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Inhibition of human neuraminidase 3 (NEU3) by *C*9-triazole derivatives of 2,3-didehydro-*N*-acetyl-neuraminic acid

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ABSTRACT

We report the synthesis of a series of *C*9 and *N*5Ac modified analogs of 2,3-didehydro-*N*-acetyl-neuraminic acid (DANA) and their inhibitory potency for the human neuraminidase 3 (NEU3) enzyme. We were able to generate a small library of compounds through the synthesis of azide derivatives of DANA, followed by Cu-catalyzed azide–alkyne cycloaddition (CuAAC) to generate triazole-containing inhibitors. Our results suggest that NEU3 can tolerate large hydrophobic groups at the *C*9 position; however, none of the derivatives made at the *N*5Ac side-chain were active. We identify three new inhibitors that have comparable potency to the best reported inhibitors of the enzyme.

The mammalian sialidase enzymes are a family of glycosyl hydrolases which cleave the terminal sialic acid residue from glycoproteins and glycolipids.[1] Dysfunction of these enzymes can result in sialic acid storage disorders, such as type I and II sialidosis.[2,3] Mammalian sialidase enzymes also play essential roles in cellular function, with reported effects on diverse processes including neurite outgrowth,[4] immune cell activation,[5] cellular communication,[6–8] signaling,[5,9–11] adhesion,[12] as well as the apoptosis[13,14] and metastasis of malignant cells.[15,16] Selective, tight-binding inhibitors of sialidase isoforms could provide an essential tool for discerning the role of individual isozymes in cell biology. However, while nanomolar inhibitors are known for pathogenic neuraminidase enzymes, most notably the influenza viral neuraminidase, no inhibitors of similar potency are known for the human enzymes.[17]

Currently identified members of the sialidase enzyme family in humans consist of NEU1, NEU2, NEU3, and NEU4. All four enzymes are classified as *exo*-sialidases (EC 3.2.1.18) and members of glycoside hydrolase family 33 in the CaZy database.[18] Among the mammalian enzymes, only the human NEU2 structure has been studied by crystallography, revealing that the enzyme adopts a six-bladed β-propeller fold.[19,20] Homology models of all four human enzymes were first proposed by Magesh et al. in 2006; however, many of the specific features of the models remain to be experimentally tested.[21] For example, the predicted catalytic residues have only been confirmed in NEU2[19] and NEU3.[22] There are few reports of inhibitors tested against the family of human NEU enzymes. A panel of 9-amino-9-deoxy-2,3-didehydro-*N*-acetyl-neuraminic acid derivatives were tested against all four enzymes; the most potent of these inhibitors showed only low micromolar activity against NEU1.[23] A library of benzoic acid derivatives have also been tested against all four isozymes.[24] The viral neuraminidase inhibitors zanamivir and oseltamivir have limited potency against all of the human sialidases.[25] However, tight-binding (<1 μM) and selective inhibitors for individual isozymes remain to be identified.

Our group has studied the substrate recognition and catalysis of human NEU3, the plasma membrane-associated sialidase, which modifies glycolipid substrates.[22] This enzyme is particularly challenging due to its hydrophobic character and propensity for aggregation. We tested the predictions of a NEU3 homology model using site-directed mutagenesis, and validated both the catalytic residues and several residues which are essential for substrate recognition. As part of this study, we performed molecular docking of a well-known sialidase inhibitor, 2,3-didehydro-*N*-acetyl-neuraminic acid (DANA). Our molecular docking studies of DANA in the NEU3 binding site revealed a relatively large hydrophobic pocket adjacent to the *C*9 position of sialic acid. We hypothesized that this region of the binding site could be used to design improved inhibitors of NEU3 based on the DANA core structure.

Abbreviations: CuAAC, Cu-catalyzed azide–alkyne cycloaddition; DANA, 2,3-didehydro-*N*-acetyl-neuraminic acid; MBP, maltose binding protein; NEU, neuraminidase; 4MU-NA, 4-methylumbelliferyl α-D-*N*-acetylneuraminic acid.

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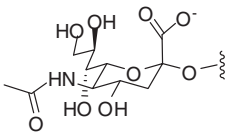
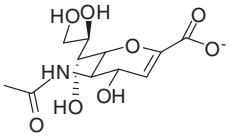
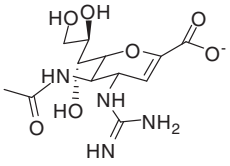
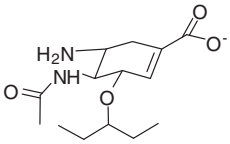
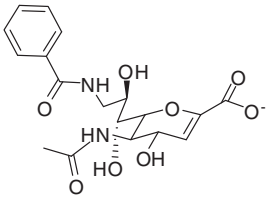
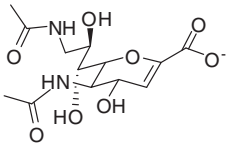
Known substrates of NEU3 include α(2,3)-linked sialosides (Table 1) found in glycolipid substrates, such as GM3.[26] Additionally, α(2,8)-linked sialosides are known to be substrates for the enzyme, and there is evidence that the enyzme has a preference for this linkage.[13] A common strategy for the design of sialidase inhibitors relies on the use of DANA as a transition state analog. The inhibition of NEU3 by DANA has been reported by several groups, and is usually found to be in the mid-micromolar range (Table 1).[22,23,25] Interestingly, zanamivir, a potent viral NEU inhibitor has been reported to have low micromolar activity against NEU3, while oseltamivir is essentially inactive against the enzyme.[25] Magesh et al. found that NEU3 could tolerate a relatively bulky phenyl-amide group at the *C*9 position of DANA, although these derivatives had relatively low potency (Table 1).[23] We considered that a potential strategy for rapidly exploring the structure activity relationships (SAR) of DANA analogs for NEU3 could revolve around a modular synthetic strategy which introduced modifications at the *C*9- and *N*5Ac-positions from a common intermediate. Li et al. first reported the use of click chemistry to generate viral NEU inhibitors from a *C*4-azido-derivative of DANA.[27] Lu and Gervay-Hague expanded on this strategy by generating *C*4- and *C*7-azido DANA analogs, which could be elaborated to triazoles and multivalent derivatives of zanamivir.[28] In a related strategy, non-hydrolyzable sialoside derivatives have been generated by incorporating an azide at *C*2 of Neu5Ac.[29] We set out to generate a small library of DANA analogs with modifications at the *C*9 and *N*5Ac as a means to explore the binding site topology of NEU3 and to identify new inhibitors of the enzyme.

Table 1
Previously reported inhibitors of NEU3

Entry	Compound	Structure	NEU3 IC_{50} (μM)
1	α-Sialoside		na
2	DANA		70 ± 15[25a] 61[23b] 43 ± 7[22b]
3	Zanamivir		7 ± 3[25a]
4	Oseltamivir		>10,000[25a]
5	**10b**[c]		320[23b]
6	**10a**[c]		>1000[23b]

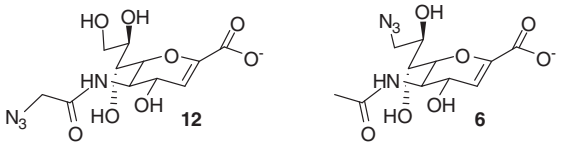
[a] Determined by inhibition of GM3 hydrolysis.
[b] Determined by inhibition of 4MU-NA hydrolysis.
[c] Nomenclature as used in Magesh et al.[23]

The basis of our strategy relied on the generation of two derivatives, the *N*5-azidoacetyl- (**6**) and 9-azido-9-deoxy (**12**) DANA derivatives (Scheme 1). We began our synthesis from *N*5-acetylneuraminic acid (Neu5Ac), which could be converted to the peracetylated methyl ester, **1**, following reported methods.[30,31] We obtained moderate yields of the acetyl-protected 2,3-didehydro analog (**2**) using triphenylphosphine hydrobromide (see Supplementary scheme SI1).[32,33] To prepare compound **6** and the related triazole derivatives, **2** was deacetylated[34] and the *O*9 position was selectively converted to a sulfonate ester, **4**.[35] The sulfonate could then be displaced with sodium azide to give the methyl ester of **6**, compound **5**.[23,35] Hydrolysis of **5** under basic conditions provided the target compound **6**. Alternatively, **5** could be used to generate triazole derivatives using the copper-catalyzed 1,3-dipolar Huisgen addition of an alkyne, also known as a Cu-catalyzed azide–alkyne cycloaddition (CuAAC).[36] We generated a series of nine triazole derivatives of **6** using standard conditions (compounds **6a–i**, Table 2).

To prepare derivatives of compound **12**, we required selective deprotection of the *N*5 side-chain. To achieve this, we first protected *N*5 with di-*tert*-butyldicarbonate (Boc_2O), followed by deacetylation to generate **7** (Scheme 2).[37] Subsequent O-acetylation provided compound **8**, which could be selectively deprotected to reveal the free amine at *N*5 (**9**). With the free amine in hand, we could introduce a modified *N*-acyl group that contained an azide handle. We employed an activated ester of azidoacetic acid,[38,39] which gave the azide **10** in excellent yield. Removal of the *O*-acetyl protecting groups provided the methyl ester **11**, which could be hydrolyzed to the target compound **12**. As before, we generated a series of triazole derivatives of **12** from **11** by CuAAC, followed by hydrolysis of the methylester (compounds **12a–i**, Table 2).

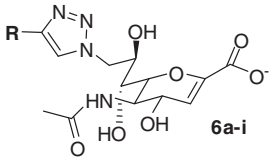
We previously reported a bacterial expression system for NEU3 which could be exploited to test the potency of inhibitors.[22] Although there are previous reports of bacterial expression of this enzyme,[40] we found that expression of the protein as an N-terminal fusion with the maltose binding protein (MBP) helped to stabilize the protein and prevent aggregation. Additionally, producing the protein in bacterial cells avoided background signal due to contaminating activity of other sialidase isoforms present in mammalian cells. Using purified MBP-NEU3, we tested the inhibitory potency of the *C*9 (compounds **6** and **6a–i**) and *N*5Ac (compounds **12** and **12a–i**) derivatives against NEU3 using a fluorescence assay based on the hydrolysis of 4-methylumbelliferyl α-D-*N*-acetylneuraminic acid (4MU-NA) (Table 2).

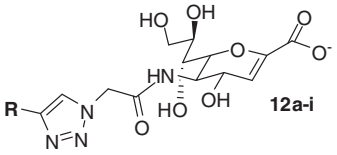
As expected, we observed that DANA was a good inhibitor of NEU3, with an IC_{50} of approximately 40 μM.[22,23,25] We found that modification of DANA at the *C*9 position by a triazole with a hydrophobic group which lacked a hydrogen bond donor lead to improved activity. The most potent compounds were the phenyl **6a** (20 ± 10 μM), hexyl **6b** (23 ± 4 μM), and phenoxymethyl **6e** (45 ± 3 μM) derivatives. Importantly, the data suggest that the triazole portion of the inhibitor is partly responsible for the increased activity, as the azide derivative **6** has slightly reduced potency when compared to DANA (70 ± 20 μM). Bulky triazole

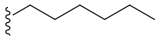
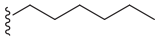
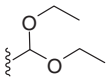
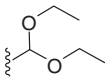
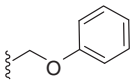
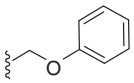
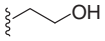
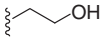
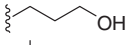
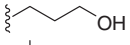


Scheme 1. DANA analog synthetic targets.

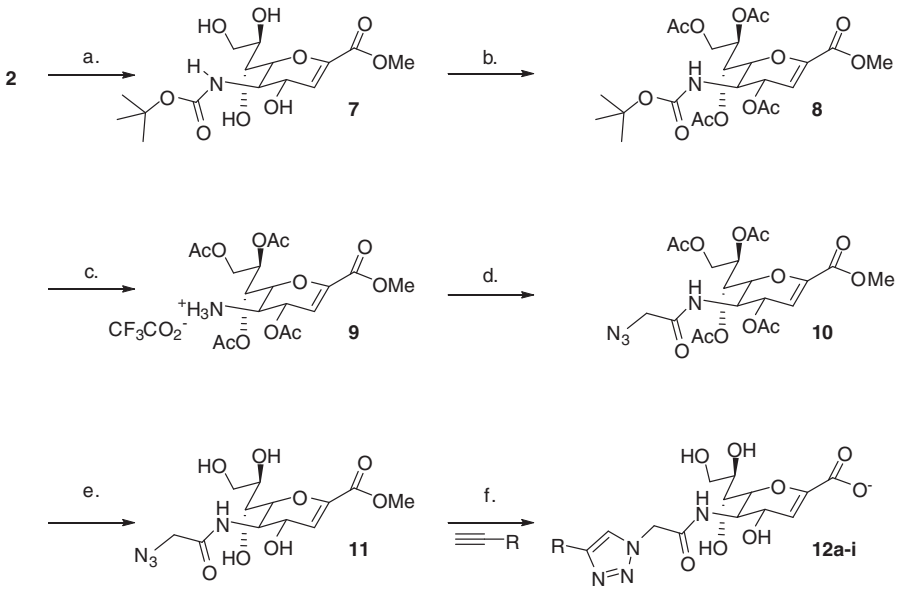
Table 2
Inhibition of NEU3





Compound	R	IC_{50}[a] (μM)	Compound	R	IC_{50} (μM)
DANA	na	48 ± 5	DANA	na	48 ± 4
6	na	70 ± 20	**12**	na	21 ± 8
6a		20 ± 10	**12a**		>500
6b		23 ± 4	**12b**		>500
6c		300 ± 200	**12c**		>500
6d		130 ± 20	**12d**		>500
6e		45 ± 3	**12e**		>500
6f		300 ± 400	**12f**		>500
6g		400 ± 600	**12g**		>500
6h		500 ± 200	**12h**		>500
6i		300 ± 500	**12i**		>500

[a] Inhibitors which did not show significant change (>50% decrease from control) were fit using the maximum inhibition value for DANA. See Supplementary data for IC_{50} curves.



Scheme 2. Synthesis of *N*5Ac-azido and -triazolyl derivatives. Reagents and conditions: (a) Boc_2O, *N*,*N*-dimethylaminopyridine, THF, 2 h (quant.); then NaOMe, MeOH (92%); (b) Ac_2O, pyridine, 12 h, 0 °C (96%); (c) TFA, CH_2Cl_2, 1.5 h; (d) *N*-hydroxysuccinimidyl-2-azidoacetate, triethylamine, 3 h, 0 °C (99% over two steps); (e) NaOMe, MeOH, 1 h, (52%); (f) sodium ascorbate, $CuSO_4$, H_2O/*t*BuOH/DCM (1:2:1); then NaOH, Amberlite IR-120(H+) (yields ranged from 31–96%).

substituents, such as **6d**, had reduced potency (130 ± 20 μM). More polar groups, including ethoxy **6c**, hydroxymethyl **6f**, hydroxyethyl **6g**, and hydroxypropyl **6h** all showed significantly reduced activity as compared to the parent DANA, or the azide **6**. Assays of compound **12** found a slightly improved potency over DANA (21 ± 8 μM), suggesting that the azide may provide some beneficial interactions. However, our data imply that the *N*5Ac pocket is relatively small, as all triazole derivatives of compound **12** tested (**12a–i**) had potencies that were significantly reduced when compared to DANA (>500 μM).

In order to understand the interactions responsible for the differences in potency among both series of compounds, we conducted molecular docking experiments using a previously reported homology model of NEU3.[22] After docking, structures were subjected to molecular dynamics and minimized (see Supplementary data). Examination of the structures of DANA and compound **6a** in the NEU3 active site suggest that a hydrophobic pocket adjacent to the glycerol-binding pocket (*C*7–C9) could contribute to the activity of compounds related to **6a** (Fig. 1). The hydrophobic pocket is made up of residues V222, V224, P247, and H277. We observed two changes to the active site residues upon binding of **6a**, D50 became reoriented to interact with *HO*7, and an apparent edge–face interaction developed between the phenyl ring and H277 (Fig. 1).[41] The model of DANA binding is consistent with the limitations we observed for the *N*5Ac pocket, which would be predicted to be unable to accommodate the bulky triazole groups found in compounds **12a–i**. Additionally, the *N*5Ac pocket is relatively hydrophobic, although a carboxylate group from N88 is positioned close enough to contact *C*4 H-bond donors.

These results support our previous study of the active site topology of NEU3 using small molecule inhibitors based on DANA.[22] The compounds tested here suggest that the *N*5Ac pocket of NEU3 is sterically limited, and cannot accommodate large groups such as the triazole derivatives tested here (**12a–i**). Importantly, we observed that modifications of the *C*9 position are well tolerated, and may have some specific requirements for potent inhibitors of the enzyme. Although previous studies have identified a tolerance for bulky groups at *C*9 (Table 1),[23] we observed that the addition of a triazole linker significantly improved activity (compounds **6a**, **6b** and **6e**). Based on our model of the active site, we propose that groups which bind in the *C*9 pocket should contain H-bond acceptors, to interact with Y181, and also hydrophobic side-chains, to interact with V222 and V224 (Fig. 1). Additional H-bond acceptors distal from *C*9 may take advantage of contacts with K195 or H277. Our results suggest that potent inhibitors of NEU3 may be developed by incorporation of a modified *C*9 side-chain of DANA analogs. Importantly, we identified compounds in this study with equivalent potency (**6a**) to the best reported

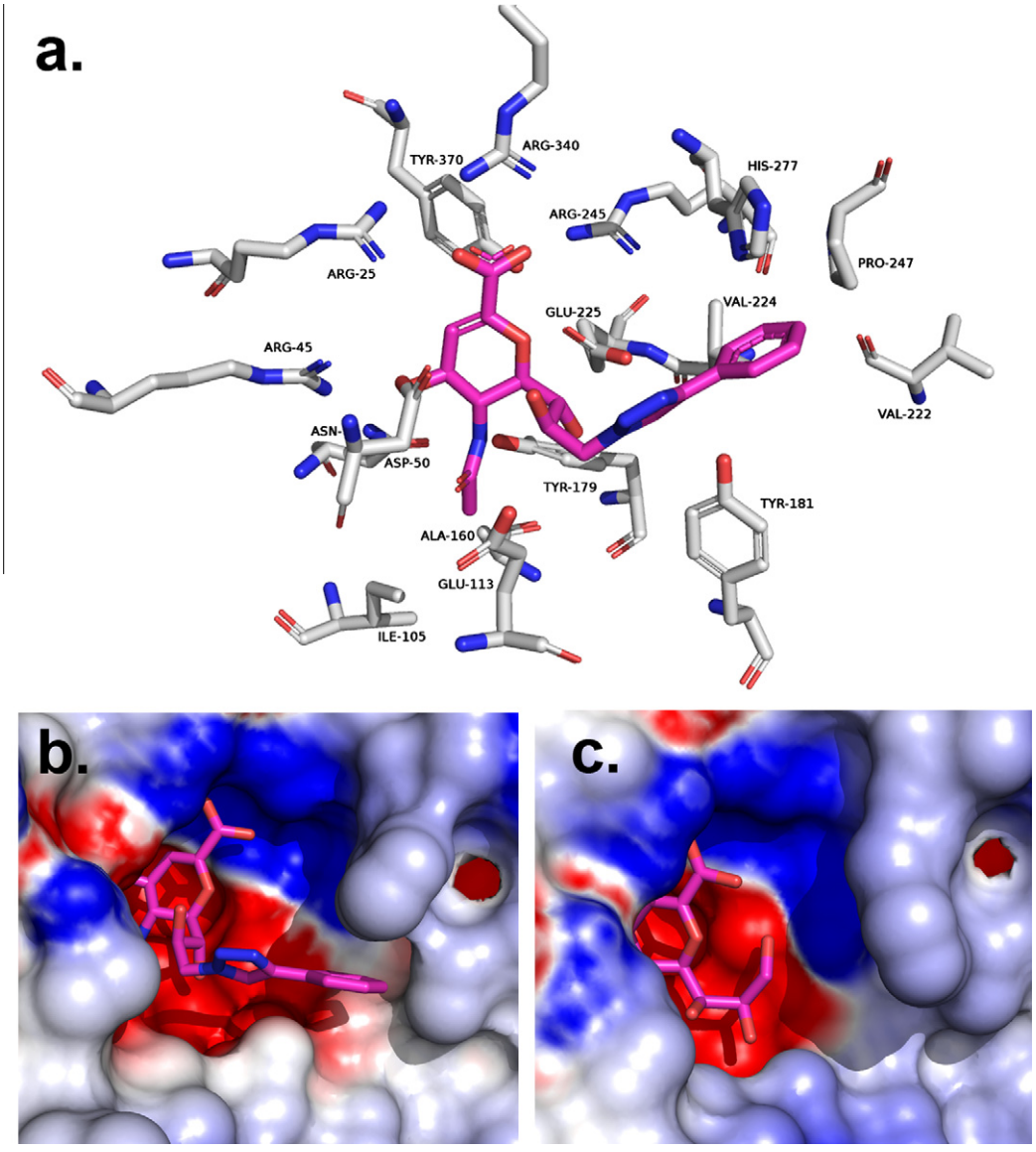


Figure 1. Molecular docking of DANA and **6a**. Inhibitors were docked and subjected to molecular dynamics using a homology model of NEU3 to determine the binding mode of the triazole derivative **6a**.[22] (a) The binding site of NEU3 has a large hydrophobic pocket adjacent to the *C*9 position including V222, V224, P247 and H277 which can accommodate hydrophobic groups, (b) an electrostatic potential map is shown for the docked structure of **6a**, and (c) DANA. Several residues have reoriented to accommodate **6a**, notably H277 is rotated to engage in an edge–face interaction with the phenyl ring of **6a**,[41] and D50 has become more solvent exposed.

inhibitors of the enzyme (zanamivir).[25] This feature of NEU3 recognition may indicate that the enzyme can tolerate unusual sialic acid modifications found at *C*9.[42] Future work will examine the specificity of these compounds for the NEU3 isoform, and test additional side-chain modifications at *C*9 that could increase potency.

Acknowledgments

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Supplementary data

Supplementary data (characterization and synthetic methods) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.111.

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