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Chemical Insight into the Emergence of Influenza Virus Strains That Are Resistant to Relenza

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Supporting Information

ABSTRACT: A reagent panel containing ten 4-substituted 4-nitrophenyl α-D-sialosides and a second panel of the corresponding sialic acid glycals were synthesized and used to probe the inhibition mechanism for two neuraminidases, the N2 enzyme from influenza type A virus and the enzyme from *Micromonospora viridifaciens*. For the viral enzyme the logarithm of the inhibition constant (K_i) correlated with neither the logarithm of the catalytic efficiency (k_{cat}/K_m) nor catalytic proficiency ($k_{cat}/K_m k_{un}$). These linear free energy relationship data support the notion that these inhibitors, which include the therapeutic agent Relenza, are not transition state mimics for the enzyme-catalyzed hydrolysis reaction. Moreover, for the influenza enzyme, a correlation (slope, 0.80 ± 0.08) is observed between the logarithms of the inhibition (K_i) and Michaelis (K_m) constants. We conclude that the free energy for Relenza binding to the influenza enzyme mimics the enzyme−substrate interactions at the Michaelis complex. Thus, an influenza mutational response to a 4-substituted sialic acid glycal inhibitor can weaken the interactions between the inhibitor and the viral neuraminidase without a concomitant decrease in free energy of binding for the substrate at the enzyme-catalyzed hydrolysis transition state. The current findings make it clear that new structural motifs and/or substitution patterns need to be developed in the search for a bona fide influenza viral neuraminidase transition state analogue inhibitor.

Recent news reports highlight the threat of an avian influenza virus strain, such as H5N1, crossing the species barrier[1] and becoming transmissible in humans. Barring the development of new therapeutic strategies, a crossover virus such as this presents a real threat of precipitating an influenza pandemic.[2] Current influenza therapeutics are designed to take advantage of the requirement of neuraminidase activity for viral pathogenicity.[3] Viral resistance to influenza therapeutics is an emerging medical problem, driven in part by the high mutational frequency of the virus.[2] Often, mutations that generate resistant strains[4−6] also attenuate viral infectivity.[7] However, continued selection pressure resulting from drug treatment results in permissive secondary mutations that allow the resistant virus to override the deleterious effects of the initial mutation.[8]

An ongoing challenge to the development of therapeutic agents for treating influenza and controlling the spread of disease[3] is the design of selective inhibitors that engender a reduced risk of viral resistance. An ideal inhibitor would precisely mimic the neuraminidase-catalyzed glycosylation[9,10] or deglycosylation[11] transition state (TS). In such a case, a mutational response by the virus to a transition state analogue (TSA) inhibitor that reduces binding avidity to the targeted viral neuraminidase must also, by virtue of the TS analogy, compromise the catalytic efficiency of this enzyme, resulting in reduced infectivity of the mutated strain of the influenza virus. If, however, the inhibitor is not a TSA, then a mutational response can lower the efficacy of the therapeutic without causing an obligatory decrease in the targeted enzymatic activity, a situation which permits the emergence of resistant strains and poses further risk to human health. Therefore, in order to design new influenza treatments with a reduced risk of provoking further viral resistance, it is imperative that we understand whether such inhibitors are true TSAs.

It is important to point out that tight-binding inhibitors are not necessarily TSAs because binding interactions that occur in the enzyme:inhibitor complex may not replicate those at an enzymatic TS. Once a tight-binding structural motif is reported, its inhibitory potency is maximized by the synthesis of structures containing different functional groups, and the resultant best inhibitor is often presumed to be the best TSA. Thus, many tight-binding inhibitors are labeled as "transition state analogues" based on chemical intuition rather than an in-depth scientific analysis. Indeed, the rapid emergence of influenza strains that are resistant to Relenza and possess catalytically competent mutant neuraminidases raises the important question of whether this drug is a genuine TSA, as has often been presumed,[12−14] or if its binding avidity to the viral enzyme is unrelated to the mechanism of catalysis. In order to test the presumed TS analogy for neuraminidase inhibitors like Neu2en5Ac (**2a**) and Relenza (**2h**), a detailed kinetic and theoretical analysis must be performed.

Scheme 1 shows the neuraminidase-catalyzed glycosylation mechanism in which the bound substrate in the accumulating Michaelis complex adopts a skew-boat conformation,[11] while the pyranosyl ring conformation of the sialosyl-enzyme intermediate is a $^2C_5$ chair.[15] To understand the individual contributions to TS stabilization—knowledge that is critical for evaluating whether an inhibitor is a TSA as well as for designing optimized TS mimics—a robust approach is to establish a linear free energy relationship (LFER) between catalysis and inhibitor binding.[16,17] Using this LFER method, inhibitors and

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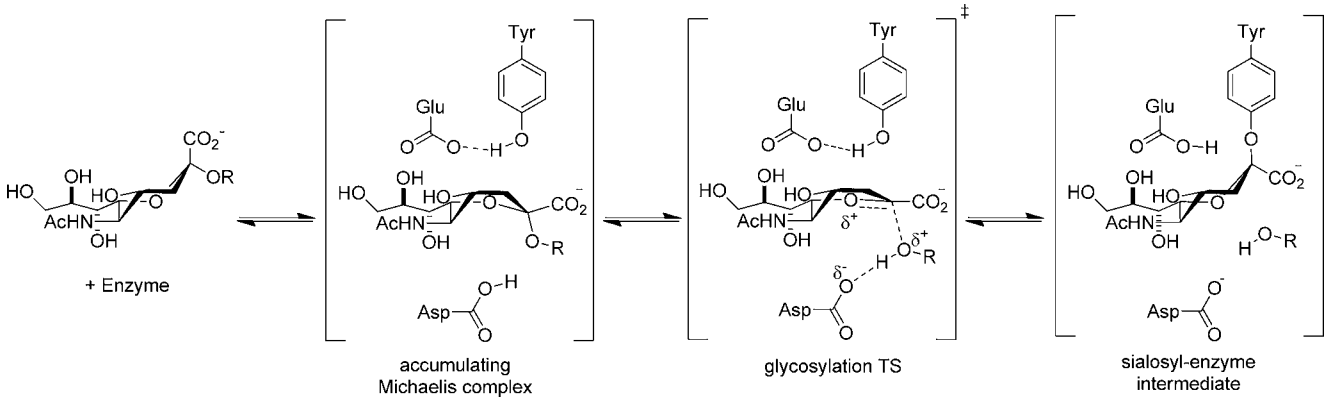
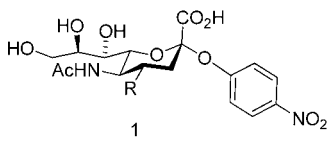
Scheme 1. Mechanism for the Neuraminidase-Catalyzed Formation of the Tyrosinyl-Bound Intermediate[a]



[a]The key active-site amino acid residues (Glu, Tyr, Asp) and the substrate are shown. The substrate is bound in a skew-boat conformation, and the sialosyl-enzyme intermediate is in a $^2C_5$ conformation.[18]

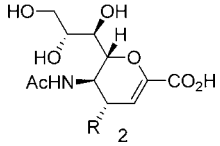
the corresponding substrates are systematically modified at a single position, and a comparison is made between the free energy for inhibitor binding to the enzyme (log K_i) and the free energy difference between the TSs for the first irreversible step and that for the uncatalyzed reaction (log $k_{cat}/K_m k_{un}$) for each substrate/inhibitor pair.[16,17] A correlation with a unit slope reveals that structural changes in the inhibitor parallel those at the TSs and that the core inhibitor structure is a genuine TSA. With these factors in mind, we aimed to test whether the ring geometry of these glycal inhibitors (including Neu2en5Ac **2a** and Relenza, **2h**), which are constrained by the ring double bond, orients the 4-substituent so that, when bound, the resultant interactions within the complex mimic those of the substrate at the glycosylation TS. We focused on these interactions because the 4-position is substituted with positively charged groups in two commercial influenza drugs, Relenza (zanamivir) and Tamiflu (oseltamivir).

To perform these studies, we synthesized a panel of ten 4-nitrophenyl α-D-sialosides (**1a** = pNP-αNeu5Ac) as substrates, and the corresponding ten 4-substituted 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acids (**2a** = Neu2en5Ac) as inhibitors (Figure 1, **1a–j** and **2a–j**). We chose to make 4-nitrophenyl substrates because of (i) synthetic accessibility, (ii) ease of monitoring kinetics, and (iii) ability to measure uncatalyzed hydrolysis rate constants (k_{un}). Sialic acid (**3**) was transformed into **4**, and then converted into oxazoline (**5**) and azido (**6**) using known procedures (Scheme 2).[19]



1

1a	R = OH
1b	R = N_3
1c	R =1-triazolyl
1d	R = NH_2
1e	R = NHAc
1f	R = NMe_2
1g	R = NHiPr
1h	R = guanidino
1i	R = H
1j	R = OMe

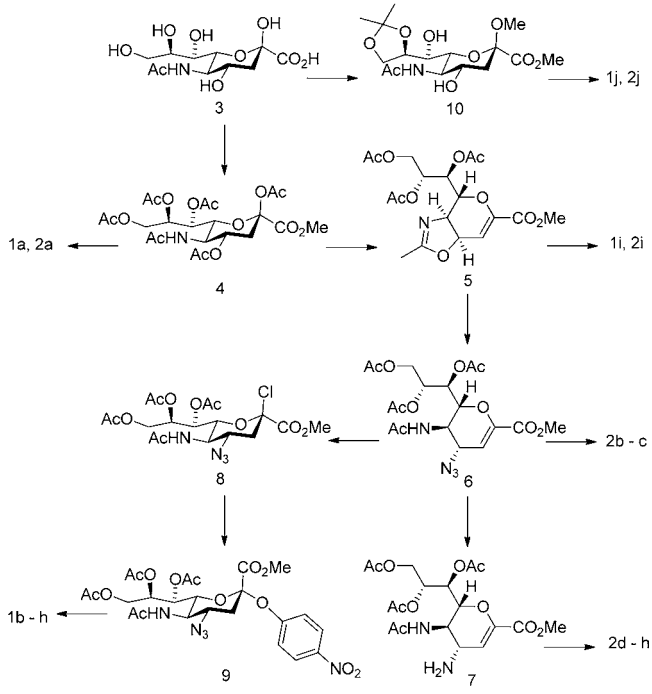


2

2a	R = OH
2b	R = N_3
2c	R =1-triazolyl
2d	R = NH_2
2e	R = NHAc
2f	R = NMe_2
2g	R = NHiPr
2h	R = guanidino
2i	R = H
2j	R = OMe

Figure 1. Synthesized substrates **1a–j** and inhibitors **2a–j**. Inhibitors Neu2en5Ac and Relenza are **2a** and **2h**, respectively.

Scheme 2. Generalized Synthetic Routes to Substrates 1a–j and Inhibitors 2a–j[a]



[a]Reagents and yields for all steps are shown in Schemes S1–S4.

Reduction of glycal **6** gave **7**, which we used to make protected **2e–h** by (i) acetylation (**2e**), (ii) reductive amination (**2f,g**), and (iii) guanidylation (**2h**). Also, azide **6** underwent a [2,3]-cycloaddition with propynoic acid, which after decarboxylation gave protected **2c**. Selective hydrogenation of oxazoline **5** gave access to glycal **2i**, while selective methylation of **10** gave the 4-methoxy compounds. Reaction of **8**, formed by HCl addition to **6**,[19] with 4-nitrophenol gave glycoside **9**. Using reactions identical to those that converted **6** into protected **2b–h**, **9** was turned into protected pNP-αNeu5Ac analogues **1b–h**. Hydrogenation of oxazoline **5** gave access to 4-deoxy compound **1i**, while careful methylation of **10** (made in two steps from sialic acid) allowed synthesis of 4-methoxy analogue **1j**. Finally, all protecting groups were removed using routine protocols to give substrates **1a–j** and inhibitors **2a–j**.

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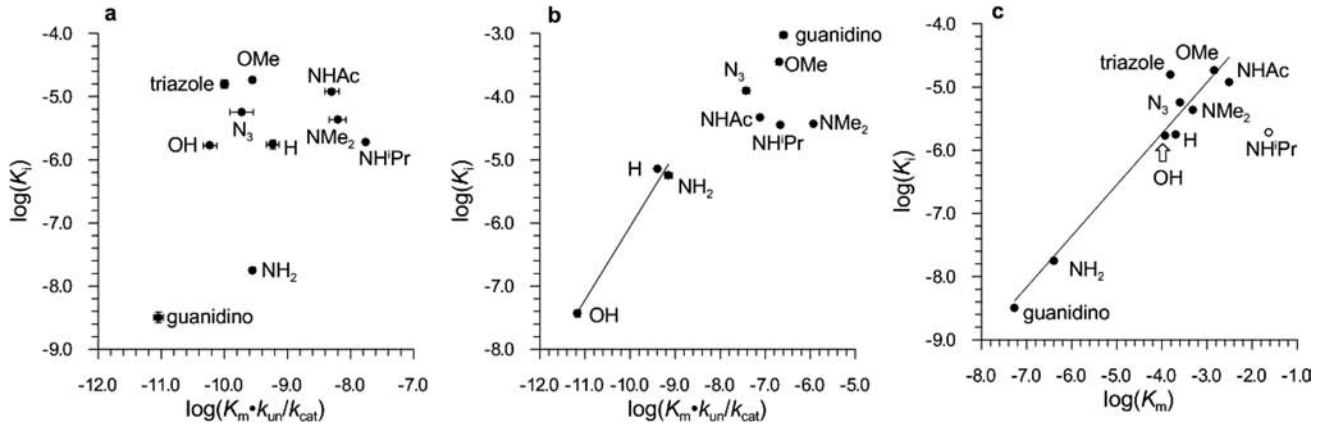

Figure 2. Neuraminidases: linear free energy correlations. Plots of $\log(K_m k_{un}/k_{cat})$ for neuraminidase-catalyzed hydrolysis of pNP 4-substituted sialosides **1a**–**j** versus $\log(K_i)$ for the corresponding glycal inhibitor (**2a**–**j**). (a) Data for the influenza N2 enzyme; (b) data for the *M. viridifaciens* neuraminidase. Error bars are shown or are encompassed within the symbol. The line shown in panel b is the best linear fit for the three smallest substituents (slope, 1.16 ± 0.18). (c) Plot of $\log(K_m)$ for the influenza-catalyzed hydrolysis of pNP 4-substituted sialosides versus $\log(K_i)$ of the correspond glycal inhibitor; the line represents the best linear fit (slope, 0.80 ± 0.08) to the data, excluding those for 4-*N*-isopropyl compounds (hollow circle).

In order to resolve whether good inhibitors are TSAs, equivalent alterations are made to a panel of substrates and inhibitors. If these modifications have identical effects on enzymatic catalysis and inhibition, then a LFER with a slope of 1 will be measured (eq 1),[17] and the inhibitor can be classified as a TSA.[17,20] In this equation, δ is the proportionality between K_i and $(K_m k_{un}/k_{cat})$, because any TSA is an imperfect mimic of the TS.[17]

$$\log(K_i) = \log(K_m k_{un}/k_{cat}) + \log(\delta) \quad (1)$$

For a linear relationship to be valid between $\log(K_i)$ and $\log(K_m/k_{cat})$, it is important that (i) enzymatic kinetic parameters are measured under conditions in which glycosylation is kinetically significant, which in these cases occurs at non-optimal pH values,[21,22] so we monitored all kinetic parameters at pH 8.03; (ii) the rate constants for the spontaneous, uncatalyzed hydrolyses (k_{un}) are identical for all substrates; and (iii) the inhibitors are kinetically stable. Although glycals are known to undergo neuraminidase-catalyzed hydration, these reactions are ~10^7-fold slower than hydrolysis.[23,24]

We conclude that our results, which are plotted in Figure S1, show that $\log(K_m/k_{cat})$ correlates with $\log(K_i)$ for neither the influenza N2 (panel a) nor the *Micromonospora viridifaciens* neuraminidase (*Mv*NA) (panel b) enzymes. However, as noted above, it is assumed that k_{un} is constant for all substrates. Consequently, we measured the rate constants for the uncatalyzed hydrolyses of **1a**–**j** (k_{un}) in order to determine whether the lack of correlation was caused by different intrinsic reactivities of the ten substrates. Notably, the spontaneous hydrolysis rate constants measured for the 4-deoxy (**1h**) through triazolyl (**1c**) substrates span more than 2 orders of magnitude (Table S4). The resultant plots of $\log(K_m k_{un}/k_{cat})$ versus $\log(K_i)$ are shown in Figure 2.

We are forced to conclude, contrary to the commonly held assumption, that sialic acid glycals, including Neu2en5Ac (**2a**) and Relenza (**2h**), are not TSA inhibitors for the influenza A N2 enzyme. That is, although the anomeric carbon of the substrate must be undergoing rehybridization toward an sp^2-like center at the glycosylation TS (Scheme 1), this does not necessarily enforce glycal inhibitors, which have sp^2 carbons at this position, to adopt a comparable ring conformation when bound to the enzyme (Figure S2). Indeed, the characteristic increase in inhibition (lower K_i) with only a moderate increase in catalytic efficiency (k_{cat}/K_m) for the guanidino compounds (**1h** and **2h**) displays the hallmarks of nonproductive binding.[25] That is, during the catalytic cycle, a component of free energy for **1h** binding to the enzyme is expressed at the Michaelis complex but not at the hydrolytic TS. Such nonproductive binding, in which the inhibitor binding mimics the enzyme–substrate interactions in the Michaelis complex, should give a correlation between $\log(K_i)$ and $\log(K_m)$.[17] A plot of $\log(K_m)$ versus $\log(K_i)$ for the influenza N2 data (Figure 2c) gives a slope of 0.80 ± 0.08 (after omitting the point for the *N*-isopropyl substituent). We conclude that Neu2en5Ac derivatives **2a**–**j**, including Relenza (**2h**), are potent ground state analogue inhibitors of influenza N2 neuraminidase in which the constrained geometry of the glycal places the 4-substitutent so that its interactions with the enzyme mimic those in the Michaelis complex.

In contrast, the data for the *Mv*NA suggest that $\log(K_m k_{un}/k_{cat})$ and $\log(K_i)$ are correlated, albeit only for the three smallest substituents (Figure 2b). Given that, in the structure of this enzyme complexed with **2a**,[26] the C4-OH group of Neu2en5Ac interacts strongly with an arginine residue (O–N distance ~3 Å), it is not surprising that interactions between substrates/inhibitors and *Mv*NA exhibit TS analogy only for small substituents. Of note, the influenza N2 and *Mv*NA enzymes are from different glycosyl hydrolase (GH) families,[27] GH34 and GH33, respectively. It is therefore not too surprising that the glycosylation TSs for these two enzymes are different.

Importantly, it has been proposed, on the basis of molecular modeling studies, that the Michaelis complex involving an influenza type A N1 enzyme and a 3- or 6-sialyllactoside substrate places the sugar ring into a $^4S_2$ or $B_{2,5}$ conformation, respectively.[28] Chan et al. used kinetic isotope effect (KIE) measurements to propose a $^6S_2$ skew-boat[11] distortion of the substrate in the accumulating Michaelis complex for the *Mv*NA-catalyzed reaction. Although deglycosylation is partly rate-limiting for influenza N2,[21,22] similar KIE experiments suggest that the Michaelis complex contains a distorted substrate.[21] The four low-energy conformations for the glycal inhibitors **2a**–**j**

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are two half-chair ($^6H_5$ and $^5H_6$) and two boat ($^{4,O}B$ and $B_{4,O}$) conformations (Figure S2). We suggest that, for the influenza enzyme, the glycal inhibitor binds in a $^6H_5$ half-chair conformation and that the interaction of the 4-substituent with the enzyme gives rise to no stabilization of the glycosylation TS as the Michaelis complex undergoes reaction to give the enzyme-bound intermediate in a $^2C_5$ chair conformation.[15]

The above analysis also explains reactivity differences for sialosyl-enzyme covalent intermediates formed between a series of influenza viral enzymes and 4-substituted 2,3-difluorosialic acids.[29] That is, the fluorinated sialosyl-enzyme intermediates (see Scheme 1 for the generic structure) containing either a guanidino or amino group at C-4 undergo deglycosylation at greatly reduced rates compared to the 4-OH derivative. This effect likely arises from nonproductive binding of the 4-substituent that is expressed at the enzyme-bound intermediate but not at the deglycosylation TS, thus resulting in the sialosyl-enzyme intermediate being longer lived for the positively charged inhibitors.[29]

In conclusion, we show that, using rigorous LFER experiments, the hypothesis that Relenza and Neu2en5Ac are TSA inhibitors for influenza N2 neuraminidase, which is based on a presumed similarity between the glycal inhibitor and the oxacarbenium ion-like TS for enzymatic hydrolysis, is incorrect. Moreover, the influenza N2 enzyme and *Mv*NA have different glycosylation TSs, and we suggest an earlier TS for *Mv*NA-catalyzed hydrolysis (less C−O bond cleavage), which is closer in structure to the Michaelis complex than is the influenza N2 TS. This difference in TS structure results in the 4-substituents of the glycal (Neu2en5Ac and Relenza), when bound to influenza N2, mimicking the interactions present in the Michaelis complex and not those at the glycosylation TS. That the addition of a guanidino group to the glycal results serendipitously in a dramatic increase in binding potency without a concomitant increase in stabilization of the glycosylation TS is ultimately the root cause for the ease with which the influenza virus generates resistant strains. Accordingly, resistant strains possess neuraminidases that have a reduced binding avidity to Relenza but are still active catalysts. Clearly, new structural motifs and/or substitution patterns need to be developed in the search for bona fide influenza neuraminidase TSAs. Such inhibitors should be less likely to provoke rapid emergence of resistant strains and their associated risk to human health.

■ ASSOCIATED CONTENT

Supporting Information

Complete ref 2; experimental procedures and characterization of compounds; plots of $\log(K_m/k_{cat})$ versus $\log(K_i)$ for the enzyme-catalyzed reactions; and conformational itinerary maps. 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.

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