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Brief Article

Complexity in Influenza Virus Targeted Drug Design: Interaction with Human Sialidases†

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With the global spread of the pandemic H1N1 and the ongoing pandemic potential of the H5N1 subtype, the influenza virus represents one of the most alarming viruses spreading worldwide. The influenza virus sialidase is an effective drug target, and a number of inhibitors are clinically effective against the virus (zanamivir, oseltamivir, peramivir). Here we report structural and biochemical studies of the human cytosolic sialidase Neu2 with influenza virus sialidase-targeting drugs and related compounds.

Introduction

Influenza virus is an RNA virus that infects avian and mammalian cells with the assistance of two glycoproteins, hemagglutinin (HA[a]) and sialidase (neuraminidase, NA). While HA is necessary for the attachment and entry of the virus to the host cell, NA facilitates the release of newly formed virions by hydrolyzing sialic acids from sialoglycoconjugates at the surface of the host cell.[1] Inhibition of NA prevents release of progeny virions and is thus a good target for drug design. Between strains, influenza virus sialidases may differ in their amino acid sequences but share an almost identical active site architecture.[2,3] Therefore, drugs targeted to the sialidase of one virus strain will most likely act on those of other strains.[4] On the basis of the knowledge of the structure of sialic acid bound in the active site of NA, three specific inhibitors with potent low nanomolar K_i values were developed for the treatment or prevention of influenza virus infection: zanamivir **1**, [5] oseltamivir (the prodrug is oseltamivir phosphate, and the active compound used here is oseltamivir carboxylate **2**),[6] and peramivir **3** [7]. **1** and particularly **2** have been stockpiled by a number of governments worldwide for treatment and prophylaxis in the event of the emergence of a new pandemic influenza strain. The avian influenza subtype H5N1 has sporadically infected humans, resulting in 442 cases as of September 2009, with 262 deaths, a mortality rate of 60%.[8] The ongoing spread of H5N1 therefore raises major concerns worldwide as a possible pandemic threat. However, it caught the world by surprise when the first influenza pandemic of the new century was derived from a swine lineage. For now both stockpiled drugs are still effective against the H1N1 pandemic strain (although there are recent reports of cases of resistance to **2**).[9] In addition, **3** has also recently received approval for emergency intravenous use.[10]

Structural and biochemical studies have yielded key insights into the mechanism of sialic acid release from a sialidase's active site, but only a few reports concerning the mammalian enzymes have been published.[11−13] Four distinct mammalian sialidases have been characterized on the basis of their subcellular localization and substrate specificities: cytosolic, lysosomal, plasma membrane-bound, and associated with mitochondria.[14,15] We have solved and reported the first three-dimensional structure of the human cytosolic sialidase Neu2 in the apo and inhibitor (2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, Neu5Ac2en or DANA **4**) bound forms and confirmed a similar organization of the enzyme's catalytic cleft when compared to influenza virus NA and sialidases from other species.[16] In addition to warnings of psychiatric side effects in patients treated with **2**, [17] side effects in mice were observed during clinical tests of **2** that might be related to murine sialidase inactivation.[18] Therefore, as **2** also modulates morphine analgesia by possible interaction with a monosialotetrahexosyl ganglioside (GM1) specific sialidase,[19,20] it is conceivable that influenza virus NA-targeted drugs act as well on human sialidases. Indeed, **1**, but not **2**, has been reported to inhibit recombinant human sialidases Neu2 and Neu3 at micromolar levels.[13] To further address this question, we have investigated human Neu2 inhibition by a range of NA inhibitors, including the three most common influenza virus sialidase inhibitors: **1**, **2**, and **3**. By a combination of enzymatic assays and crystal structure determination of Neu2 in complex with the inhibitors, the present studies confirm significant levels of inhibition of Neu2 by several influenza virus NA-targeting inhibitors with the exception of **2** and provide useful information for structure-based drug design of next generation inhibitors against influenza virus NA or mammalian sialidases.

†Coordinates and structure factors have been deposited in the PDB with accession codes 2f0z, 2f10, 2f11, 2f12, and 2f13 for Neu2 in complex with inhibitors **1**, **3**, **5**, **6**, and **7**, respectively.

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[a]Abbreviations: HA, hemagglutinin; NA, sialidase or neuraminidase; DANA, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid; GM1, monosialotetrahexosyl ganglioside; IEM, isobutyl ether DANA mimetic; HEM, 3-hydroxypropyl ether DANA mimetic; DEM, 2,3-dihydroxypropylether DANA mimetic; PDB, Protein Data Bank; MU-NeuAc, 4-methylumbelliferyl α-*N*-acetyl-D-neuraminic acid; WHO, World Health Organization.

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Materials and Methods

Cloning, Protein Expression, Purification, and Crystallization. Cloning and protein production of Neu2 have been described elsewhere.[16] Crystals of the uncomplexed sialidase were grown from a reservoir solution containing 0.8 M Na/KPO_4, 0.1 M HEPES buffer, pH 8.0. Single crystals appeared after microseeding experiments from initial agglomerates of multicrystals. Soaking experiments were optimized for each inhibitor in terms of concentration and soaking time. All the inhibitors were dissolved in water prior to soaking experiments. The complexes with **1** (GlaxoSmithKline) or **3** (GlaxoSmithKline) were solved from crystals soaked in 10 mM **1** for 20 min or 30 mM **3** for 10 min. For the inhibitor **2** (GlaxoSmithKline or Dr. Keith Watson, WEHI, Australia), 1−20 mM inhibitor-containing solutions were prepared and crystals soaked for 1−30 min. Unfortunately, this resulted in no electron density for any ligand in the protein active site, cracked crystals, or disruption of the crystals. The other three inhibitors, glucuronide-based DANA mimetics,[21] isobutyl ether mimetic (IEM **5**), 3-hydroxypropyl ether mimetic (HEM **6**), and 2,3-dihydroxypropyl ether mimetic (DEM **7**) were prepared at 10 mM, and crystals were soaked for 3 min.

Data Collection, Structure Determination, and Refinement. Neu2/inhibitor complex structures were solved by the molecular replacement method using human Neu2 coordinates (Protein Data Bank accession number 1snt) as a search model. Data for the complex crystals were collected at beamlines of the Photon Factory (Japan). The crystals were flash-cooled in a 100 K dry nitrogen stream with 25% glycerol as a cryoprotectant. Data collection and refinement statistics are summarized in Supporting Information Table 1.

Enzymatic Assay. The sialidase activity of Neu2 was determined essentially by the same procedure as previously described.[22] Briefly, the reaction mixtures (final volume of 100 μL) containing 27 ng of recombinant Neu2, 20 μg of bovine serum albumin, and various amounts of fluorescent artificial substrate 4-methylumbelliferyl α-*N*-acetyl-D-neuraminic acid (MU-NeuAc) were incubated up to 10 min at 310 K with shaking. The reactions were stopped by the addition of 1.5 mL of 0.2 M glycine buffered with NaOH at pH 10.2. Fluorescence experiments were measured with excitation at 365 nm and emission at 445 nm, using 4-methylumbelliferone to set up a calibration curve. K_m and apparent V_{max} values were determined by the method of Lineweaver and Burk using five different MU-NeuAc concentrations, from 0.04 to 0.21 mM. The results are the mean of at least two experiments carried out in triplicate.

To measure the inhibitory constants K_i, typical V_o/[S] experiments described above were carried out in the presence of three different inhibitor concentrations. In the case of **2**, no inhibition was detected using up to 5 mM at 310 K, whereas preincubation of the drug with the sialidase for 15 min at 277 K prior to the addition of the substrate at 310 K showed some minor inhibitory effects (data not shown). To obtain a value for K_i, the kinetic data were fitted to the standard equation for competitive inhibition.

Results

Inhibition Assays against Neu2. Six potential NA-targeting inhibitors were compared with **4** for their ability to inhibit the sialidase activity of recombinant Neu2. While **1** and **3** clearly showed competitive inhibition with K_i of 0.017 and 0.33 mM, respectively, **2** did not inhibit Neu2 activity even at concentrations as high as 5 mM (Figure 1, Table 1). Additionally, the three DANA mimetics **5**, **6**, and **7** reacted moderately with Neu2, with higher K_i values compared to **4** (K_i = 0.14 mM) (Table 1).

Crystal Structures of Neu2/Inhibitor Complexes. We successfully solved the structures of Neu2 coordinating five of the influenza virus NA-targeting inhibitors investigated in this study (Supporting Information Table 1); however, no

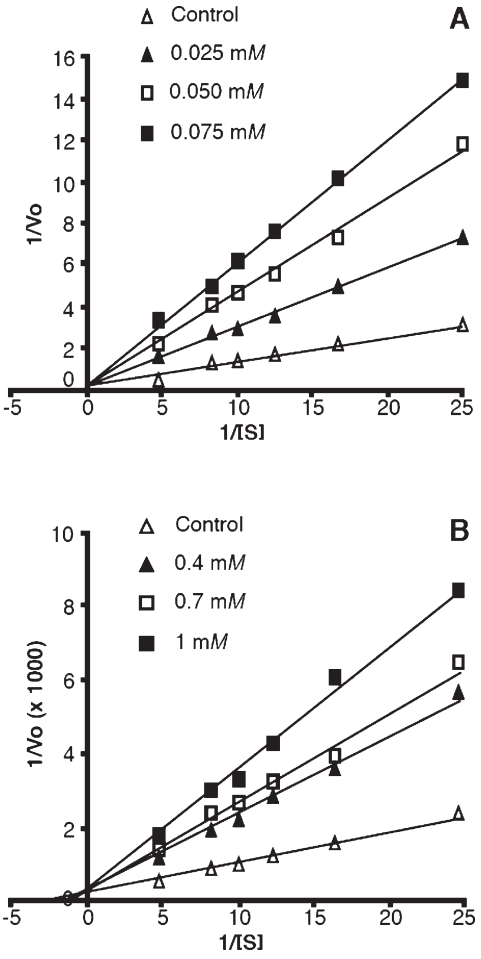
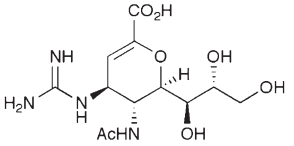
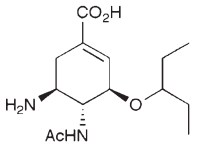
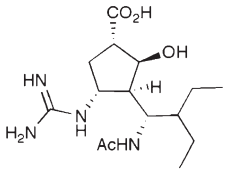
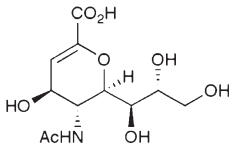
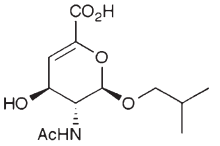
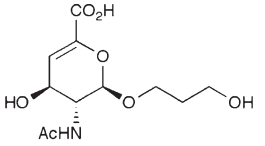
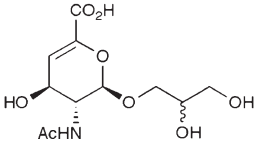


Figure 1. Inhibition assays of Neu2. Sialidase activity of Neu2 in the presence of indicated concentrations of **1** (A) and **3** (B), respectively. The plotted values are the mean of two experiments carried out in triplicate.

Neu2/**2** complex was observed. Whereas **1** is more tightly coordinated by Neu2 residues than **4** [16], **3** fits in the active site mainly through H-bonds with its carboxyl headgroup (Figure 2A and Figure 2C). More precisely, the C-4 functionalization in **1** and **3**, absent in **4**, favors the formation of additional hydrogen bonds with Asn86 and Glu39 side chains. The cyclopentane core in **3** favors the placement of the guanidinium group for interaction with these two residues (Figure 2C). However, the total number of hydrogen bonds stabilizing **3** is reduced compared to **1** due to differences in the exact positioning of the guanidinium group (arising from the different core structures) and due to the substitution of the glycerol side chain by a lipophilic group. To accept the lipophilic moiety of **3**, small shifts in Tyr179 (∼0.8 Å) and Leu217 (∼0.7 Å) side chains contribute to shape a hydrophobic pocket (Figure 2C).

In the cases of **5**, **6**, and **7** the C-6 glycerol side chain of **4** is replaced with a lipophilic moiety (**5**), reminiscent of **2**, or with functionalized ether groups (**6**, **7**; Table 1). As indicated above, unlike **2**, all three DANA mimetics inhibit Neu2 weakly, the first indication of a possible physical interaction of the inhibitors with the sialidase. The Neu2/inhibitor complex crystal structures clarified the recognition pattern by Neu2 active site residues, showing the three compounds coordinated mostly through their carboxyl headgroup, a common feature of all sialidase inhibitors. Small side chain

Table 1. Enzymatic Sialidase Assay of Neu2 with Various NA-Targeting Inhibitors[c]

	Name		Struture	K_i (m*M*)	Observed H-bonds
Zmr	Zanamivir	**1**		0.017	12
OC	Oseltamivir carboxylate	**2**		n.d.[a]	–
Bcz	Peramivir	**3**		0.33	8
DANA	Neu5Ac2en	**4**		0.14	14[b]
IEM	isobutylether mimetic	**5**		0.88	6
HEM	3-hydroxypropylether mimetic	**6**		0.74	7
DEM	2,3-dihydroxypropylether mimetic	**7**		1.4	8

[a] n.d.: not determined. [b] From ref 16. [c] The indicated "observed H-bonds" numbers correspond to the present structural data analysis.

reorganizations assist Neu2 in molding a molecular surface that would properly accept the C-6 substituents.

Discussion

While the current H1N1 influenza pandemic appears to be a milder disease than anticipated for a new pandemic strain, there have still been thousands of fatalities, and the pandemic potential of the H5N1 virus remains a serious concern. Because of delays of several months in preparing a new pandemic vaccine, governments around the world are stockpiling large quantities of the influenza virus NA-targeted drugs oseltamivir **2** and zanamivir **1**. Although still undergoing clinical testing, peramivir **3** has also been approved for emergency intravenous use. The monitoring of adverse reactions to these drugs in humans is critical, as highlighted by the increasing number of side effects such as neuropsychiatric disorders and severe skin reactions in patients treated with **2**.[17] In an attempt to explain these symptoms, Li and co-workers hypothesized that a specific interaction between oseltamivir carboxylate and the human sialidase Neu2 could be at the origin of the reported syndromes.[23] However, this human sialidase was found to be resistant to inhibition by oseltamivir carboxylate in a report by Hata et al.[13] The recent fast tracking of approval of **3** for emergency intravenous use[10] reinforces the needs to clarify the possible interactions of these molecules with human sialidases. To gain further insights into the inhibitory activity of influenza virus NA-targeting drugs against the human cytosolic sialidase Neu2, we attempted in this work to relate enzymatic assays and X-ray crystal structures of the complexes with the inhibitors, which permitted us to compare the effects of different functionalization in these molecules.

We previously reported that Neu2 tightly coordinates the naturally occurring inhibitor DANA **4** through a network of 14 hydrogen bonds, in addition to some hydrophobic interactions.[16] In this early study, it was shown that Neu2 offered

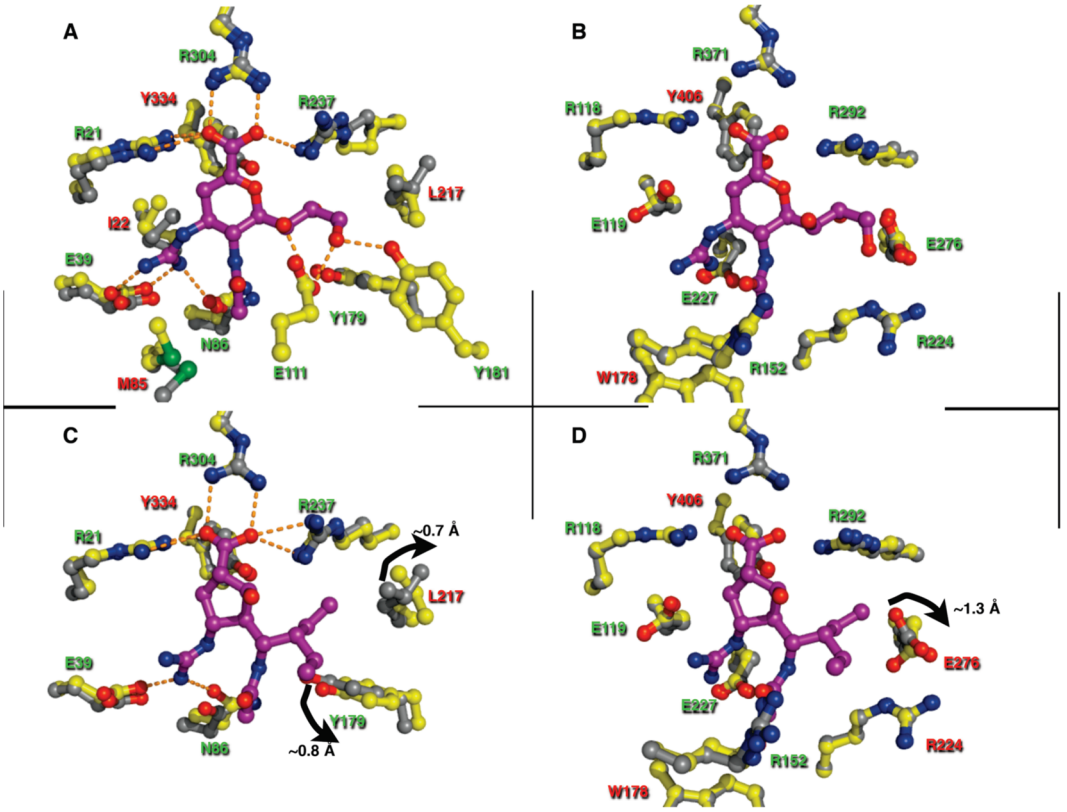


Figure 2. Coordination patterns of **1** and **3**: structures of the human Neu2/zanamivir **1** (A) and Neu2/peramivir **3** (C) complexes (ball-and-stick model, yellow and purple) superimposed with the native structure of Neu2 (gray); structures of the influenza NA/zanamivir **1** (B) and NA/peramivir **3** (D) complexes (ball-and-stick model, yellow and purple), superimposed with the native structure of influenza NA (gray). The influenza NA structures were extracted from coordinates 1nnc (Zmr), 1l7f (Bcz), and 7nn9, respectively. Hydrogren bond networks are shown as orange dotted lines, and hydrophobic-interacting residues are labeled in red. Black arrows indicate the changes in side chain conformations occurring after complex formation with **3**.

residues at the "bottom" of its active site of a different nature than the ones encountered in bacterial or viral NAs, attributes initially assumed to favor substrate selection. On the other hand, highly comparable features can also be noticed in influenza virus NA and human Neu2. Similar to what has been reported for influenza NA, the coordination of **1** by Neu2 is extended through the formation of additional hydrogen bonds to the C-4 guanidino group of 1 (Figure 2A and Figure 2B). This increased interaction was proposed to explain partly the greater effectiveness of **1** against influenza virus NA.[24] Consistent with the tighter coordination by Neu2 residues, the inhibitory effect of **1** was found to be 8-fold higher than that of **4**.

While **1** sits fully in the catalytic cleft of the enzyme, **3** appears to be stabilized mostly by its carboxyl headgroup, resulting in fewer hydrogen bonds generated upon complex formation (Figure 2C). Additionally, the strain for Neu2 to reach the energy barrier needed to shift the side chains of Tyr179 and Leu217 to accommodate the lipophilic substitution in **3** is a tempting explanation for the weaker inhibitory effect of the drug against the human sialidase. Similarly in influenza A virus NAs, it has been shown that a small reorganization of the Glu276 side chain was necessary for **3** to fit in the active site[25](Figure 2D).

Structural analysis showed that a major conformational change was not observed in the case of **2** bound in an influenza B NA[26,27] and is proposed to account for the lower efficacy[28] of **2** against influenza B NAs compared to influenza A NAs.[26] With the absence of a noticeable interaction between **2** and Neu2 in the present study, these data suggest that the active site of the human cytosolic sialidase might also not be flexible enough to accept the drug, leading to a higher specificity of **2** for influenza virus NA.

To complement this analysis, the capacity of Neu2 to recognize a novel series of DANA mimetics that inhibit influenza virus sialidase at micromolar levels was also investigated. The three inhibitors present longer or bulkier C-6 substituents when compared with **4** or **1**, with as a direct consequence lower inhibitory effects against Neu2. Consistent with binding of **3**, small conformational changes are observed in the complex structures between Neu2 and **5**, **6**, and **7**, respectively (Supporting Information Figures 1−3). By analogy then, the partial movement of the active site residues' side chains, notably of Tyr179 and Leu217, would raise the binding energy of the molecules in Neu2, leading to reduced affinity as shown by higher K_i values for these inhibitors. The resulting variations in the inhibitory efficacy of various inhibitors versus disparate sialidases might therefore be explained partly as differing extents of the hydrogen bond networks responsible for the coordination of the molecules, paralleled with the relative adaptability of the enzyme active site for binding the inhibitors. These observations provide valuable insights for the design of new anti-influenza drugs that may better minimize the inhibition of human sialidases.

With the global spread of the H1N1 seasonal influenza resistant to oseltamivir and already cases of oseltamivir resistant pandemic H1N1 influenza emerging,[9] there is an ongoing need for the development of new influenza inhibitors.

In the present study, five such molecules were shown to physically interact with the human cytosolic sialidase Neu2 and to inhibit its activity. As the concentrations of **1** required to inhibit Neu2 are in the micromolar range and the cytosolic levels of **1** are estimated to be up to 200-fold lower than the concentration that inhibits Neu2,[29] the clinical relevance of binding of **1** is not clear. However, when **3** is administered intravenously at 300 mg per day, plasma concentrations are in the range 10000−20000 ng/mL, corresponding to ∼30−60 μM.[30] The current dosing is 600 mg, which could result in plasma levels of **3** only 2- to 3-fold lower than the K_i, so the clinical consequences could be of more concern. Our enzymatic and structural data of Neu2 in complex with two of the clinically used drugs zanamivir and peramivir highlight the need for thorough investigations in regard to the potential effects NA-targeted drugs might have on other human sialidases.

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Supporting Information Available: Data collection and refinement statistics table; figures of the coordination patterns of the complexes between Neu2 and **5**, **6**, and **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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