to O-4H of the sialic acid residues (51%–54% of the simulation) and another to O-2H of Gal (40–45% of the simulation). However, for **8**, the H-bond to Neu5,9Ac$_2$ decreased to only 2% occupancy and that to Gal decreased to 38%.

CONCLUSION

Our substrate specificity studies of hNEU indicate that naturally occurring 9-*O*-acetyl (Neu5,9Ac$_2$) and 5-*N*-glycolyl (Neu5Gc) sialic acid modifications have a significant impact on

hydrolysis by hNEU. Substrate specificity studies with 4MU substrates **1** and **2** indicated that the hNEU isoenzymes exhibited discrimination between Neu5Ac and Neu5,9Ac_2 substrates. While NEU4 preferred 4MU-Neu5,9Ac_2, NEU1, NEU2, and NEU3 were substantially less active against 9-*O*-Ac modified substrates. Notably, NEU2 was essentially inactive (100 times lower activity) on Neu5,9Ac_2 substrate **2**. These data confirm that 9-*O*-acetylation of sialic acid had a substantial and isoenzyme-specific impact on hNEU activity. By optimizing a known assay for the detection of free sialic acid, we were able to study hNEU kinetics on trisaccharide substrates with a hydrophobic aglycone which acted as improved mimics of natural hNEU substrates. All three isoenzymes tested (NEU2, NEU3, and NEU4) had a 2-fold preference for Neu5Ac over Neu5Gc octyl sialyllactosides. Sialic acid hydrolysis by all hNEU isoenzymes discriminated against the 9-*O*-acetylation of sialic acid in the trisaccharide substrates. Consistent with the 4MU substrates, NEU2 was inactive (100-times lower activity) on Neu5,9Ac_2 substrates. Data for NEU3 was ambiguous by the malononitrile assay; however, an ESI-MS assay confirmed that NEU3 had a 7-fold preference for Neu5Ac over Neu5,9Ac_2 substrates. In contrast to the 4MU substrates, NEU4 demonstrated a 2-fold preference for Neu5Ac over Neu5,9Ac_2 octyl sialyllactoside substrates, indicating that the aglycone had an influence over observed activity. In general, we observed that substrate tolerance for NEU2, NEU3, and NEU4 followed a trend with Neu5Ac > Neu5Gc >> Neu5,9Ac_2. Furthermore, the presence of modified sialoside residues (Neu5Gc, Neu5,9Ac_2) accentuated hNEU preferences for the α(2→3) glycosidic linkage. We propose that a full understanding of the role of the 9-*O*-Ac modification of sialic acid will require additional study of hNEU activity on a variety of sialoglycoconjugates to account for the effects of reducing end sugars and aglycone on human neuraminidase specificity. Specifically, future study should include investigation of hNEU activity towards Neu5,9Ac_2 polysaccharides and glycoprotein substrates.

Ultimately, the development of chemical tools to study the effects of 9-*O*-acetylation on hNEU activity towards complex glycoconjugates in cells will be essential for elucidating the role of this sialic acid modification in biological systems. Several examples suggest an important physiological role for 9-*O*-Ac metabolizing enzymes SOAT and SIAE, which have impacts in autoimmune diseases and cancers.[15, 17] Our findings strongly suggest that the 9-*O*-Ac modification of sialic acids could be important in the regulation of hNEU activity and may provide a biochemical link between SIAE, SOAT, and hNEU enzymatic activity.

METHODS

General Methods. All reagents were purchased from commercial sources and used without further purification unless otherwise noted. Reaction solvents were purified by successive passage through columns of alumina and copper under an argon atmosphere using Innovative Technology, Inc. PURE SOLV (SPS-400-7). Reactions were monitored by analytical TLC on silica gel 60-F254 (0.25 nm, Silicycle). Visualization was achieved using UV fluorescence and/or by charring with 5% sulfuric acid in ethanol. Organic solvents were evaporated under reduced pressure at 40 °C. Reaction products were purified by column chromatography on silica gel (230-400 mesh, Silicycle) unless otherwise noted. When the eluent system required greater than 10% methanol, Iatrobeads 6RS-8060 (Shell-USA Inc.) were used. HPLC was performed with a Waters Delta 600 pump, and a Waters 600 controller with Empower 2 software. Eluted peaks were detected with a Waters 2420 evaporative light scattering (ELS) detector or a Waters 2996 photodiode array (PDA) detector (Waters Ltd.). NMR experiments were conducted on Varian 400, 500, 600, and 700 MHz instruments. Chemical shifts are reported relative to deuterated solvent peaks or 3-(trimethylsilyl)-propionic-2,2,3,3,-d_4 acid sodium salt in D_2O as an internal standard. The ESI mass spectra were recorded on Agilent Technologies 6220 TOF after dissolving samples in $CHCl_3$ or CH_3OH and adding NaCl.

Enzyme Preparation. Enzymes for the one-pot sialylation reactions were prepared as described previously.[37, 42] The aldolase was *E. coli* sialic acid aldolase[54] expressed with a $(His)_6$ tag in *E. coli* strain BL21(DE3) and was purified using a Ni-NTA column then used at a concentration of 4.6 mg mL^{-1}. The CMP-Neu5Ac synthetase was *Neisseria meningitides* CMP-Neu5Ac synthetase (NmCss)[54] expressed in *E. coli* strain AD202 grown to 2.4 x 10^8 cells mL^{-1} and used in a crude enzyme mixture. The α(2→3) sialyltransferase was *Campylobacter jejuni* α(2→3) sialyltransferase (CstII)[66] expressed in *E. Coli* strain AD202 grown to 2.4 x 10^8 cells mL^{-1} and used in a crude enzyme mixture, and the α(2→6) sialyltransferase was *Photobacterium damsela* α(2→6) sialyltransferase (Pd2,6ST) expressed with a $(His)_6$ tag in E. Coli strain Nova Blue (DE3). and was purified using a Ni-NTA column then used at a concentration of 3.0 mg mL^{-1}.[42, 67] Human neuraminidase enzymes NEU2 and NEU3 were expressed as fusion proteins with maltose-binding protein. Human neuraminidase enzyme NEU4 was expressed as a fusion protein with glutathione-S-transferase protein. Isoenzymes NEU2-4 were purified as described.[37, 65, 68] Specific activity of the neuraminidase enzymes was determined against 4-methylumbelliferyl α-$_D$-N-acetylneuraminic acid (4MU-NANA) in comparison to a standard curve of neuraminidase from *Clostridium perfringens.*

Human neuraminidase enzyme NEU1 was produced from HEK293E cells. HEK293E cells (transduced with a Lentivirus expressing Cathepsin A and NEU1 with an N-terminal $(His)_6$ tag).[69] The highest expressing stable cell line was selected from the transduced cell mixture (40 % transducing efficiency), using Amphotericin B (10 X). When at least 10 plates (10 cm^2) of this highest expressing cell line was grown, the cells were washed three times with 1 mL PBS buffer. The cells were harvested with 300 µL of PBS buffer per plate. The harvested mixture was sonicated (5 x 10 s, 60 W, on ice). Cells were lysed with 4 x 75 µL lysis buffer (1 mM EDTA, 0.2M NaCl,

20 mM MOPS, 0.1 % Triton X-100 pH 7.2) and shaken for 1 hr at 4 °C. NEU1 was used in further assays as a crude cell lysate, in which the majority of the neuraminidase activity could be accounted for by NEU1 (Figure S4).

Solution-phase kinetics assay with 4MU substrates. Substrate (**1** or **2**), (30 uL, 0.5 mM in H_2O) was incubated at 37 °C for 15 min in 60 µL 0.1 M sodium acetate buffer at the enzyme's optimum pH (4.5 for NEU1, NEU3 and NEU4, 5.6 for NEU2). Enzyme was added (30 µL, 3.33 x 10^{-5} U μL^{-1}) and the assay mixture was incubated at 37 °C. At timepoints of 0, 10, 20, 30, and 40 minutes, a sample of assay mixture (20 µL) was removed and quenched in 100 µL of 0.2 M Na glycine buffer (pH 10.2). Fluorescence was measured on a SpectraMax M2^e plate reader (Molecular Devices, excitation 365 nm, emission 445 nm). Relative rates were determined, after background subtraction of fluorescence at time = 0, by linear regression forcing through time = 0 using Graphpad Prism and are an average of three runs. Relative rates are relative to a matched Neu5Ac control (4MU-Neu5Ac, **1**)

Kinetics assay employing malononitrile for the detection of free sialic acid. Enzymes were diluted to 15 µL in 20 mM MOPS with 0.2 M NaCl, pH 7.2. Substrates (**4**-**9**) (2.5 x 10^{-8} mol in 45 µL water) were incubated at 37 °C for 15 min in 120 µL 0.1 M sodium acetate buffer, followed by addition of the appropriate enzyme to a final pH at the enzyme's optimum (pH 5.6 for NEU2, pH 4.5 for NEU1, NEU3, and NEU4; 0.003 U for NEU1, 0.0013 U for NEU2, 0.00075 U for NEU3, 0.0029 U for NEU4). At each time point, 30 µL of the assay solution was removed and quenched into 50 µL 0.2 M sodium borate (pH 9.5), and 15 µL of 0.8% (w/v) malononitrile was added to the solution followed by heating to 100 °C for 20 min. Fluorescence was measured on a SpectraMax M2^e plate reader (Molecular Devices, excitation 357 nm, emission 434 nm). Relative rates were determined by linear regression of three average runs and are reative to a matched Neu5Ac control

(α(2,3) Neu5Ac octyl sialyllactoside, **4**). Points were corrected for background and fitting was forced through the zero point using Graphpad Prism. Data were evaluated to exclude any outliers identified by applying Dixon's Q Test.[70] .

ESI-MS kinetic measurements. All ESI-MS measurements were carried out in negative ion mode using a Synapt G2S instrument (Waters) equipped with a nano-ESI source. Nanoflow ESI (nanoESI) tips were produced from borosilicate capillaries (1.0 mm o.d., 0.68 mm i.d.), which were pulled in-house using a P-1000 micropipette puller (Sutter Instuments). To carry out nanoESI, a voltage of -0.8 kV was applied to a platinum wire inserted into the nanoESI tip. For all experiments, a source temperature of 60 °C was used. All data were processed using Mass Lynx software v.4.1 (Waters).

Enzymatic studies were performed by rapid manual mixing of hNEU3 (0.0002 units) with a 5 μL sample of **4**, **6**, and **8** (100 μM in 200 mM ammonium acetate buffer, pH 4.8) followed by immediate introduction into a nanoESI tip for time-resolved ESI-MS measurements. Data points were taken at reaction times ranging from 5 min to 60 min. *N*-acetyl-9-azido-9-deoxy-neuraminic acid (IS1, 334.28 Da) and leucine enkephalin (IS2, 555.62 Da) served as internal standards.[37] As a negative control, ESI mass spectra were acquired in identical conditions but in the absence of NEU3. The ion abundance (*Ab*) ratio of substrate (SX)-to-IS or product (PSX)-to-IS was monitored as a function of reaction time, as shown in equations (1) and (2)

$$\frac{SX}{IS} = \frac{Ab(SX)}{Ab(IS)} \qquad (1)$$

$$\frac{PSX}{IS} = \frac{Ab(PSX)}{Ab(IS)} \qquad (2)$$

The reported relative rates correspond to the average of the values determined with IS1 and IS2.

Molecular dynamics (MD) simulations. MD simulations were run for the trisasccharide of octyl-GM3 analogs **4**,**6**, and **8** bound to NEU2 and NEU3. We used the crystal structure of NEU2 bound to DANA (PDB: 1VCU),[64] and aligned carbons of the sialic acid in the three trisaccharides to the corresponding carbon atoms in DANA. We used a similar procedure for NEU3, starting from our homology model. The ordered waters found in the NEU2 crystal structure[64] were kept during system set up and were added to the NEU3 systems. All simulations were run in AMBER 14[71] using *pmemd.cuda* (GPU acceleration) on Nvidia GeForce GTX 980 GPUs. The *ff14SB* force field[72] was used for NEU2 and NEU3 and GLYCAM06[73] was used for **4**, **6**, and **8**. We set the initial ϕ and ψ angles for **4**, **6**, and **8** to those described by DeMarco and Woods.[74] Each complex was neutralized with the addition of Na+ ions and was solvated in a box of TIP3P water[75] with 10 Å between the solute and the edges of the box in all dimensions.

For all six systems, the water was first minimized using 100 steps of steepest decent, followed by 4900 steps of conjugate gradient. Then the entire system was minimized with 100 steps of steepest decent, followed by 4900 steps of conjugate gradient. The systems were then further equilibrated by heating from 5 K to 300 K over 50 ps, followed by cooling back to 5 K over an additional 50 ps. After the annealing step, the systems were again heated from 5 K to 300 K over 100 ps, then allowed to run at 300 K for 100 ps before the production simulations were started. Production was run for 25 ns. The timestep was 2 fs, bonds to hydrogen were constrained with the SHAKE[76] algorithm, and the cutoff for non-bonded interactions was 8.0 Å. The temperature was maintained with the Berendsen thermostat[77] (*ntt* = 1) with velocities rescaled every 1 ps.

Synthetic methods

Chemoenzymatic synthesis of octyl sialyllactosides. Enzymatic reactions were performed with stirring at 37 °C. Reaction was monitored by TLC using 6:3:3:2 ethyl acetate: acetic acid: methanol: H_2O as an eluent system and charring with 5 % sulfuric acid in ethanol. Upon completion, ethanol was added and the reaction mixture was centrifuged at 17,000 rpm for 1 hour. The supernatant was collected and lyophilized. Crude product was purified with a Sep-pack C-18 reverse phase cartridge. The product was eluted with MeOH:H_2O (1:2).

Method A: For Neu5Ac octyl sialyllactosides (4, 5). Neu5Ac (2.90 mg, 9.4 μmol), cytidine triphosphate disodium salt (4.95 mg, 9.4 μmol, 1 M $MgCl_2$ (80 μL), and deionized H_2O (600 μL) were dissolved in 1 M Tris-HCl (400 μL, pH 8.8). The reaction was charged with CMP-Neu5Ac synthetase (200 μL), β-octyl lactoside (**SI4**) (2.5 mg, 6.2 μmol), sialyltransferase (200 uL), and deionized H_2O (600 μL). The reaction proceeded overnight.

Method B: For Neu5Gc octyl sialyllactosides (6, 7). N-glycolyl-D-mannosamine (ManGc, 2.25 mg, 9.4 μmol), cytidine triphosphate disodium salt (4.95 mg, 9.4 μmol, 1 M $MgCl_2$ (80 μL), and deionized H_2O (600 μL) were dissolved in 1 M Tris-HCl (400 μL, pH 8.8). The reaction was charged with sialic acid aldolase, CMP-Neu5Ac synthetase, β-octyl lactoside (**SI4**) (2.5 mg, 6.2 μmol), sialyltransferase (300 uL), and deionized H_2O (600 μL). The reaction proceeded overnight.

Method C: For Neu5,9Ac$_2$ octyl sialyllactosides (8, 9). Neu5,9Ac_2 (**SI6**) (2.5 mg, 8.5 mmol), cytidine triphosphate disodium salt (4.95 mg, 9.4 μmol, 1 M $MgCl_2$ (80 μL), and deionized H_2O (600 μL) were dissolved in 1 M Na HEPES (400 μL, pH 7.2). The reaction was charged with CMP-Neu5Ac synthetase (200 μL), β-octyl lactoside (**SI4**) (2.0 mg, 5.0 μmol), sialyltransferase (200 μL), and deionized H_2O (600 μL). The reaction proceeded for 3 hours.

4-methylcoumarin-7-yl 5-acetamido-9-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid (2). A solution of 4-MU-NANA (**1**, in 500 µL dry DMF), prepared as previously reported[78] (5 mg, 0.01 mmol), was charged with glacial acetic acid (5 µL) and trimethyl orthoacetate (20 uL, 0.157 mmol). The reaction was stirred for four hours and then dried under reduced pressure. The crude mixture was purified by HPLC on a C-18 reversed phase Waters (10 µm, 10 x 250 mm) column. Pure **2** was eluted with a linear gradient of 0-70% acetonitrile in water over 30 minutes, with a flow rate of 7 mL/min to give a 5 % yield as a white solid. ^{1}H NMR data was consistent with previous reports.[46] ^{13}C NMR (125 MHz, D_2O) δ 177.9 ($NH\underline{C}OCH_3$), 177.2 ($O\underline{C}OCH_3$), 175.8 (C-1), 167.6 (C-2'), 160.1 (C-8'), 159.3 (C-10'), 156.3 (C-5'), 129.1 (C-6'), 120.3 (C-7'), 119.1 (C-4'), 114.6 (C-3'), 110.7 (C-9'), 105.2 (C-2), 76.6, 72.2, 71.5, 70.6, 68.9 (C-9), 54.6, 43.7 (C-3), 24.9 ($NHCO\underline{C}H_3$), 23.1 ($OCO\underline{C}H_3$), 20.9 (C-4'-$\underline{C}H_3$). ESI-MS calculated for $C_{23}H_{27}NO_{12}$ $[M-H]^-$ 508.1460 found: 508.1462.

***O*-(5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)- (2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-octanol (4).** Compound **4** was prepared using Method A to yield 4 mg (87 %) as a white solid. ^{1}H and ^{13}C NMR data were consistent with previous reports.[37, 79]

***O*-(5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2→6)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-octanol (5).** Compound **5** was prepared using Method A to yield 3.8 mg (83 %) as a white solid. ^{1}H NMR (700 MHz, D_2O) δ 4.49 (d, J = 8.2 Hz, 1H, H-1'), 4.43 (d, J = 8.3 Hz, 1H, H-1''), 4.00-3.52 (H-5', H-6a', H-6b', H-5'', H-6a'', H-6b'', H-7''', H-8''', H-9a''', H-9b'''), 3.95 (1H, H-4''', from TOCSY), 3.93 (1H, $OC\underline{Ha}Hb(CH_2)_6CH_3$, from TOCSY, 3.87 (1H, H-5''', from TOCSY), 3.70 (1H, $OCHa\underline{Hb}(CH_2)_6CH_3$, from TOCSY), 3.69 (1H, H-3'', from TOCSY), 3.67 (1H, H-4''', from

COSY), 3.65 (1H, H-3', from TOCSY), 3.54 (1H, H-2'', from COSY), 3.34 (t, *J* = 8.6 Hz, 1H, H-2'), 2.72 (dd, *J* = 12.6, 4.7 Hz, 1H, H-3eq'''), 2.04 (s, 3H, NHCOC$\underline{H}_3$), 1.75 (t, *J* = 12.5 Hz, 1H, H-3ax'''), 1.66-1.61 (m, 2H, $OCH_2C\underline{H}_2(CH_2)_5CH_3$), 1.39-1.25 (m, 10H, $OCH_2CH_2(C\underline{H}_2)_5CH_3$), 0.87 (t, *J* = 6.6 Hz, 3H, $O(CH_2)_7C\underline{H}_3$). ^{13}C NMR (175 MHz, D_2O) δ 177.7 ($NH\underline{C}OCH_3$, from HMBC), 175.8 (<u>C</u>OO-, from HMBC), 104.5 (C-1'', from HSQC), 103.0 (C-1', from HSQC), 102.9 (C-2''', from HMBC), 82.0 (C-2'', from HMBC), 78.5 (C-3''', from HMBC), 76.7, 74.7, 73.91, 73.86 (C-2', from HSQC), 73.1, 73.0 (C-4''', from HSQC), 72.7, 70.2 ($O\underline{C}H_2(CH_2)_6CH_3$, from HMBC), 69.3 (C-4'', from HSQC), 68.9, 64.5, 63.6, 62.8, 61.4, 53.2 (C-5''', from HSQC), 40.5 (C-3''', from HSQC), 29.8 ($OCH_2\underline{C}H_2(CH_2)_5CH_3$, from HSQC), 29.5, 26.7, 26.5 ($OCH_2CH_2\underline{C}H_2(CH_2)_4CH_3$, from HMBC), 25.7 ($O(CH_2)_6\underline{C}H_2CH_3$, from HMBC), 23.4 ($NHCO\underline{C}H_3$, from HSQC), 22.4, 14.5 ($O(CH_2)_7CH_3$, from HSQC). ESI-MS calculated for $C_{31}H_{55}NO_{19}$ $[M-H]^-$ 744.3296 found: 744.3300.

***O*-(5-glycolylamido-3,5,-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-octanol (6).** Compound **6** was prepared using Method B to yield 2.5 mg (53%) as a white solid. ^{1}H NMR (700 MHz, D_2O) δ 4.54 (d, *J* = 7.8 Hz, 1H, H-1''), 4.49 (d, *J* = 8.1 Hz, 1H, H-1'), 4.14 (d, *J* = 3.2 Hz, 1H, H-3''), 4.13 (s, 2H, $NHCOC\underline{H}_2OH$), 4.01-3.53 (H-4', H-5', H-6a', H-6b',H-5'', H-6a'', H-6b'', H-7''', H-8''', H-9a''', H-9b'''), 3.97 (1H, H-4'', from COSY), 3.95 (1H, H-5''', from TOCSY), 3.80 (1H, H-4''', from COSY), 3.78 (1H, H-6''', from TOCSY), 3.66 (1H, H-3', from HSQC), 3.60 (1H, H-2'', from COSY), 3.31 (t, *J* = 8.5 Hz), 1H, H-2'), 2.79 (dd, *J* = 12.4, 4.7 Hz, 1H, H-3e'''), 1.83 (t, *J* = 12.2 Hz, 1H, H-3a'''), 1.63 (m, 2H, $OCH_2C\underline{H}_2(CH_2)_5CH_3$), 1.39-1.26 (m, 10H, $OCH_2CH_2(C\underline{H}_2)_5CH_3$), 0.87 (t, *J* = 7.0 Hz, 3H, $O(CH_2)_7C\underline{H}_3$. ^{13}C NMR (175 MHz, D_2O) δ 176.5 (NH-<u>CO</u>-CH_2OH, from HMBC), 175.3 (<u>C</u>OO-, from HMBC), 100.4 (C-2''', from HMBC), 103.4

(C-1’’, from HSQC), 102.7 (C-1’, from HSQC), 79.0, (C-2’’, from HMBC), 77.1, 76.8 (C-3’, from HMBC), 76.4 (C-3’’, from HSQC), 74.5, 74.2 (C-6’’’, from HSQC), 73.6, 72.0 (C-2’, from HMBC), 70.4, 69.1 (C-4’’’, from HMBC), 68.8, 68.6 (C-4’’, from HSQC), 63.0, 62.8, 61.7 (CH_2OH, from HSQC), 52.3 (C-5’’’, from HSQC), 52.3, 40.4 (C-3’’’, from HSQC), 32.6, 29.8 ($OCH_2\underline{C}H_2(CH_2)_5CH_3$, from HSQC), 29.3, 28.5 ($OCH_2CH_2\underline{C}H_2(CH_2)_4CH_3$, from HMBC), 27.0, 24.4 ($O(CH_2)_6\underline{C}H_2CH_3$, from HMBC), 14.5 ($O(CH_2)_7\underline{C}H_3$, from HSQC). ESI-MS calculated for $C_{31}H_{55}NO_{20}$ $[M-H]^-$ 760.3245 found: 760.3245.

***O*-(5-glycolylamido-3,5,-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2→6)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-octanol (7).** Compound **7** was prepared using Method B to yield 3.2 mg (68%) as a white solid. ^{1}H NMR (700 MHz, D_2O) δ 4.49 (d, *J* = 8.3 Hz, 1H, H-1’), 4.44 (d, *J* = 8.1 Hz, 1H, H-1’’), 4.13 (s, 2H, CH_2OH), 4.01-3.53 (H-4’, H-5’, H-6a’, H-6b’, H-4’’, H-5’’, H-6a’’, H-6b’’, H-5’’’, H-6’’’, H-7’’’, H-8’’’, H-9a’’’, H-9b’’’) 3.81 (1H, H-4’’’, from COSY), 3.71 (2H, $OCH_2(CH_2)_6CH_3$, from COSY), 3.70 (1H, H-3’, from COSY), 3.57 (H-2’’, from COSY), 3.34 (t, *J* = 8.7 Hz, 1H, H-2’), 2.74 (dd, *J* = 12.4, 4.6 Hz, 1H, H-3eq’’’), 1.77 (t, *J* = 12.3 Hz, 1H, H-3ax’’’), 1.66-1.61 (m, 2H, $OCH_2C\underline{H_2}(CH_2)_5CH_3$), 1.39-1.25 (m, 10H, $OCH_2CH_2(C\underline{H_2})_5CH_3$), 0.87 (t, *J* = 7.3 Hz, 3H, $O(CH_2)_7C\underline{H_3}$. ^{13}C NMR (175 MHz, D_2O) δ 176.8 ($NH\underline{C}OCH_2OH$, from HMBC), 173.8 ($\underline{C}OO$-, from HMBC), 105.6 (C-1’’, from HSQC), 104.3 (C-1’, from HSQC), 101.6 (C-2’’’, from HMBC), 80.9 (C-2’’, from HMBC), 79.8, 76.4, 75.6, 75.3, 73.2, 72.9, 71.5, 71.3, 70.6, 70.2, 69.9 (C-4’’’, from HMBC), 66.2, 64.9, 64.5, 63.5, 62.1 ($\underline{C}H_2OH$, from HSQC), 53.5, 40.9 (C-3’’’, from HSQC), 31.4, 29.7, 29.6 ($OCH_2\underline{C}H_2(CH_2)_5CH_3$, from HSQC), 26.4, 25.3 ($O(CH_2)_6\underline{C}H_2CH_3$, from HMBC), 23.3, 14.8

($O(CH_2)_7\underline{C}H_3$, from HSQC). ESI-MS calculated for $C_{31}H_{55}NO_{20}$ $[M-H]^-$ 760.3245 found: 760.3242.

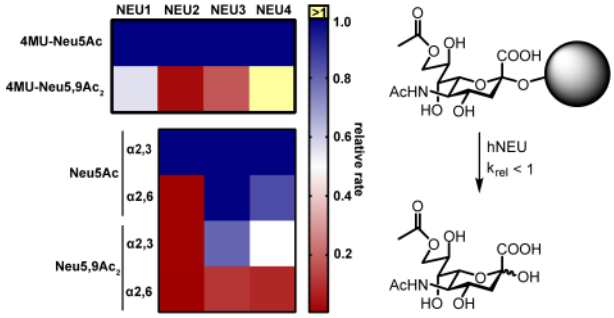
***O*-(5-Acetamido-9-acetoxy-3,5,9-trideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-octanol (8).** Compound **8** was prepared using Method C to yield a mixture of **4**:**8** 1:2. To yield pure product **8**, the crude mixture was separated using HPLC on a C-18 reversed-phase Waters Xterra (3.5 μm, 4.8 x 150 mm) column. The trisaccharides were eluted with a linear gradient of 20–50% methanol in H_2O over 30 min, with a flow rate of 0.7 mL/min, to yield 0.4 mg (10%) of **8** as a white solid. ^{1}H NMR (700 MHz, D_2O) δ 4.54 (d, *J* = 7.8 Hz, 1H, H-1''), 4.49 (d, *J* = 8.1 Hz, 1H, H-1'), 4.42 (dd, *J* = 11.8, 2.2 Hz, 1H, H-9a'''), 4.20 (dd, *J* = 11.8, 6.4 Hz, 1H, H-9b'''), 4.13-4.09 (m, 2H, H-8''', H-3'', from COSY, TOCSY), 4.02-3.54 ($OC\underline{H}_2(CH_2)_6CH_3$, H-3', H-4', H-5', H-6a', H-6b', H-5'', H-6a'', H-6b', H-6'''), 3.97 (1H, H-4'', from TOCSY), 3.87 (1H, H-5''', from TOCSY), 3.69 (1H, H-4''', from COSY), 3.66 (1H, H-7''', from TOCSY), 3.59 (1H, H-2'', from COSY), 3.31 (t, *J* = 8.3 Hz, 1H, H-2'), 2.77 (dd, *J* = 12.8, 4.8 Hz, 1H, H-3e'''), 2.15 (s, 3H, $OCOC\underline{H}_3$), 2.05 (s, 3H, $NHCOC\underline{H}_3$), 1.81 (t, *J* = 12.2 Hz, 1H, H-3a'''), 1.66-1.61 (m, 2H, $OCH_2C\underline{H}_2(CH_2)_5CH_3$), 1.39-1.25 (m, 10H, $OCH_2CH_2(C\underline{H}_2)_5CH_3$), 0.87 (t, *J* = 7.0 Hz, 3H, $O(CH_2)_7C\underline{H}_3$. ^{13}C NMR (175 MHz, D_2O) δ 176.1 ($NH\underline{C}OCH_3$, from HMBC), 175.4 ($O\underline{C}OCH_3$, from HMBC), 175.0 (<u>C</u>OO-, from HMBC), 103.3 (C-1'', from HSQC), 103.0 (C-1', from HSQC), 101.2 (C-2''', from HMBC), 79.6 (C-2'', from HMBC), 76.6, 76.3, 76.1, 76.1 (C-7''', from HMBC), 75.9, 74.8, 73.4 (C-2', from HMBC), 72.4, 68.9, 66.8 (C-9''', from HSQC), 63.4, 62.2, 62.2, 61.6 (C-4'', from HSQC), 60.7, 53.6, 40.6 (C-3''', from HSQC), 30.2 ($OCH_2\underline{C}H_2(CH_2)_5CH_3$, from HSQC), 29.5-25.0 ($OCH_2CH_2(\underline{C}H_2)_4CH_2CH_3$ 23.9 ($OCH_2(CH_2)_5\underline{C}H_2CH_3$, from HMBC),

23.8 (NHCO<u>C</u>H_3, from HSQC), 21.5 (OCOCH_3, from HSQC), 14.8 (O$(CH_2)_7$<u>C</u>H_3, from HSQC). ESI-MS calculated for $C_{33}H_{57}NO_{20}$ $[M-H]^-$ 786.3401 found: 786.3403.

***O*-(5-Acetamido-9-acetoxy-3,5,9-trideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2→6)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-octanol (9).** Compound **9** was prepared using Method C to yield 1.3 mg (33%) as a white solid. ^{1}H NMR (700 MHz, D_2O) δ 4.49 (d, *J* = 7.8 Hz, 1H, H-1'), 4.44 (d, *J* = 7.8 Hz, 1H, H-1''), 4.42 (dd, *J* = 12.0, 2.2 Hz, 1H, H-9a'''), 4.21 (dd, *J* = 12.0, 5.8 Hz, 1H, H-9b'''), 4.12 (ddd, *J* = 9.6, 5.8, 2.2 Hz, 1H, H-8'''), 4.01-3.52 (OC<u>H</u>$_2$$(CH_2)_6CH_3$, H-6a', H-6b', H-5'', H-6a'', H-6b'', H-5''', H-6'''), 4.00 (1H, H-5', from TOCSY), 3.95 (1H, H-4'', from TOCSY), 3.81 (1H, H-4', from TOCSY), 3.68 (1H, H-3', from COSY), 3.68 (H-4''', from COSY), 3.67 (1H, H-3'', from COSY), 3.62 (1H, H-7''', from COSY), 3.55 (1H, H-2'', from COSY), 3.34 (t, *J* = 8.7 Hz, 1H, H-2'), 2.72 (dd, *J* = 12.6, 4.7 Hz, 1H, H-3eq'''), 2.14 (s, 1H, OCOC<u>H</u>$_3$), 2.05 (s, 1H, NHCOC<u>H</u>$_3$), 1.75 (t, *J* = 12.1 Hz, 1H, H-3ax'''), 1.67-1.61 (m, 2H, OCH_2C<u>H</u>$_2$$(CH_2)_5CH_3$), 1.39-1.25 (m, 10H, $OCH_2CH_2(C$<u>H</u>$_2)_5CH_3$), 0.87 (t, *J* = 6.8 Hz, 3H, O$(CH_2)_7C$<u>H</u>$_3$). ^{13}C NMR (175 MHz, D_2O) δ 175.4 (NH<u>C</u>OCH_3, from HMBC), 175.0 (O<u>C</u>OCH_3, from HMBC), 174.4 (<u>C</u>OO-, from HMBC), 103.9 (C-1'', from HSQC), 103.0 (C-1', from HSQC), 101.8 (C-2''', from HMBC), 76.7 (C-3', from HMBC), 75.1, 74.9, 74.26, 74.25, 73.7, 73.6 (C-2', from HSQC), 71.8, 71.7 (C-2'', from HSQC), 70.4 (C-8''', from HSQC), 69.3 (C-4''', from HMBC), 68.8 (C-4'', from HSQC), 67.4 (C-7''', from HMBC), 66.9 (C-9''', from HSQC), 63.5, 63.1, 52.8, 41.1 (C-3''', from HSQC), 32.6, 30.0, 29.7 (OCH_2<u>C</u>$H_2(CH_2)_5CH_3$, from HSQC), 26.9, 26.3 (O$(CH_2)_6$<u>C</u>H_2CH_3, from HMBC), 23.4, 23.3 (NHCO<u>C</u>H_3, from HSQC), 21.5 (OCO<u>C</u>H_3, from HSQC), 14.4 (O$(CH_2)_7$<u>C</u>H_3, from HSQC). ESI-MS calculated for $C_{33}H_{57}NO_{20}$ $[M-H]^-$ 786.3401 found: 786.3396.

Figures and Tables

Graphical Abstract



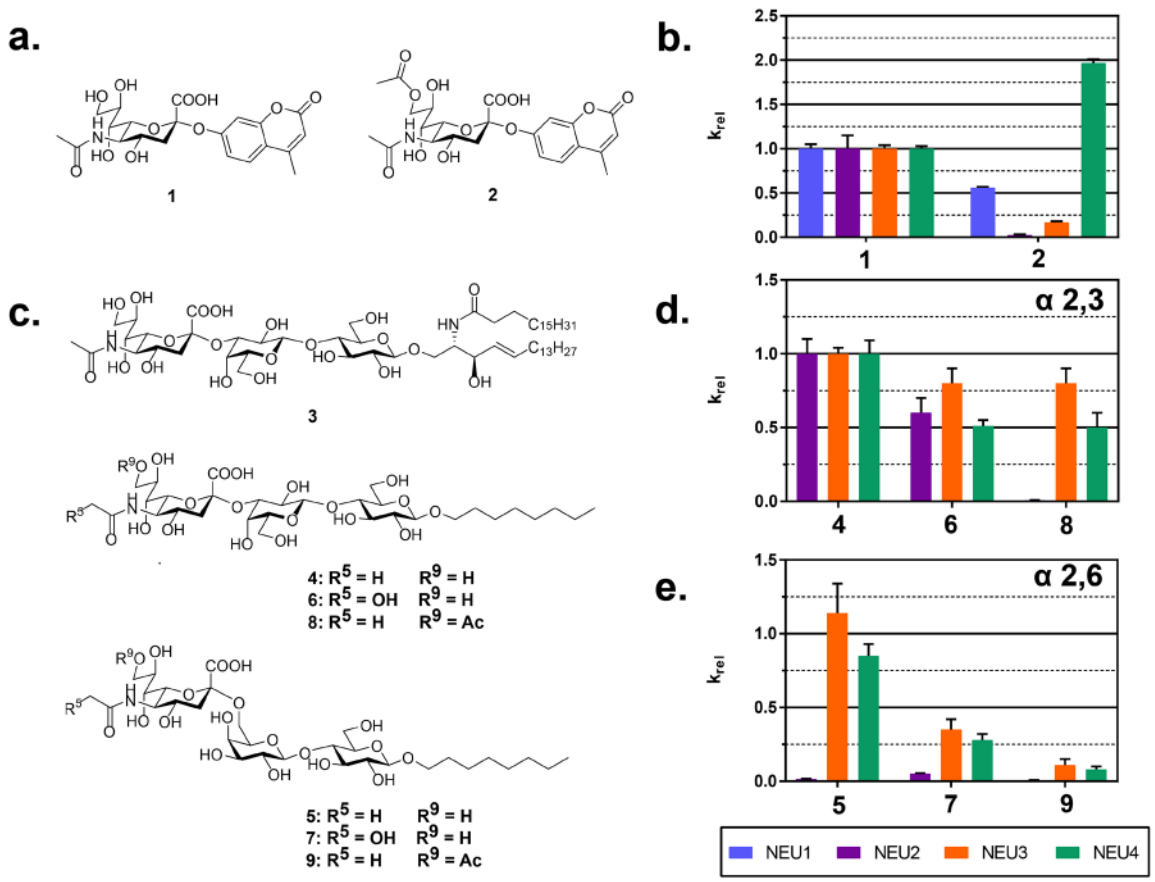


Figure 1 Human neuraminidase substrate activity assays. a) Structure of fluorogenic hNEU substrates 4MU-Neu5Ac **1** and 4MU-Neu5,9Ac$_2$ **2**. b) Effect of sialic acid 9-*O*-acetylation on hNEU hydrolysis of 4MU-substrates. Rates were obtained by linear regression of triplicate experiments. Data is presented as k_{rel} and normalized to 4MU-Neu5Ac **1**, and error bars correspond to one standard deviation. c) Structures of hNEU substrate GM3 **3** and octyl sialyllactoside targets containing α(2→3) (**4**, **6**, **8**) or α(2→6) (**5**, **7**, **9**) linkages. d) Substrate specificity of hNEU towards octyl sialyllactoside substrates containing an α(2→3) linkage to galactose. Data are given as relative rates, normalized to α(2→3) Neu5Ac **4** substrate. Data for Neu5Ac and Neu5Gc α(2→3) octyl sialyllactosides **4** and **6** were taken every one minute for 4 minutes. Rates for substrate **8** were obtained by taking points every 10 min over 40 min in triplicate experiments and error is shown for one standard deviation. e) Substrate specificity of hNEU towards octyl sialyllactoside substrates containing an α(2→6) linkage to galactose. Data are given as relative rates, normalized to α(2→3) Neu5Ac **5** substrate. Rates were obtained by taking points every 10 min over 40 min in triplicate experiments and error is shown as one standard deviation. NEU1 cleavage of octyl sialyllactosides **4** and **5** was too slow for detection and is not shown.

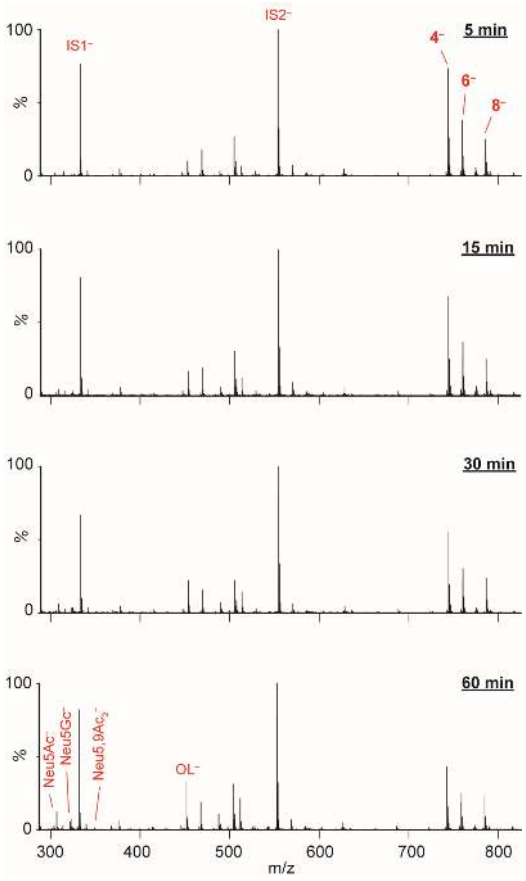


Figure 2. Time-resolved ESI-MS data acquired for NEU3 cleaving α(2→3) linked substrates**.** Mass spectra were measured in negative ion mode for 200 mM aqueous ammonium acetate solutions (pH 4.8, 22 °C) of **4**, **6**, and **8** (100 mM each) and NEU3 (0.0002 units). OL^- is the octyl lactoside anion; the internal standards are *N*-acetyl-9-azido-9-deoxy-neuraminic acid (IS1) and leucine enkephalin (IS2).

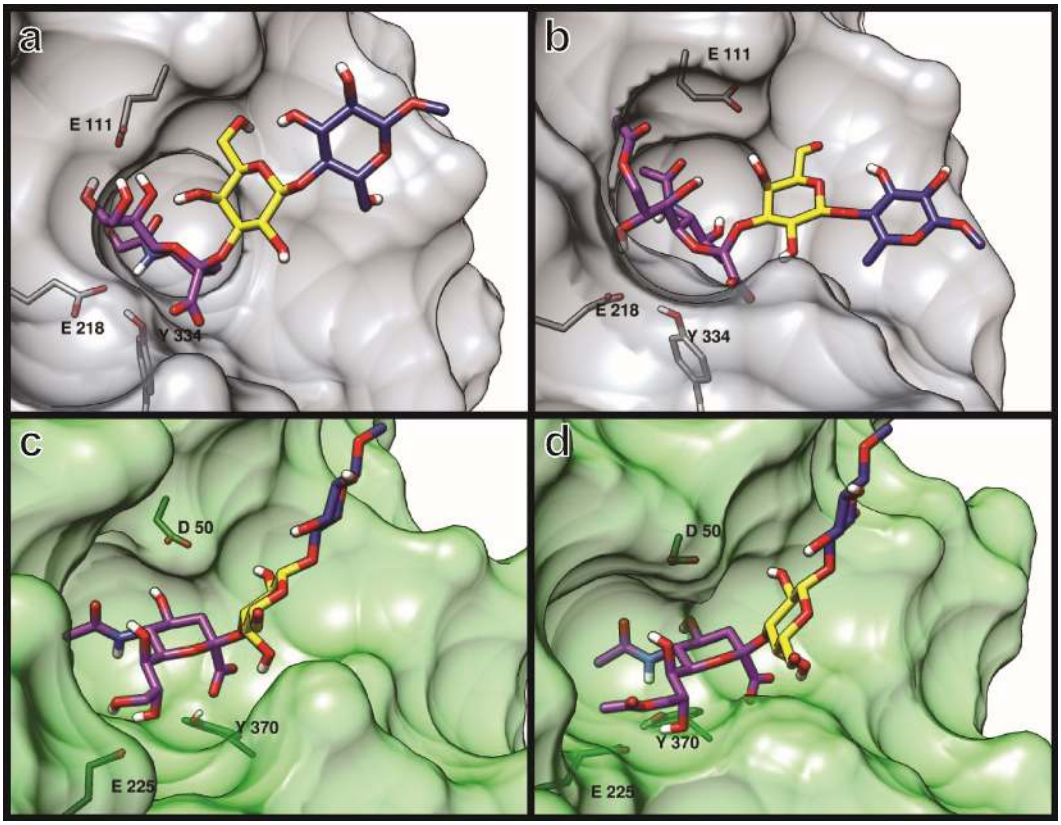


Figure 3. Models of substrate binding to NEU2 and NEU3**.** View of the active site of a) NEU2 with α(2→3) Neu5Ac-Lac-CH_3 (methyl sialyllactoside analogue of **4**) bound to the active site. b) NEU2 with α(2→3) Neu5,9Ac_2-Lac-CH_3 (methyl sialyllactoside analogue of **8**) bound to the active site. c) NEU3 with methyl sialyllactoside analogue of **4** bound to the active site d) NEU3 with methyl sialyllactoside analogue of **8** bound to the active site.

Table 1. Results of time-resolved ESI-MS data for NEU3 cleaving α(2→3) linked octyl sialyllactosides.

		Single Substrate[a]		Mixture[a]	
Substrate		Substrate depletion	Product formation	Substrate depletion	Product formation
4	Neu5Ac	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.1	1.00 ± 0.2
6	Neu5Gc	0.6 ± 0.1	0.42 ± 0.07	0.37 ± 0.07	0.44 ± 0.08
8	$Neu5,9Ac_2$	0.17 ± 0.06	0.06 ± 0.03	0.25 ± 0.02	0.11 ± 0.03

a. Results are reported as relative rates (k_{rel}) using the mean of three runs, and errors corresponding to one standard deviation.

Acknowledgements. HEK293 cells expressing NEU1 were a kind gift from A. V. Pshezhetsky, Department of Pediatrics and Biochemistry, University of Montreal, Montreal, Quebec. C. Hunter acknowledges support from an NSERC CGSD Scholarship. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Alberta Glycomics Centre.

Supporting information. Supporting information is available including plots of the kinetic data for substrates, additional details regarding substrate synthesis, and details of molecular dynamics simulations. This material is available free of charge *via* the internet at http://pubs.acs.org.

References

1. Angata, T., and Varki, A. (2002) Chemical diversity in the sialic acids and related α-keto acids: an evolutionary perspective, *Chem. Rev. 102*, 439-470.
2. Varki, N. M., and Varki, A. (2007) Diversity in cell surface sialic acid presentations: implications for biology and disease, *Lab. Invest. 87*, 851-857.
3. Schwarzkopf, M., Knobeloch, K.-P., Rohde, E., Hinderlich, S., Wiechens, N., Lucka, L., Horak, I., Reutter, W., and Horstkorte, R. (2002) Sialylation is essential for early development in mice, *Proc. Natl. Acad. Sci. U. S. A. 99*, 5267-5270.
4. Shi, W.-X., Chammas, R., Varki, N. M., Powell, L., and Varki, A. (1996) Sialic Acid 9-O-Acetylation on Murine Erythroleukemia Cells Affects Complement Activation, Binding to I-type Lectins, and Tissue Homing, *J. Biol. Chem. 271*, 31526-31532.
5. Rogers, G. N., Herrler, G., Paulson, J., and Klenk, H. (1986) Influenza C virus uses 9-O-acetyl-N-acetylneuraminic acid as a high affinity receptor determinant for attachment to cells, *J. Biol. Chem. 261*, 5947-5951.
6. Sjoberg, E. R., Powell, L. D., Klein, A., and Varki, A. (1994) Natural ligands of the B cell adhesion molecule CD22 beta can be masked by 9-O-acetylation of sialic acids, *J. Cell Biol. 126*, 549-562.
7. Birks, S. M., Danquah, J. O., King, L., Vlasak, R., Gorecki, D. C., and Pilkington, G. J. (2011) Targeting the GD3 acetylation pathway selectively induces apoptosis in glioblastoma, *Neuro Oncol 13*, 950-960.
8. Klein, A., and Roussel, P. (1998) O-Acetylation of sialic acids, *Biochimie 80*, 49-57.
9. Cheresh, D. A., Varki, A. P., Varki, N. M., Stallcup, W. B., Levine, J., and Reisfeld, R. A. (1984) A monoclonal antibody recognizes an O-acylated sialic acid in a human melanoma-associated ganglioside, *J. Biol. Chem. 259*, 7453-7459.
10. Cheresh, D. A., Reisfeld, R. A., and Varki, A. P. (1984) O-acetylation of disialoganglioside GD3 by human melanoma cells creates a unique antigenic determinant, *Science 225*, 844-846.
11. Khedri, Z., Xiao, A., Yu, H., Landig, C. S., Li, W., Diaz, S., Wasik, B. R., Parrish, C. R., Wang, L.-P., Varki, A., and Chen, X. (2016) A chemical biology solution to problems with studying biologically important but unstable 9-*O*-acetyl sialic acids, *ACS Chem. Biol.*
12. Mandal, C., Chatterjee, M., and Sinha, D. (2000) Investigation of 9-O-acetylated sialoglycoconjugates in childhood acute lymphoblastic leukaemia, *Br. J. Haematol. 110*, 801-812.
13. Varki, A., Muchmore, E., and Diaz, S. (1986) A sialic acid-specific O-acetylesterase in human erythrocytes: possible identity with esterase D, the genetic marker of retinoblastomas and Wilson disease, *Proc. Natl. Acad. Sci. U. S. A. 83*, 882-886.
14. Zhu, H., Chan, H. C., Zhou, Z., Li, J., Zhu, H., and Yin, L. (2004) A gene encoding sialic-acid-specific 9-O-acetylesterase found in human adult testis, *BioMed Res. Int. 2004*, 130-136.
15. Surolia, I., Pirnie, S. P., Chellappa, V., Taylor, K. N., Cariappa, A., Moya, J., Liu, H., Bell, D. W., Driscoll, D. R., Diederichs, S., Haider, K., Netravali, I., Le, S., Elia, R., Dow, E., Lee, A., Freudenberg, J., De Jager, P. L., Chretien, Y., Varki, A., MacDonald, M. E., Gillis, T., Behrens, T. W., Bloch, D., Collier, D., Korzenik, J., Podolsky, D. K., Hafler, D., Murali, M., Sands, B., Stone, J. H., Gregersen, P. K., and Pillai, S. (2010) Functionally defective germline variants of sialic acid acetylesterase in autoimmunity, *Nature 466*, 243-247.

16. Mandal, C., Mandal, C., Chandra, S., Schauer, R., and Mandal, C. (2012) Regulation of O-acetylation of sialic acids by sialate-O-acetyltransferase and sialate-O-acetylesterase activities in childhood acute lymphoblastic leukemia, *Glycobiology 22*, 70-83.
17. Fougeray, S., Fleurence, J., Faraj, S., Bahri, M., Cochonneau, D., Terme, M., Leclair, M.-D., Thébaud, E., Paris, F., and Birklé, S. (2016) O-acetylated gangliosides: Structure, biosynthesis, immunogenicity, functions and their potential for cancer immunotherapy, *J. Cancer Res. Ther. 4*, 21-30.
18. Parameswaran, R., Lim, M., Arutyunyan, A., Abdel-Azim, H., Hurtz, C., Lau, K., Müschen, M., Robert, K. Y., von Itzstein, M., and Heisterkamp, N. (2013) O-acetylated N-acetylneuraminic acid as a novel target for therapy in human pre-B acute lymphoblastic leukemia, *J. Exp. Med. 210*, 805-819.
19. Higa, H. H., Butor, C., Diaz, S., and Varki, A. (1989) O-acetylation and de-O-acetylation of sialic acids. O-acetylation of sialic acids in the rat liver Golgi apparatus involves an acetyl intermediate and essential histidine and lysine residues--a transmembrane reaction?, *J. Biol. Chem. 264*, 19427-19434.
20. Baumann, A.-M. T., Bakkers, M. J., Buettner, F. F., Hartmann, M., Grove, M., Langereis, M. A., de Groot, R. J., and Mühlenhoff, M. (2015) 9-O-Acetylation of sialic acids is catalysed by CASD1 via a covalent acetyl-enzyme intermediate, *Nat. Comm. 6*, 7673.
21. Monti, E., Bassi, M., Papini, N., Riboni, M., Manzoni, M., Venerando, B., Croci, G., Preti, A., Ballabio, A., Tettamanti, G., and Borsani, G. (2000) Identification and expression of NEU3, a novel human sialidase associated to the plasma membrane, *Biochem. J. 349*, 343-351.
22. Miyagi, T., Wada, T., Iwamatsu, A., Hata, K., Yoshikawa, Y., Tokuyama, S., and Sawada, M. (1999) Molecular cloning and characterization of a plasma membrane-associated sialidase specific for gangliosides, *J. Biol. Chem. 274*, 5004-5011.
23. Seyrantepe, V., Landry, K., Trudel, S., Hassan, J. A., Morales, C. R., and Pshezhetsky, A. V. (2004) Neu4, a novel human lysosomal lumen sialidase, confers normal phenotype to sialidosis and galactosialidosis cells, *J. Biol. Chem. 279*, 37021-37029.
24. Pshezhetsky, A. V., Richard, C., Michaud, L., Igdoura, S., Wang, S., Elsliger, M.-A., Qu, J., Leclerc, D., Gravel, R., Dallaire, L., and Potier, M. (1997) Cloning, expression and chromosomal mapping of human lysosomal sialidase and characterization of mutations in sialidosis, *Nat. Genet. 15*, 316-320.
25. Cairo, C. W. (2014) Inhibitors of the human neuraminidase enzymes, *Med. Chem. Commun. 5*, 1067–1074.
26. Tringali, C., Papini, N., Fusi, P., Croci, G., Borsani, G., Preti, A., Tortora, P., Tettamanti, G., Venerando, B., and Monti, E. (2004) Properties of Recombinant Human Cytosolic Sialidase HsNEU2: The enzyme hydrolyzes monomerically dispersed GM1 ganglioside molecules *J. Biol. Chem. 279*, 3169-3179.
27. Bonten, E., van der Spoel, A., Fornerod, M., Grosveld, G., and d'Azzo, A. (1996) Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis, *Genes Dev. 10*, 3156-3169.
28. Sasaki, A., Hata, K., Suzuki, S., Sawada, M., Wada, T., Yamaguchi, K., Obinata, M., Tateno, H., Suzuki, H., and Miyagi, T. (2003) Overexpression of plasma membrane-associated sialidase attenuates insulin signaling in transgenic mice, *J. Biol. Chem. 278*, 27896-27902.
29. Michalski, J.-C., Corfield, A. P., and Schauer, R. (1986) Properties of human liver lysosomal sialidase, In *Biol. Chem.*, pp 715-722.

30. Li, Y., Cao, H., Yu, H., Chen, Y., Lau, K., Qu, J., Thon, V., Sugiarto, G., and Chen, X. (2011) Identifying selective inhibitors against the human cytosolic sialidase NEU2 by substrate specificity studies, *Mol. BioSyst. 7*, 1060-1072.
31. Davies, L. R. L., Pearce, O. M. T., Tessier, M. B., Assar, S., Smutova, V., Pajunen, M., Sumida, M., Sato, C., Kitajima, K., Finne, J., Gagneux, P., Pshezhetsky, A., Woods, R., and Varki, A. (2012) Metabolism of Vertebrate Amino Sugars with N-Glycolyl Groups: Resistance of α2–8-linked N-glycolylneuraminic acid to enzymatic cleavage *J. Biol. Chem. 287*, 28917-28931.
32. Schauer, R. (1978) Characterization of sialic acids, *Methods Enzymol. 50*, 64-89.
33. Oehler, C., Kopitz, J., and Cantz, M. (2002) Substrate Specificity and Inhibitor Studies of a Membrane-Bound Ganglioside Sialidase Isolated from Human Brain Tissue, *Biol. Chem. 383*, 1735-1742.
34. Nagai, T., and Yamada, H. (1988) Characterization of mouse liver sialidase and partial purification of the lysosomal sialidase, *Chem. Pharm. Bull. 36*, 4008-4018.
35. Li, W., Xiao, A., Li, Y., Yu, H., and Chen, X. (2017) Chemoenzymatic synthesis of Neu5Ac9NAc-containing α2–3-and α2–6-linked sialosides and their use for sialidase substrate specificity studies, *Carbohydr. Res. 451*, 51-58.
36. Khedri, Z., Muthana, M. M., Li, Y., Muthana, S. M., Yu, H., Cao, H., and Chen, X. (2012) Probe sialidase substrate specificity using chemoenzymatically synthesized sialosides containing C9-modified sialic acids, *Chem. Comm. 48*, 3357-3359.
37. Sandbhor, M. S., Soya, N., Albohy, A., Zheng, R. B., Cartmell, J., Bundle, D. R., Klassen, J. S., and Cairo, C. W. (2011) Substrate recognition of the membrane-associated sialidase NEU3 requires a hydrophobic aglycone, *Biochemistry 50*, 6753-6762.
38. Magesh, S., Moriya, S., Suzuki, T., Miyagi, T., Ishida, H., and Kiso, M. (2008) Design, synthesis, and biological evaluation of human sialidase inhibitors. Part 1: Selective inhibitors of lysosomal sialidase (NEU1), *Bioorg. Med. Chem. Lett. 18*, 532–537.
39. Albohy, A., Zhang, Y., Smutova, V., Pshezhetsky, A. V., and Cairo, C. W. (2013) Identification of selective nanomolar inhibitors of the human neuraminidase, NEU4, *ACS Med. Chem. Lett. 4*, 532-537.
40. Ogura, H., Furuhata, K., Sato, S., Anazawa, K., Itoh, M., and Shitori, Y. (1987) Synthesis of 9-O-acyl- and 4-O-acetyl-sialic acids, *Carbohydr. Res. 167*, 77-86.
41. Yu, H., Cheng, J., Ding, L., Khedri, Z., Chen, Y., Chin, S., Lau, K., Tiwari, V. K., and Chen, X. (2009) Chemoenzymatic synthesis of GD3 oligosaccharides and other disialyl glycans containing natural and non-natural sialic acids, *J. Am. Chem. Soc. 131*, 18467-18477.
42. Yu, H., Huang, S., Chokhawala, H., Sun, M., Zheng, H., and Chen, X. (2006) Highly efficient chemoenzymatic synthesis of naturally occurring and non-natural α2,6-linked sialosides: A P. damsela α2,6-sialyltransferase with extremely flexible donor substrate specificity, *Angew. Chem., Int. Ed. 118*, 4042-4048.
43. Rauvolfova, J., Venot, A., and Boons, G.-J. (2008) Chemo-enzymatic synthesis of C-9 acetylated sialosides, *Carbohydr. Res. 343*, 1605-1611.
44. Kleineidam, R. G., Furuhata, K., Ogura, H., and Schauer, R. (1990) 4-Methylumbelliferyl-α-glycosides of partially O-acetylated N-acetylneuraminic acids as substrates of bacterial and viral sialidases, *Biol. Chem. Hoppe-Seyler 371*, 715-720.
45. Chokhawala, H. A., Yu, H., and Chen, X. (2007) High-throughput substrate specificity studies of sialidases by using chemoenzymatically synthesized sialoside libraries, *ChemBioChem 8*, 194-201.

46. Furuhata, K., and Ogura, H. (1989) Studies on sialic acids. XIX. Syntheses of partially *O*-acetylated 4-methylcoumarin-7-yl 5-acetamido-3, 5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosidonic acids, *Chem. Pharm. Bull. 37*, 2037-2040.
47. Houliston, R. S., Endtz, H. P., Yuki, N., Li, J., Jarrell, H. C., Koga, M., van Belkum, A., Karwaski, M.-F., Wakarchuk, W. W., and Gilbert, M. (2006) Identification of a Sialate O-Acetyltransferase from Campylobacter jejuni - Demonstration of direct transfer to the C-9 position of terminal α-2, 8-linked sialic acid, *J. Biol. Chem. 281*, 11480-11486.
48. Zamora, C. Y., d'Alarcao, M., and Kumar, K. (2013) Fluorogenic sialic acid glycosides for quantification of sialidase activity upon unnatural substrates, *Bioorg. Med. Chem. Lett. 23*, 3406-3410.
49. Smutova, V., Albohy, A., Pan, X., Korchagina, E., Miyagi, T., Bovin, N., Cairo, C. W., and Pshezhetsky, A. V. (2014) Structural basis for substrate specificity of mammalian neuraminidases, *PLoS One 9*, e106320.
50. Tangvoranuntakul, P., Gagneux, P., Diaz, S., Bardor, M., Varki, N., Varki, A., and Muchmore, E. (2003) Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid, *Proc. Natl. Acad. Sci. U. S. A. 100*, 12045-12050.
51. Samraj, A. N., Pearce, O. M., Läubli, H., Crittenden, A. N., Bergfeld, A. K., Banda, K., Gregg, C. J., Bingman, A. E., Secrest, P., and Diaz, S. L. (2015) A red meat-derived glycan promotes inflammation and cancer progression, *Proc. Natl. Acad. Sci. U. S. A. 112*, 542-547.
52. Alisson-Silva, F., Kawanishi, K., and Varki, A. (2016) Human risk of diseases associated with red meat intake: Analysis of current theories and proposed role for metabolic incorporation of a non-human sialic acid, *Mol. Aspects Med. 51*, 16-30.
53. Yu, H., Chokhawala, H. A., Huang, S., and Chen, X. (2006) One-pot three-enzyme chemoenzymatic approach to the synthesis of sialosides containing natural and non-natural functionalities, *Nat. Protoc. 1*, 2485-2492.
54. Yu, H., Yu, H., Karpel, R., and Chen, X. (2004) Chemoenzymatic synthesis of CMP–sialic acid derivatives by a one-pot two-enzyme system: comparison of substrate flexibility of three microbial CMP–sialic acid synthetases, *Bioorganic Med. Chem. 12*, 6427-6435.
55. Crich, D. (2011) Methodology development and physical organic chemistry: A powerful combination for the advancement of glycochemistry, *J. Org. Chem. 76*, 9193-9209.
56. Boons, G.-J., and Demchenko, A. V. (2000) Recent advances in O-sialylation, *Chem. Rev. 100*, 4539-4566.
57. Allevi, P., Anastasia, M., Costa, M. L., and Rota, P. (2011) Two procedures for the syntheses of labeled sialic acids and their 1,7-lactones, *Tetrahedron: Asymmetry 22*, 338-344.
58. He, N., Yi, D., and Fessner, W. D. (2011) Flexibility of substrate binding of cytosine-5′-monophosphate-N-acetylneuraminate synthetase (CMP-sialate synthetase) from *Neisseria meningitidis*: An enabling catalyst for the synthesis of neo-sialoconjugates, *Adv. Synth. Catal. 353*, 2384-2398.
59. Yu, H., Chokhawala, H. A., Varki, A., and Chen, X. (2007) Efficient chemoenzymatic synthesis of biotinylated human serum albumin-sialoglycoside conjugates containing O-acetylated sialic acids, *Org. Biomol. Chem. 5*, 2458-2463.
60. Markely, L. R. A., Ong, B. T., Hoi, K. M., Teo, G., Lu, M. Y., and Wang, D. I. C. (2010) A high-throughput method for quantification of protein sialylation, *Anal. Biochem. 407*, 128-133.

61. Zou, Y., Albohy, A., Sandbhor, M., and Cairo, C. W. (2010) Inhibition of human neuraminidase 3 (NEU3) by C9-triazole derivatives of 2, 3-didehydro-N-acetyl-neuraminic acid, *Bioorg. Med. Chem. Lett. 20*, 7529-7533.
62. Mozzi, A., Mazzacuva, P., Zampella, G., Forcella, M. E., Fusi, P. A., and Monti, E. (2012) Molecular insight into substrate recognition by human cytosolic sialidase NEU2, *Proteins: Struct., Funct., Bioinf. 80*, 1123-1132.
63. Magesh, S., Suzuki, T., Miyagi, T., Ishida, H., and Kiso, M. (2006) Homology modeling of human sialidase enzymes NEU1, NEU3 and NEU4 based on the crystal structure of NEU2: Hints for the design of selective NEU3 inhibitors, *J. Mol. Graphics and Modell. 25*, 196–207.
64. Chavas, L. M. G., Tringali, C., Fusi, P., Venerando, B., Tettamanti, G., Kato, R., Monti, E., and Wakatsuki, S. (2005) Crystal structure of the human cytosolic sialidase Neu2: Evidence for the dynamic nature of substrate recognition, *J. Biol. Chem. 280*, 469–475.
65. Albohy, A., Li, M. D., Zheng, R. B., Zou, C., and Cairo, C. W. (2010) Insight into recognition and catalysis of the mammalian neuraminidase 3 (NEU3) through molecular modeling and site directed mutagenesis, *Glycobiology 20*, 1127-1138.
66. Gilbert, M., Brisson, J.-R., Karwaski, M.-F., Michniewicz, J., Cunningham, A.-M., Wu, Y., Young, N. M., and Wakarchuk, W. W. (2000) Biosynthesis of ganglioside mimics in *Campylobacter jejuni* OH4384: Identification of the glycosyltransferase genes, enzymatic synthesis of model compounds, and characterization of nanomole amounts by 600-MHz 1H and 13C NMR analysis, *J. Biol. Chem. 275*, 3896-3906.
67. Teo, C.-F., Hwang, T.-S., Chen, P.-H., Hung, C.-H., Gao, H.-S., Chang, L.-S., and Lin, C.-H. (2005) Synthesis of sialyl TN glycopeptides – enzymatic sialylation by α2,6-sialyltransferase from Photobacterium damsela, *Adv. Synth. Catal. 347*, 967-972.
68. Zhang, Y., Albohy, A., Zou, Y., Smutova, V., Pshezhetsky, A. V., and Cairo, C. W. (2013) Identification of selective inhibitors for human neuraminidase isoenzymes using C4, C7-modified 2-deoxy-2, 3-didehydro-N-acetylneuraminic acid (DANA) analogues, *J. Med. Chem. 56*, 2948-2958.
69. Pshezhetsky, A. V., and Potier, M. (1996) Association of N-acetylgalactosamine-6-sulfate sulfatase with the multienzyme lysosomal complex of β-galactosidase, cathepsin A, and neuraminidase: possible implication for intralysosomal catabolism of keratan sulfate, *J. Biol. Chem. 271*, 28359-28365.
70. Rorabacher, D. B. (1991) Statistical treatment for rejection of deviant values: critical values of Dixon's" Q" parameter and related subrange ratios at the 95% confidence level, *Analytical Chemistry 63*, 139-146.
71. Case, D. A., Babin, V., Berryman, J. T., Betz, R. M., Cai, Q., Cerutti, D. S., T.E. Cheatham, I., Darden, T. A., Duke, R. E., Gohlke, H., Goetz, A. W., Gusarov, S., Homeyer, N., Janowski, P., Kaus, J., Kolossváry, I., Kovalenko, A., Lee, T. S., LeGrand, S., Luchko, T., Luo, R., Madej, B., Merz, K. M., Paesani, F., Roe, D. R., Roitberg, A., Sagui, C., Salomon-Ferrer, R., Seabra, G., Simmerling, C. L., Smith, W., Swails, J., Walker, R. C., Wang, J., Wolf, R. M., Wu, X., and Kollman, P. A. (2014) AMBER 14, University of California, San Francisco, CA.
72. Hornak, V., Abel, R., Okur, A., Strockbine, B., Roitberg, A., and Simmerling, C. (2006) Comparison of multiple Amber force fields and development of improved protein backbone parameters, *Proteins 65*, 712–725.

73. Kirschner, K. N., Yongye, A. B., Tschampel, S. M., González-Outeiriño, J., Daniels, C. R., Foley, B. L., and Woods, R. J. (2008) GLYCAM06: A generalizable biomolecular force field. Carbohydrates, *J. Comput. Chem. 29*, 622–655.
74. DeMarco, M. L., and Woods, R. J. (2009) Atomic-resolution conformational analysis of the GM3 ganglioside in a lipid bilayer and its implications for ganglioside–protein recognition at membrane surfaces, *Glycobiology 19*, 344-355.
75. Jorjensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W., and Klein, M. L. (1983) Comparison of simple potential functions for simulating liquid water, *J. Chem. Phys. 79*, 926–935.
76. Ryckaert, J.-P., Ciccotti, G., and Berendsen, H. J. C. (1977) Numerical integration of the cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes, *J. Comp. Phys. 23*, 327–341.
77. Berendsen, H. J. C., Postma, J. P. M., van Gunsteren, W. F., DiNola, A., and Haak, J. R. (1984) Molecular dynamics with coupling to an external bath, *J. Chem. Phys. 81*, 3684–3690.
78. Myers, R. W., Lee, R. T., Lee, Y. C., Thomas, G. H., Reynolds, L. W., and Uchida, Y. (1980) The synthesis of 4-methylumbelliferyl α-ketoside of N-acetylneuraminic acid and its use in a fluorometric assay for neuraminidase, *Anal. Biochem. 101*, 166-174.
79. Albohy, A., Richards, M. R., and Cairo, C. W. (2015) Mapping substrate interactions of the human membrane-associated neuraminidase , NEU3, using STD NMR, *Glycobiology 25*, 284-293.