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Identification of Selective Nanomolar Inhibitors of the Human Neuraminidase, NEU4

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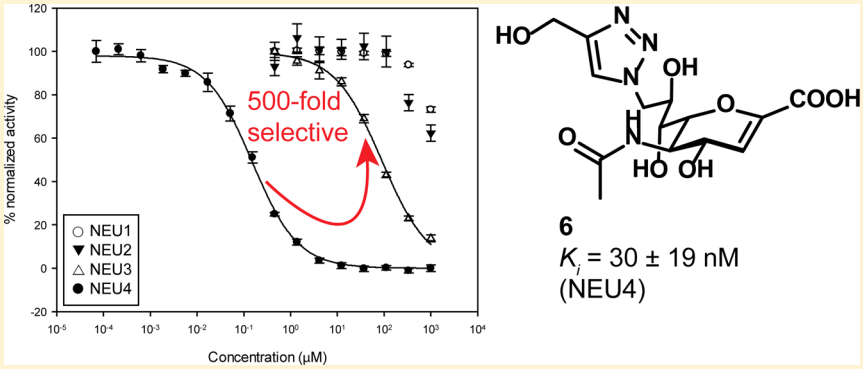
Supporting Information



ABSTRACT: The human neuraminidase enzymes (hNEU) play important roles in human physiology and pathology. The lack of potent and selective inhibitors toward these enzymes has limited our understanding of their function and the development of therapeutic applications. Here we report the evaluation of a panel of compounds against the four human neuraminidase isoenzymes. Among the compounds tested, we identified the first selective, nanomolar inhibitors of the human neuraminidase 4 enzyme (NEU4). The most potent NEU4 inhibitor (5-acetamido-9-[4-hydroxymethyl[1,2,3]triazol-1-yl]-2,3,5,9-tetradeoxy-D-glycero-D-galacto-2-nonulopyranosonic acid) was found to have an inhibitory constant (K_i) of 30 ± 19 nM and was 500-fold selective for its target over the other hNEU isoenzymes tested *in vitro* (NEU1, NEU2, and NEU3). This is the first report of any inhibitor of hNEU with nanomolar potency, and this confirms that the 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid (DANA) scaffold can be exploited to develop new, potent, and selective inhibitors that target this important family of human enzymes.

KEYWORDS: *Neuraminidase, sialidase, NEU1, NEU2, NEU3, NEU4, inhibitors, sialic acid, glycosyl hydrolase*

Although significant effort has been devoted to the development of potent inhibitors for the viral neuraminidase enzymes (vNEU),[1] the family of human neuraminidase enzymes (NEU1−NEU4) have not enjoyed as much attention. One reason for this disparity may be the lack of specific pathologies associated with hNEU. Genetic deficiency of hNEU1 leads to systemic disorders, sialidosis, and galactosialidosis caused by lysosomal storage of sialoglycoconjugates.[2] While the hNEU family has been explored for nearly two decades, evidence for their specific biochemical roles has emerged more slowly.[3−5] In particular, specific isoenzymes of hNEU have been recently reported to play important roles in inflammation,[6,7] platelet clearance,[8] insulin signaling,[9] and cancer,[10] thus highlighting the need for new tools to explore their role in human physiology.[5] Thus, chemical inhibitors for this class of enzymes are needed to explore a range of pathologies.[11] Importantly, these new chemical tools must provide selective targeting of the enzymes in order to avoid potentially toxic side effects and to discriminate between hNEU isoenzymes.

There are few reports of selective inhibitors against hNEU, and the most potent inhibitors currently known are of mid- to low-micromolar activity. Magesh et al. reported compounds selective for NEU1 (IC_{50} ∼ 10 μM).[12] Inhibitors which target hNEU over bacterial NEU have been reported.[13,14] Potent inhibitors of vNEU have only moderate activity against hNEU, with zanamivir being the most potent of these against NEU2 (IC_{50} ∼ 16 μM).[15] Our group has previously examined the activity of oseltamivir derivatives that show mild selectivity for NEU3 over NEU4.[16] To address this lack of selective inhibitors, we recently tested a series of *C*4, *C*7-modified DANA-analogs designed to target specific isoenzymes of hNEU. This study identified compounds with 12- to 40-fold selectivity against NEU2 and NEU3, respectively.[17] However, all these previous reports of inhibitors for hNEU have failed to

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identify compounds with sufficient potency or selectivity to be used *in vivo*.

In a previous report, we designed a series of *C*9-modified derivatives of DANA and tested their activity against NEU3.[18] After our recent development of assays for the remaining three hNEU isoenzymes,[17] we retested members of the original *C*9-compound series. These experiments led to the identification of compounds with unprecedented potency and selectivity for the NEU4 isoenzyme.

Our library of *C*9-modified DANA derivatives exploited Cu catalyzed azide−alkyne cycloaddition (CuAAC) between a panel of alkynes with *C*9 or *N*5-azido forms of DANA. Since initial testing of this library identified inhibitors with improved activity against NEU3 relative to the parent DANA structure, we hypothesized that some of these compounds could have potency against other isoenzymes. Therefore, we tested a subset of the original library against the four hNEU isoenzymes (Figure 1).

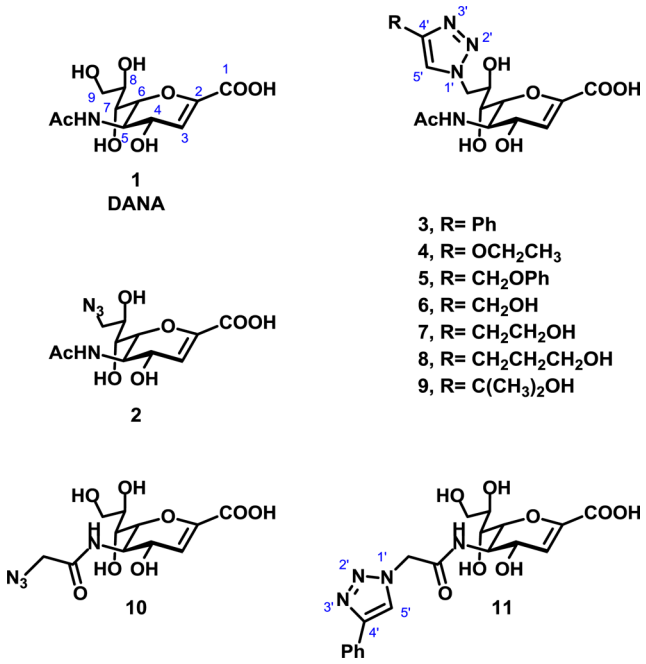

Figure 1. Structures of the compounds used in this study (**1**−**11**).

The identity of samples used in this study was confirmed by high resolution mass spectrometry, and HPLC was used to confirm that all samples were >95% purity (see Supporting Information). We then proceeded to test compounds for their ability to inhibit NEU1, NEU2, NEU3, and NEU4 enzymes.[18]

The results of enzyme inhibition assays are summarized in Table 1. We confirmed that the parent compound, DANA, had broad activity against all four isoenzymes (7−90 μM inhibition).[12,15,17] Modification of DANA to the *C*9-azido derivative (**2**) resulted in decreased activity against all isoenzymes tested, with the greatest loss in potency observed against NEU1. The *C*4′-phenyl-*C*9-triazole derivative of DANA (**3**) had similar potency for NEU3 (4.6 ± 0.2 μM) and NEU4 (2.3 ± 0.2 μM) but little activity against NEU1 and NEU2 (>200 μM). The addition of a hydrogen bond acceptor in the *C*9-triazole moiety (**4**) resulted in moderate selectivity for NEU4 (3.6 ± 0.3 μM) over other isoenzymes (25-fold selectivity). The *C*4′-methylphenoxy-*C*9-triazole analog of DANA (**5**) was over 200-fold selective for NEU3 (5.5 ± 0.6 μM) and NEU4 (1.7 ± 0.2 μM) isoenzymes. Truncation of the *C*4′-side chain to a hydroxymethyl group (**6**) provided the most potent compound in our study. Compound **6** was found to have nanomolar potency for NEU4 (0.16 ± 0.01 μM) yet maintained only micromolar activity against other isoenzymes (500-fold). A similar, though not as drastic, trend was observed for the *C*4′-hydroxyethyl derivative **7** (NEU4, 0.81 ± 0.04 μM). Further extension of the *C*4′ side chain to the hydroxypropyl derivative, **8**, decreased potency for NEU4 (2.1 ± 0.1 μM), whereas activity against NEU3 remained only moderate (39 ± 1 μM). The branched *C*4′-side chain of compound **9** provided a minor improvement in selectivity for NEU4; however, potency against the enzyme dropped by approximately 2-fold relative to that of compound **8** (4.0 ± 0.3 μM).

The *N*5-azidoacetate derivative of DANA (**10**) had previously been reported to have improved activity against NEU3[18] and NEU2.[14] In this study we tested the compound against all four hNEU isoenzymes and found that it was most potent against NEU3 (4.7 ± 0.3 μM) and NEU4 (4.5 ± 0.1 μM) and had approximately 6-fold lower activity against NEU1 (29 ± 0.5 μM) and NEU2 (37 ± 5 μM). Testing of a DANA analog which included a triazole group at the *N*5-position (**11**) revealed that only the NEU2 isoenzyme could tolerate this bulky side chain (9 ± 1 μM). Importantly, compound **11** was approximately 30-fold selective for NEU2 over the other isoenzymes and was the most potent compound for NEU2 among those tested here.

The remarkable potency of compound **6** against NEU4 led us to examine its activity in more detail. An overlay of the inhibition curves for compound **6** against hNEU showed a clear separation between its activity for NEU4 and all other isoenzymes, establishing that the selectivity of the inhibitor was at least 500-fold over its next most active target (NEU3; Figure 2). We then determined the inhibition constant (K_i) for compounds **6** and **7** against NEU4 (Supporting Information). These experiments found that compounds **6** and **7** had K_i values against NEU4 of 30 ± 19 nM and 60 ± 16 nM, respectively, making them the most potent and selective inhibitors reported for any hNEU enzyme. In order to understand the observed selectivity of these compounds in more detail, we developed a molecular model of the interaction of compound **6** with its target, NEU4. No crystal structure data has been reported for NEU4 to date, so we used homology modeling with NEU2 as a template (see Experimental Procedures).[19] After docking of compound **6** to the active site of the NEU4 homology model and subsequent molecular dynamics, we obtained a binding model (Figure 3) which maintained most of the expected contacts with the key features of the DANA core. Interestingly, we observed that the 4′-hydroxymethyl group of compound **6** was able to engage in H-bond contacts with the carbonyl groups of S243 and W274 and the side chain of R242 (Figure 3b). Thus, our model suggests that the precise placement of the 4′-hydroxymethyl group is responsible for the remarkable activity of this compound against NEU4. This conclusion is also consistent with the activity of compounds **7** and **8**, which both show successive drops in potency with the homologation of methylene groups at the 4′ position. Our model of NEU4 also suggests that the lack of specificity for other isoenzymes, such as NEU2, is the result of differences in the glycerol side chain binding pocket. An alignment of the NEU4 model to NEU2 finds a large conformational change between a loop of the enzyme that forms half of the binding pocket (see Supporting Information).

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Table 1. Inhibition of Neuraminidase Isoenzymes

Compound	Relative activity[b]		IC_{50} (μM)[a]							
			NEU1		NEU2		NEU3		NEU4	
	Target	Selectivity	IC_{50}	±	IC_{50}	±	IC_{50}	±	IC_{50}	±
DANA (1)	n/a	n/a	76	6	90	10	6.3	0.5	13	1
2	NEU3	3	620	10	240	20	19.7	2.3	60	20
3	NEU3,4	72	200	30	250	70	4.6	0.2	2.3	0.2
4	NEU4	25	170	20	130	20	90	10	3.6	0.3
5	NEU3,4	220	>1000	-	800	70	5.5	0.6	1.7	0.2
6	NEU4	500	>1000	-	>1000	-	80	10	0.16	0.01
7	NEU4	47	>1000	-	>1000	-	38	2	0.81	0.04
8	NEU4	19	660	20	>1000	-	39	1	2.1	0.1
9	NEU4	45	>1000	-	>1000	-	180	40	4.0	0.3
10	NEU3,4	6	29.0	0.5	37	5	4.7	0.3	4.5	0.1
11	NEU2	28	>1000	-	9	1	>1000	-	250	40

[a]Coloring of potency values is by relative ranking within each isoenzyme. Darker shades of red indicate higher potency compounds with activity below 500 μM, or which are ranked 1−7 among compounds tested. Darker shades of blue indicate weaker potency, with activities typically above 500 μM or which are ranked 8−11 among compounds tested. [b]Relative activity was determined by dividing the potency of the compound for its next weakest target by that of its primary target. For cases where multiple isoenzymes are listed as the target, the average of these was used.

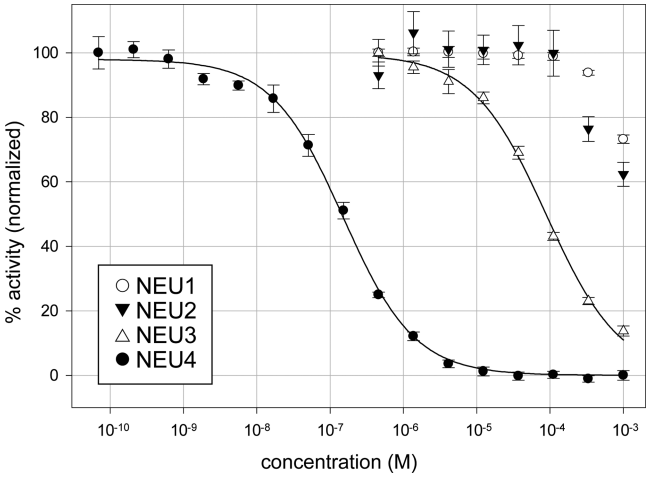

Figure 2. Potency and selectivity of compound **6** against hNEU. The potency of compound **6** was determined using the 4-MU-NANA assay. IC_{50} curves of the compound with NEU1, NEU2, NEU3, and NEU4 are overlaid. The IC_{50} against NEU4 was 160 ± 10 nM, with a selectivity of at least 500-fold over the other three isoenzymes.

We attribute the difference in activity of compound **6** to this conformational change.

Our results suggested that **6** was a potent competitive inhibitor of NEU4; however, the above assays exploit the fluorogenic substrate, 4MU-NANA, which is not a native substrate of the enzyme. To confirm that this class of inhibitors could, indeed, inhibit the cleavage of native NEU4 substrates, we adapted a reported assay which detects the generation of free sialic acid.[20] We tested our most potent NEU4 inhibitor, compound **6**, for its ability to inhibit enzyme cleavage of GM3, a known glycolipid substrate of NEU4 (Figure 4).[21] We found that the IC_{50} of **6** with GM3 as a substrate was in the high nanomolar range (740 ± 70 nM) and was 350-fold more potent than DANA. These data confirmed that compound **6** is a potent inhibitor of NEU4 cleavage of native glycolipid substrates.

In this report, we have identified inhibitors which selectively target isoenzymes of hNEU. Our most potent compounds, **6** and **7**, are active against NEU4, with nanomolar K_i values. Several of the compounds are also specific against NEU4, with selectivities that ranged from 50-fold (**9** and **7**) to 500-fold (**6**). These inhibitors were also demonstrated to act as nanomolar inhibitors of NEU4 processing of the ganglioside substrate, GM3. We also observed that DANA analogs containing a bulky *N*5 group resulted in selective inhibition of NEU2 (28-fold, **11**). These results provide evidence that minor modifications of the DANA scaffold can generate potent and selective inhibitors of hNEU. Additionally, the range of activity and selectivity found among the panel of C9-modified compounds (**2**−**9**) supports our hypothesis that the binding sites for the glycerol side chain of Neu5Ac are diverse among hNEU.[16,18] NEU4 was identified following the sequencing of the human genome, almost two decades later than other hNEU, but multiple studies have already implicated it in vital functions, such as processing of brain gangliosides,[22] neuronal development,[23] and cancer.[24] The development of specific and potent small molecule inhibitors of this enzyme will therefore provide a vital tool for future studies of pathways controlled by NEU4 and reveal new avenues of therapeutic intervention.

EXPERIMENTAL PROCEDURES

Characterization of Inhibitors. Inhibitors used in this study were prepared as described previously.[18] Compound purity was determined by HPLC and confirmed to be ≥95% for all compounds tested. Compound identity was confirmed by high resolution mass spectrometry (see Supporting Information for details).

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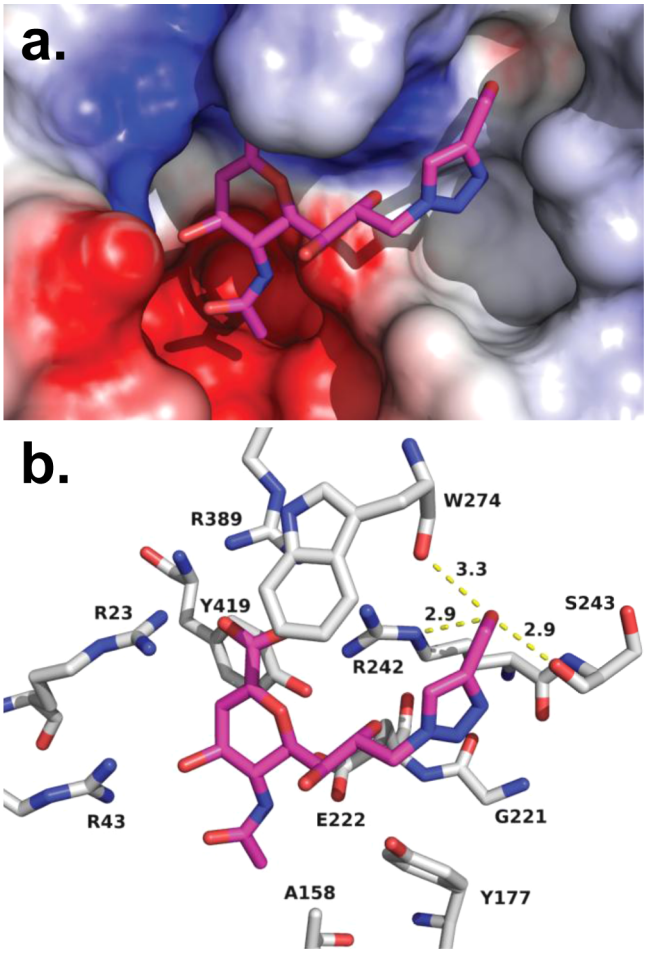

Figure 3. Molecular model of compound **6** in the active site of NEU4. Using our homology model of NEU4, compound **6** was docked to the active site and then subjected to molecular dynamics (10 ns). (a) An electrostatic surface representation of the active site is shown with compound **6** bound. (b) The general binding mode observed for DANA derivatives observed for NEU2 was preserved in our model, including contacts with the arginine triad (R23, R389, and R242). H-bond contacts are maintained between O4 and R43, as well as the glycerol side chain O8 with R242. The C4′−CH_2OH has multiple H-bond contacts which include the backbone carbonyls of S243 (2.9 Å) and W274 (3.3 Å) and the N_ε of R242 (2.9 Å).

Inhibition Assays. NEU3 and NEU2 were expressed in *E. coli* as N-terminal MBP fusion proteins and purified as described previously.[25] NEU4 was expressed as a GST fusion protein and purified as described.[16] NEU1 was purified as previously described.[26] Assays were conducted in 0.1 M sodium acetate buffer at the enzyme optimum pH (4.5 for NEU1, NEU3, and NEU4; 5.5 for NEU2), using a similar amount of enzymatic activity for all four proteins, as determined by assay with 4MU-NANA. Inhibitors were subjected to 3-fold serial dilutions starting from a final concentration of 1 mM. Dilutions were performed in reaction buffer (20 μL). The mixture was then incubated for 15 min at 37 °C. Fluorogenic substrate (4MU-NANA, 50 μM final concentration) was added to the reaction buffer (20 μL) and incubated at 37 °C for 30 min. The reaction was quenched with 200 μL of 0.2 M sodium glycinate buffer pH = 10.7, and enzyme activity was determined by measuring fluorescence (λ_{ex} = 365 nm; λ_{em} = 445 nm) in a 384 well plate using a plate reader (Molecular Devices, Sunnyvale, CA). Assays were performed with four replicates for each point; error bars indicate the standard deviation. Reported IC_{50} values were determined by nonlinear regression using SigmaPlot 12. For curves which showed less than a 50% decrease in signal, fits were conducted using the maximum inhibition values found for DANA.

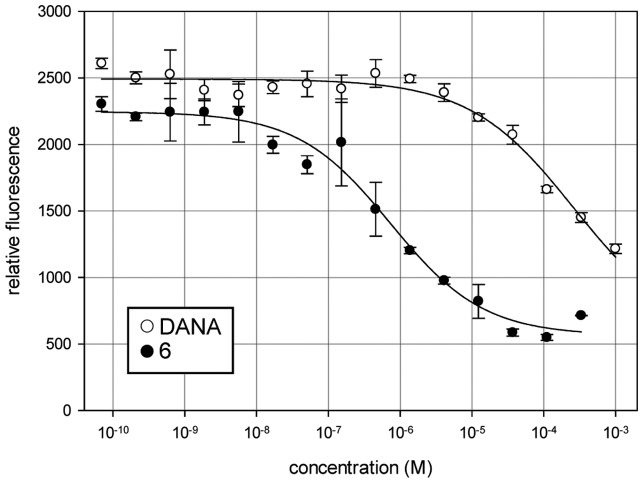

Figure 4. Compound **6** inhibits NEU4 cleavage of a glycolipid substrate, GM3. An IC_{50} curve demonstrating the inhibition of glycolipid hydrolysis catalyzed by NEU4 with compound **6**.[20] The determined IC_{50} for **6** was 0.74 ± 0.07 μM, and that for DANA was 260 ± 40 μM, demonstrating an approximately 350-fold difference in activity. Assays were performed with four replicates for each point; error bars indicate the standard deviation.

To test the potency of inhibitors against a glycolipid substrate, we adapted a known assay for the detection of free sialic acid.[20] The assay was conducted in 0.1 M sodium acetate buffer (pH 4.5) with NEU4. The reaction mixture was incubated for 30 min at 37 °C. Substrate (GM3, 500 μM final concentration) was added to the reaction mixture and incubated at 37 °C for 1 h. The reaction was quenched with 200 μL of 0.2 M sodium borate buffer (pH 9.5). A fluorescent product was developed by the addition of malononitrile (40 μL of 0.8% solution) and heating at 80 °C for 20 min. Enzyme activity was determined by measuring fluorescence (λ_{ex} = 357 nm excitation; λ_{em} = 443 nm) in a 384 well plate. Reported IC_{50} values were determined by nonlinear regression.

***K_i* Determinations.** Solutions of 4MU-NANA in sodium acetate buffer (0.1 M) at the optimum pH of the target enzyme were prepared with concentrations of 20, 40, 60, 80, and 100 μM. Each substrate solution (25 μL) was mixed with an equal volume of a solution containing serial concentrations of the inhibitor and the target enzyme. The inhibitor concentrations were selected as a range which included the determined IC_{50} value. The reaction mixture (50 μL) was transferred to a 384-well plate. The rate of the product formation at 37 °C was followed by measuring the fluorescence (λ_{ex} = 365 nm; λ_{em} = 445 nm) every 30 s for 60 min using a plate reader (Molecular Devices, Sunnyvale, CA). Pseudo-first-order rates were determined using the initial linear part of the resulting curves. The double reciprocal plot (1/rate versus 1/[S]) for each inhibitor concentration was used to determine a slope, and a plot of the slopes versus inhibitor concentration was fit to determine K_i.

Molecular Modeling. The NEU2 structure reported by Chavas et al. was used to develop a homology model of NEU4 (PDB ID: 1VCU).[19] NEU4 and NEU2 sequences (accession numbers: Q8WWR8 and Q9Y3R4, respectively) were aligned using ClustalW2[27] and found to have 43% homology. Although the extended sequence of NEU4L (residues 287−374) did not align with any parts of NEU2, the full sequence was used to generate a preliminary homology model of NEU4 using SWISS-MODEL.[28] The sequence between residues 287 and 374 remained as an unorganized loop in the initial model. The model was first minimized and then DANA was docked to the active site, providing the initial structure for molecular dynamics calculations using MacroModel 9.9. The protein−ligand complex was equilibrated for 100 ps at 300 K, followed by 10 ns of dynamics at constant temperature with 1.5 fs time steps (see Supporting Information). The

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force field used was AMBER*, with aqueous solvent modeled using GB/SA continuum solvation.[29] The final model of the complex was obtained after unconstrained minimization to convergence. After removing DANA from the active site, compound **6** was docked using Autodock 4.2 with a 60^3 Å grid centered on the active site (0.375 Å resolution.)[30,31] The 200 lowest energy ligand poses were evaluated by cluster analysis, and the lowest energy conformer which maintained key contacts to the active site was selected. The resulting complex was again subjected to molecular dynamics following the same protocol described above to obtain a final model of the interaction of compound **6** with NEU4. Final structures were visualized in PyMOL,[32] and protein surfaces were calculated using DelPhi.[33]

■ ASSOCIATED CONTENT

Supporting Information

IC_{50} curves, K_i determinations, and characterization of inhibitors are available in supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

DANA, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid; GST, glutathione S-transferase acid; MBP, maltose binding protein; 4MU-NANA, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid; NEU, neuraminidase; NMR, nuclear magnetic resonance; HRMS, high resolution mass spectrometry; HPLC, high performance liquid chromatography

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