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Inhibitor selectivity of a new class of oseltamivir analogs against viral neuraminidase over human neuraminidase enzymes

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ABSTRACT

The viral neuraminidase enzyme is an established target for anti-influenza pharmaceuticals. However, viral neuraminidase inhibitors could have off-target effects due to interactions with native human neuraminidase enzymes. We report the activity of a series of known inhibitors of the influenza group-1 neuraminidase enzyme (N1 subtype) against recombinant forms of the human neuraminidase enzymes NEU3 and NEU4. These inhibitors were designed to take advantage of an additional enzyme pocket (known as the 150-cavity) near the catalytic site of certain viral neuraminidase subtypes (N1, N4 and N8). We find that these modified derivatives have minimal activity against the human enzymes, NEU3 and NEU4. Two compounds show moderate activity against NEU3, possibly due to alternative binding modes available to these structures. Our results reinforce that recognition of the glycerol side-chain is distinct between the viral and human NEU enzymes, and provide experimental support for improving the selectivity of viral neuraminidase inhibitors by exploiting the 150-cavity found in certain subtypes of viral neuraminidases.

1. Introduction

The development of anti-viral drugs to combat influenza infection continues to be a major area of medicinal chemistry research.[1] The most successful small molecule anti-influenza strategies have targeted the viral neuraminidase enzyme (vNEU), a member of glycosyl hydrolase family 34 which cleaves terminal *N*5-acetyl-neuraminic acid (Neu5Ac) residues from host glycoproteins (EC 3.2.1.18).[2] The most successful vNEU inhibitors are transition-state mimics based on 2,3-didehydro-*N*5-acetylneuraminic acid (DANA), including zanamivir, and cyclohexene analogs such as oseltamivir (Chart 1).[1]

Anti-influenza drugs which target vNEU slow the release of budding viral particles and can reduce the severity of infection.[1] However, by their nature these drugs have the potential to interfere with human enzymes which recognize similar substrates.

Sialic acids, and the enzymes which regulate them, are critical for eukaryotic cell function;[3,4] thus, specificity of anti-influenza drugs for their intended target is essential. The family of human neuraminidase (hNEU) enzymes is currently known to consist of four isoforms: NEU1, NEU2, NEU3, and NEU4.[5] These enzymes are classified as members of the glycosyl hydrolase family 33.[2] Among them, NEU1 and NEU3 are found at the plasma and lysosomal membranes; NEU2 is a cytosolic protein; and NEU4 is associated with mitochondria.[5] NEU3 is a peripheral membrane protein thought to be specific for ganglioside substrates;[6] and is known to play a role in cellular signaling through the regulation of

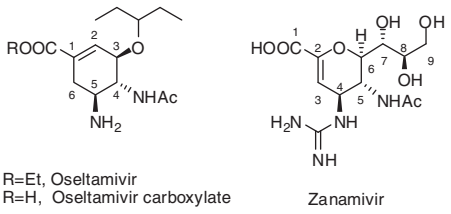

Chart 1.

Abbreviations: DANA, 2,3-didehydro-2-deoxy-*N*-acetyl-neuraminic acid; MBP, maltose binding protein; NEU, neuraminidase; 4MU-NA, 4-methylumbelliferyl α-D-*N*-acetylneuraminic acid.

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membrane glycolipid composition.[7,8] This function is critical for a variety of signaling pathways, for example, overexpression of NEU3 in transgenic mice leads to a diabetic phenotype.[9] Additionally, mutations in NEU1 can result in sialidosis, a lysosomal storage disorder.[10] Although eukaryotic and viral neuraminidase enzymes are not completely homologous, they share a common enzymatic mechanism—raising the possibility of common inhibition by transition-state mimics.[11]

The nine influenza type A neuraminidase enzyme isoforms are classified into two groups. Interestingly, members of group-1 contain an additional pocket adjacent to the substrate binding site which is not found in group-2 enzymes, known as the 150-pocket (Fig. 1a, b).[12] This observation has led to the design of compounds which can take advantage of this feature to improve inhibitor affinity.[13–15] Such strategies may help to avoid viral resistance by providing alternative lead compounds.[16] Structure-based drug design of inhibitors against hNEU has relied on the crystal structure of NEU2,[17–21] which remains the only member of the family which has been crystallized.[20,22] The high homology within the family has been used to construct models of the remaining three isoforms.

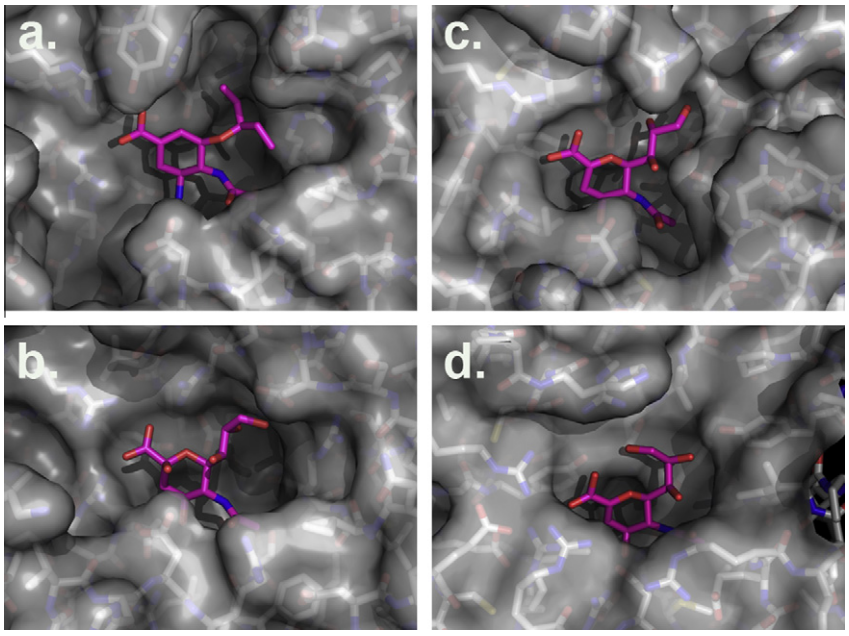


Figure 1. Active site topology of NEU enzymes. The active site topology of viral and mammalian neuraminidases show distinct differences. (a) The viral N8 isoform can adopt an open conformation which exposes the 150-pocket adjacent to the C5 position. The enzyme is shown in complex with oseltamivir (PDB: 2HT7).[12] (b) The viral N2 enzyme is shown in complex with sialic acid (PDB: 2BAT).[37] Note that the 150-pocket is not present. (c) The mammalian NEU2 enzyme is shown in complex with DANA (PDB: 1VCU).[22] (d) A homology model of NEU3 is shown in complex with DANA.[26]

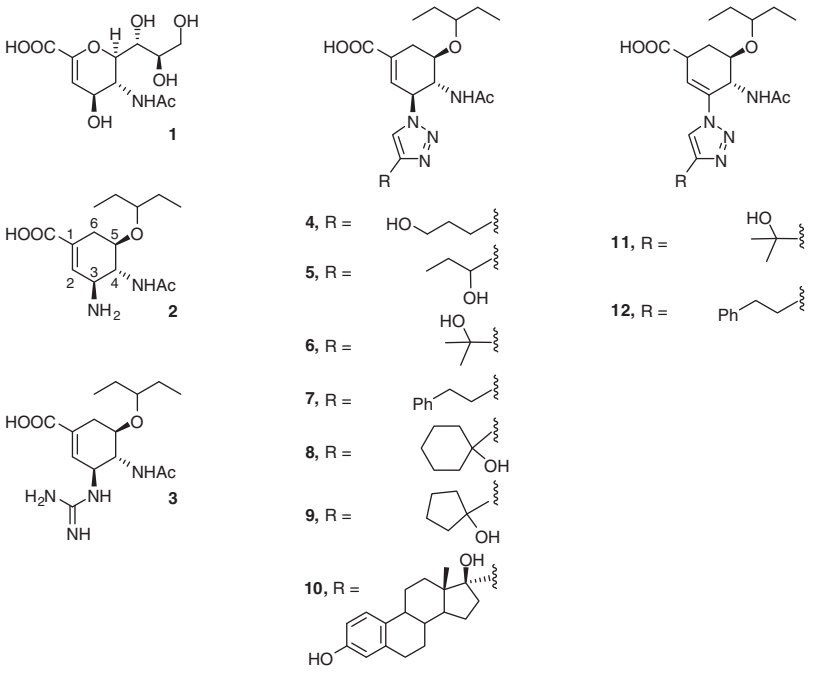


Chart 2.

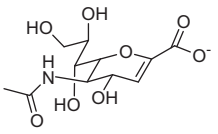
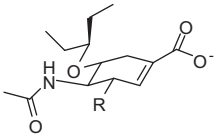
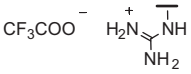
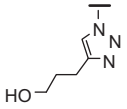
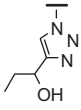
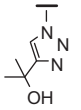
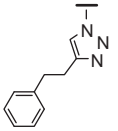
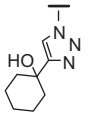
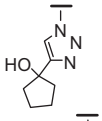
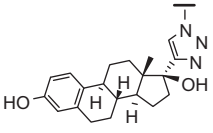
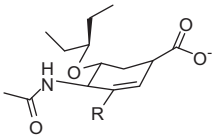
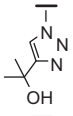
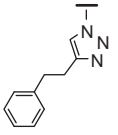
A number of mutations in NEU1 lend support to the predictions of the homology model,[10,23,24] and the NEU3 model has been tested directly using site-directed mutagenesis.[25,26]

There have been few studies which have tested the activity of synthetic inhibitors against the hNEU, and fewer still to compare activity between the eukaryotic and viral enzymes. The activity of oseltamivir, zanamivir, and DANA against the hNEU isoforms has been previously examined.[27] Zanamivir was found to be a micromolar inhibitor of NEU2 and NEU3, and DANA showed micromolar activity against NEU2, NEU3 and NEU4. Importantly, oseltamivir was found to be inactive against all of the hNEU enzymes. This finding suggested that the *C*7–*C*9 binding pocket of the human enzymes are distinct from that of the viral enzymes. Synthetic derivatives of DANA which incorporate *C*9 modifications have been found to be active against NEU1[17] and NEU3.[19]

Inhibitors of vNEU have recently been designed to take advantage of the 150-pocket found in group-1 enzymes. Wen et al. designed inhibitors which included modifications of the *C*4 group of zanamivir with IC_{50} values of ~2 μM.[14] Mohan et al. have recently reported a series of oseltamivir analogs which incorporated a 1,2-olefin and a series of *C*3 substituents (Chart 2, Table 1).

When tested against vNEU, the best of the *C*3 triazole-modified compounds showed slightly reduced potency (**5**, K_i = 72 nM) as compared to zanamivir (K_i = 0.2 nM). Critically, these results

Table 1
Inhibition of NEU3

Parent	R	Compd	NEU3 IC_{50} (μM)	NEU4 IC_{50} (μM)
	na	DANA **1**	30 ± 3	22 ± 15
		**2**	>1000	>1000
		**3**	>1000	>1000
		**4**	>1000	>1000
		**5**	>1000	>1000
		**6**	>1000	>1000
		**7**	>1000	>1000
		**8**	>1000	>1000
		**9**	>1000	>1000
		**10**	350 ± 100	800 ± 400
		**11**	640 ± 210	>1000
		**12**	>1000	>1000

confirmed that one could access the 150-cavity through the replacement of the *C*5-amino group with functionalized triazole substituents on the oseltamivir-like template and still maintain high potency. These compounds provide, therefore, an essential new lead for vNEU inhibitor design. It was unclear if this new class of modified vNEU inhibitors would still be tolerated by the hNEU enzymes. Therefore, we set out to test the hypothesis that vNEU inhibitors which interact with the 150-pocket of group-1 vNEU would also have reduced off-target activity against hNEU enzymes.

2. Results and discussion

We tested the inhibitory potency of the *C*3-modified oseltamivir analogs reported by Mohan et al.[13] against two recombinant human neuraminidase enzymes, NEU3 and NEU4. We produced NEU3 as an N-terminal MBP-fusion protein as described previously.[26] NEU4 was produced as an N-terminal GST-fusion protein and affinity-purified. Characterization of NEU4 confirmed that the enzyme had an acidic pH optimum (pH 4.5–5.0) and its activity was not dependent on the presence of Ca^{2+} or Mn^{2+} metals, or EDTA. As expected, the addition of copper salts partially inactivated the enzyme.[28] NEU4 activity was slightly reduced in the presence of sodium cholate; however, a neutral surfactant had no significant effect on activity (see Supplementary data). These results suggest that recombinant NEU3 and NEU4 have similar properties.[26]

To test the activity of inhibitors against hNEU, enzyme and inhibitor were incubated with the fluorogenic substrate, 4-methylumbelliferyl α-D-*N*-acetylneuraminic acid (4MU-NA), to monitor enzyme activity. Results were compared to DANA as a known inhibitor.[26] The results of the assay are summarized in Table 1. As expected, we observed that derivatives which were closely related to the structure of oseltamivir, such as compound **2**, had no activity against NEU3 or NEU4.[27] Previous work by Hata et al. had found that zanamivir, which contains a *C*3-guanidino modification, was a potent inhibitor of NEU3.[27] In contrast, we found that the modification of the *C*3 position of an oseltamivir analog to a guanidino group (**3**) did not improve activity. This finding, therefore, suggests that the interactions common between zanamivir and **3** (namely those of the carboxylate, the guanidino, and NHAc groups) are insufficient for potency against the human enzymes in the absence of polar contacts on the glycerol side-chain.

The majority of the compounds tested showed no activity against hNEU, supporting their selectivity for vNEU. Compounds **4**–**9** did not exhibit measurable inhibition below 1 mM concentrations. A similar trend was found for the related derivative, **12**. If one assumes these compounds adopt a similar binding mode to zanamivir in the NEU3 and NEU4 active site, this observation may indicate that the binding pocket cannot accommodate these larger groups. However, replacement of the triazole with smaller polar groups at *C*3 did not lead to significant inhibition in compounds **2** and **3**. Interestingly, **11** was able to inhibit NEU3 (IC_{50} = 640 ± 210 μM), but had no measurable activity against NEU4 (Fig. 2). Compounds **6** and **11** differ only in the placement of the endocyclic double bond, thus the activity of compound **11** could be the result of an altered ring conformation or placement within the active site. This ring conformation of **11** may permit an alternative binding mode in the NEU3 active site which is not available to compound **6**. Finally, compound **10** showed significant inhibitory activity against NEU3 (IC_{50} = 350 ± 100 μM), but barely measurable activity against NEU4 (IC_{50} = 800 ± 400 μM). It seems unlikely that the steroid group of this derivative would be accommodated in the expected binding mode of zanamivir. Instead, we propose these results indicate that an alternative binding mode is available which maintains contact with the *C*1-carboxylate and *C*5-NHAc groups to the binding site, but which flips the orientation of the ring to place large *C*3-substitutents in the binding site for the glycerol side-chain.[29] This is consistent with previous findings that have identified potent NEU3 inhibitors containing hydrophobic groups at the *C*9 position of DANA.[19] Modeling studies support that this binding mode would require a significant shift of the ring orientation, and may explain the low potency of this compound (see Supplementary data).

The binding pocket of hNEU which recognizes the glycerol side-chain has distinct features from that of vNEU (Fig. 1c, d). The glycerol binding pocket of hNEU presents an array of polar residues, forming a H-bond network which complexes *O*7, *O*8, and *O*9; in NEU2 these residues are E111, Y179, Y181, and R237.[22] The corresponding interaction in vNEU involves only the Glu276 residue, which simultaneously contacts both *O*8 and *O*9 of Neu5Ac, and

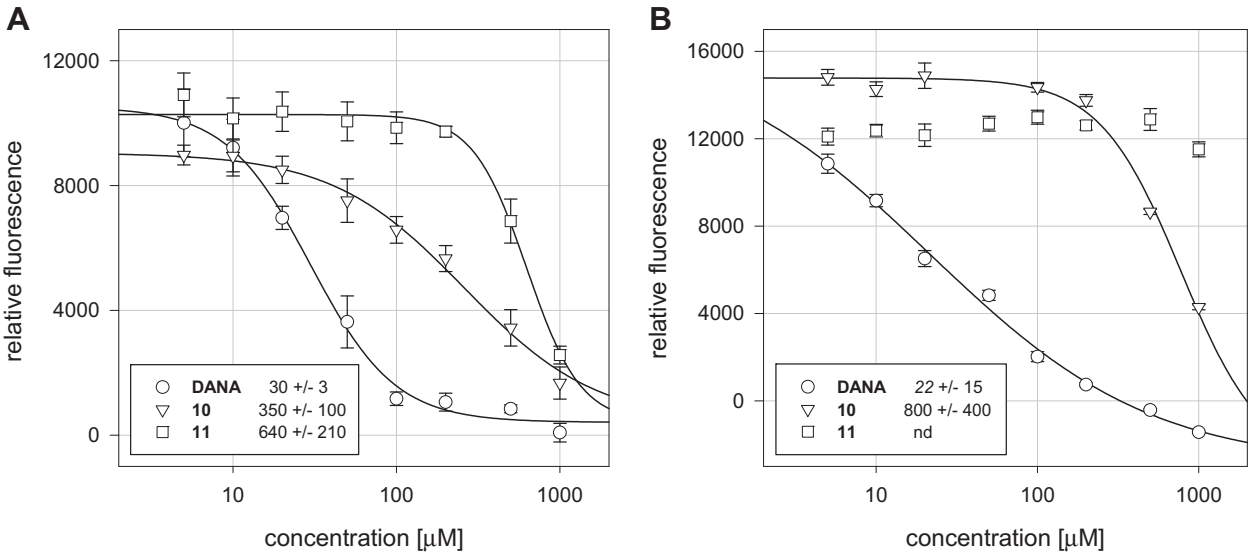


Figure 2. Inhibition of NEU3 and NEU4. Inhibition of hNEU was determined by monitoring the conversion of a fluorogenic substrate, 4MU-NA, by the enzymes.[26] All compounds shown in Table 1 were tested against NEU3 (A) and NEU4 (B). Compounds which showed significant inhibition are plotted, including **DANA**, **1** (○), **10** (▽), and **11** (□). See Supplementary data for additional data.

which can undergo a conformational change to accommodate the hydrophobic side chain of oseltamivir.[30,31] Thus, this region of the binding site (the *C*7–*C*9 pocket) is a distinguishing feature of recognition in these two classes of enzymes, and helps explain the selectivity of oseltamivir for vNEU. Inhibitors of hNEU with comparable potency to the best vNEU inhibitors remain elusive. However, strategies for the design of selective inhibitors of hNEU are emerging. Modifications of the *C*9 position of DANA have yielded NEU1,[17] NEU2,[20] and NEU3[19] inhibitors. Two groups have independently examined the activity of the *N*5-azidoacetate analog of DANA for NEU2[21] and NEU3,[19] and found that this modification resulted in improved activity against both enzymes. It is interesting to note that modifications of the *O*9 position of sialic acid can play an important role in human sialobiology,[32] and that native lectins, such as the siglecs, are extremely sensitive to modifications in this region of the substrate.[33] Thus, hNEU function may rely on tolerance of larger groups in this region of the binding site.

3. Conclusion

Antiviral therapies which target vNEU have the potential for off-target effects against hNEU enzymes. Indeed, polymorphisms that alter the susceptibility of hNEU to inhibition by oseltamivir[34,35] have been proposed to play a role in observed side effects.[36] Our results help establish molecular features of vNEU inhibitors which can be used to reduce off-target interactions with hNEU. First, the *C*7–*C*9 pocket of hNEU plays a critical role in the recognition of substrate. Previous studies of hNEU inhibition by oseltamivir analogs also found limited activity for derivatives with a non-polar group (3-pentyl) occupying the *C*7–*C*9 pocket.[27] While hydrophobic groups in this position did not improve activity against hNEU, work by Zou et al. suggested that a side-chain which retains the *O*7 and *O*8 groups of Neu5Ac can still maintain potency against NEU3.[19] Second, the recently recognized 150-pocket of group-1 vNEU enzymes provides another important handle for inhibitor selectivity. While several of the compounds tested here had activity in the nanomolar range against vNEU, even the best vNEU inhibitors were inactive against hNEU. Thus, the lack of inhibition of the human NEU enzymes by compounds tested here supports the hypothesis that vNEU inhibitors which contain a non-polar side-chain in the *C*7–*C*9 pocket are poor inhibitors of hNEU. Additionally, the incorporation of a guanidino or other large group at the *O*4 pocket does not improve the potency of these analogs for hNEU. Our results may also suggest that analogs of compounds **10** and **11** take advantage of the larger *C*7–*C*9 binding pocket of NEU3 through an alternative binding mode.

4. Materials and methods

4.1. Neuraminidase inhibitors

Reagents for the neuraminidase assay, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (DANA) and 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4MU-NA), were purchased from Sigma–Aldrich (Oakville, Ontario). Oseltamivir analogs were prepared as described by Mohan et al.[13]

4.2. Neuraminidase enzymes

NEU3 was expressed in *Escherichia coli* and purified as an MBP-NEU3 fusion protein (pMAL-c2x) as previously reported.[26] To generate a NEU4 fusion protein, the optimized gene of *Neu4* (DNA2.0, Menlo Park, CA) was synthesized and subcloned into vector pGEX-4T-1, and the resulting plasmid containing Neu4 as an *N*-terminal Glutathione S-Transferase fusion (*GST-Neu4*) was transformed into *E. coli* BL21(DE3) pLySs (Novagen, Gibbstown, NJ) by adding 50 ng plasmid to 50 μL of competent cells. Cells were incubated on ice for 15 min followed by heat shock (30 s, 42 °C), and then returned to ice for 2 min. The transformation reactions were plated on LB agar plates with final concentrations of 35 μg/mL chloramphenicol and 100 μg/mL ampicillin. Clones were selected and grown in LB medium with antibiotics (35 μg/mL chloramphenicol and 100 μg/mL ampicillin) for 7 h, after which 50% glycerol was added to a final concentration of 15%, and these stocks were stored at −80 °C.

To express the protein, 20 mL of overnight turbid culture was inoculated into 1 L of LB medium containing 1% glucose and antibiotics (35 μg/mL chloramphenicol and 100 μg/mL ampicillin) at 30 °C. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 100 μM to induce expression at 20 °C with shaking (225 rpm for 20 h). Cells were harvested by centrifugation. The pellet was resuspended (50 mL per liter of medium) in buffer (PBS, 5 mM DTT, 10% glycerol, 1% Triton X-100, and 1 mM EDTA, pH 7.4) supplemented with a complete protease inhibitor tablet (Roche, Laval, Quebec). The cell suspension was passed through a cell disruptor once at 20,000 psi and then immediately pelleted by centrifugation at 105,000×*g* for 60 min at 4 °C. The supernatant was loaded onto a glutathione agarose column (Agarose Bead Technologies, Tampa, FL) equilibrated with the equilibration buffer (PBS, 5 mM DTT, 10% glycerol, 0.1% Triton X-100, pH 7.4). The purified GST-fusion protein was eluted with the equilibration buffer containing 20 mM reduced L-glutathione (GSH). The eluted protein was stored at −80 °C.

4.3. Characterization of NEU4

GST-NEU4 protein was purified as above and diluted to a concentration of 0.1 mg/mL. The neuraminidase solution was incubated with 500 μM 4MU-NA for 60 min at 37 °C, the reaction was stopped using quenching buffer (50 μL of 0.2 M glycine/NaOH pH 10.7) and activity was determined by fluorescence (365 nm excitation; 445 nm emission). Enzyme activity was measured at pH 3.7, 4.0, 4.25, 4.50, 4.75, 5.0, 5.5 (0.25 M sodium acetate buffer) and 5.25, 6.0, 6.5, 7.0, 7.5, 8.0 (0.1 M sodium phosphate buffer).

Purified GST-NEU4 was tested for effects of additives by dissolving the protein in 0.1 M sodium acetate buffer (pH 4.5), at a protein concentration of 0.25 mg/mL as determined by A_{280}. Results were normalized to buffer control. Additive solutions were: $CaCl_2$ (7 mM), $CaCl_2$ (0.5 mM), $MnCl_2$ (7 mM), $MnCl_2$ (0.5 mM), EDTA (1 mM), DTT (5 mM), Triton X-100 (0.07%), sodium cholate (0.15%), $CuSO_4$ (2 mM), $CuSO_4$ (late addition, 2 mM), $CuSO_4$ (0.1 mM), and $CuSO_4$ (late addition, 0.1 mM). Conditions noted as a late addition had additive treatment at the end of incubation period to test for any influence of the additive on the assay itself. The reaction was prepared by adding 4MU-NA (500 μM final concentration), additive (to the indicated final concentration) and purified enzyme in a total volume of 0.04 mL. The mixture was incubated at 37 °C for 1 h, and stopped using quenching buffer (50 μL of 0.2 M glycine/NaOH pH 10.7).

4.4. Enzyme inhibition assay

Compounds were tested for their inhibition of the purified neuraminidase enzymes. The enzymes were tested by adding 0.5 μL of a serial dilution (five replicates for each reaction) of the inhibitor to 4.5 μL of enzyme (0.1 mg/mL) to achieve final inhibitor concentrations of 5, 10, 20, 50, 100, 200, 500 and 1000 μM. The mixture was incubated at 37 °C for 30 min. After incubation, 5 μL of 1 mM 4MU-NA (final concentration 500 μM) in 0.1 M sodium acetate buffer (NEU3, pH 5.0; NEU4, pH 4.5) was added to the reaction and incubated for 1 h. The reactions were stopped using quenching buffer (50 μL of 0.2 M glycine/NaOH pH 10.2).

Supernatant (50 μL) was removed from each reaction to a 384 well plate. Fluorescence was then measured on a Spectramax M2e Microplate Reader (Molecular Devices) with excitation 365 nm and emission 445 nm. The data were fit to a four parameter logistic curve using the following equation:

$$f = \frac{\min + (\max - \min)}{1 + (x/\mathrm{IC}_{50})^{-Hillslope}}$$

Acknowledgments

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Supplementary data

Supplementary data (assay data, methods, and enzyme characterization) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.03.039.

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