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Inhibition of sialidases from viral, bacterial and mammalian sources by analogues of 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid modified at the C-4 position*

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The inhibition of sialidase activity from influenza viruses A and B, parainfluenza 2 virus, *Vibrio cholerae*, *Arthrobacter ureafaciens*, *Clostridium perfringens*, and sheep liver by a range of 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid analogues modified at the C-4 position has been studied. All substitutions tested resulted in a decrease in the degree of inhibition of the bacterial and mammalian sialidases. For sialidases from influenza viruses A and B, on the other hand, most of the substitutions tested either had no significant effect on binding or, in the case of the basic amino and guanidino substituents, resulted in significantly stronger inhibition. The results for parainfluenza 2 virus sialidase were mostly intermediate, in that inhibition was neither significantly increased nor decreased by most of the modifications. We conclude that only the influenza A and B sialidase active sites possess acid groups correctly positioned to participate in charge–charge interactions in the region of C-4 of bound substrate, and that the C-4 binding pockets of the bacterial and mammalian sialidases examined are considerably smaller than is observed for either the influenza virus or parainfluenza virus sialidases.

Keywords: sialidase, sialic acid, *N*-acetylneuraminic acid, inhibition, viral, bacterial, mammalian

Introduction

Sialidases (EC 3.2.1.18) are found throughout the eukaryotes and are also present in a number of bacterial and viral sources [1, 2]. These enzymes are implicated in a variety of biological roles (reviewed in [2]) including the facilitation of release of virions from cells infected with influenza virus [3–5] and removal of sialic acids acting as biological masks for various glycoconjugates leading to the exposure of receptors, for example, in mammalian systems [6].

The sialidase from influenza virus is a target for drug design. Using the x-ray crystallographic structure of the enzyme [7, 8] as a starting point, our group has carried out a series of molecular graphics and kinetic studies [9–11] which have assisted in the rational design and synthesis of inhibitors of sialidase as potential anti-influenza drugs [11]. It is important, given the involvement of endogenous sialidases in a range of metabolic processes, that anti-influenza sialidase agents exhibit selectivity for the viral over the host mammalian enzyme. We have reported elsewhere [11, 12] the design and synthesis of 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu5Ac2en) analogues modified at the C-4 position which exhibit selectivity for the viral over mammalian sialidase. A great deal of recent interest has also been shown by other workers in inhibitor studies of sialidases [13–16], some of which may also be suitable candidates for drug design but whose three-dimensional structures are currently unknown. Given the availability of a number of structurally related inhibitors synthesized in our laboratory, we report here the use of these inhibitors to 'map' the interactions around the C-4 position of the sialic acid template for several sialidases from viral, bacterial and mammalian sources.

Materials and methods

Enzyme

Sialidase purified from influenza virus (Tokyo/3/67, N2) was obtained from Dr J. McKimm-Breschkin, CSIRO Division of Biomolecular Engineering, Parkville, Australia. Culture

* This paper is dedicated to the memory of Professor Dr E. Zbiral.

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media containing influenza B virus and parainfluenza 2 virus were generously supplied by Dr M. Kennett, Fairfield Hospital, Fairfield, Australia. Virus was disrupted and sialidase activity extracted from these culture media by the addition of 1 vol 4% Triton X-100 in 2 M KCl. Sialidase from *Vibrio cholerae* was obtained from Calbiochem (La Jolla, CA, USA). Sialidases from *Clostridium perfringens* and *Arthrobacter ureafaciens* were purchased from Boehringer-Mannheim, Germany.

A crude extract of sheep liver was prepared as follows. Liver in 3 vol ice-cold 0.25 M sucrose containing 1 μg ml^{-1} Pepstatin, 1 μg ml^{-1} Leupeptin, 5 mM EDTA and 2 mM phenylmethylsulfonyl fluoride (PMSF) was homogenized in a Waring blender. The homogenate was centrifuged at 4000 × **g** for 10 min. The supernatant (S1) was then centrifuged at 100 000 × **g** for 60 min to yield a second supernatant (S2). The required material with sialidase activity was then precipitated by stirring slowly at 4 °C overnight in 20 mM Tris, pH 7.0, containing 25% polyethyleneglycol. The precipitate was then pelleted by centrifugation at 1200 × **g** for 10 min. The pellet was resuspended in 20 mM Tris-HCl buffer, pH 7.0, against which it was then dialysed exhaustively. This preparation was found to be stable for several weeks at 4 °C. The sialidase activity from sheep liver was shown to follow Michaelis–Menten kinetics. Preliminary characterization of this enzyme has been reported [17]. All the enzyme preparations used in this work showed a linear relationship between enzyme concentration and activity as well as following Michaelis–Menten kinetics.

Assay

Sialidase activity was assayed using the fluorimetric assay of Potier *et al.* [18], as modified in [19]. The substrate, 4-methylumbelliferyl Neu5Ac, was synthesized in our laboratory by the method of Myers *et al.* [20]. The buffer used for all experiments was 50 mM sodium acetate, 6 mM $CaCl_2$, pH 5.5. All the sialidases studied were found to be active under these conditions, although the pH was not necessarily optimal for all enzymes studied. After preliminary experiments to estimate the order of magnitude of the K_i for each compound, K_i values were determined by assaying for sialidase activity in the presence of varying concentrations of compound, and at substrate concentrations of 20 and 80 μM. At least four compound concentrations ranging from 0.5 to 5 times the estimated K_i value were used, in addition to assaying in the absence of any compound at each substrate concentration. However, in some cases only the preliminary estimate could be carried out due to insufficient quantity of a compound. Assays were performed in at least duplicate. The K_i was determined routinely graphically using Dixon plots. The competitive nature of the inhibition was confirmed using Hanes–Woolf plots [21].

Sialidase inhibitors

Neu5Ac2en (**1**) was prepared according to [22]. Inhibitors **6** and **8** were prepared in our laboratory (unpublished results). All other inhibitors were prepared according to [12].

Results and discussion

As expected from the literature, Neu5Ac2en (**1**) exhibited K_i values in the range 10^{-5}–10^{-6} M for all enzymes studied (Table 1). This proposed transition-state analogue [9] therefore provides something of a benchmark template against which to compare the effect of a range of modifications at the C-4 position (Fig. 1) on the inhibition of each sialidase. We have found previously [11] that 4-amino- (**3**) and 4-guanidino-Neu5Ac2en (**2**) inhibit sialidase from influenza A (N2) with apparent K_i values of 10^{-8} M and 10^{-9} M, respectively. Compound **2** also exhibits slow, tight-binding inhibition of influenza A (N2) sialidase (unlike the remaining enzymes studied) with the tightly bound form having a K_i of 2×10^{-10} M [11]. Since this particular compound shows promise as an *in vivo* inhibitor of influenza virus replication, it was of interest to assess the specificity of inhibition with respect to sialidases from other sources – particularly mammalian sialidase. From Table 1 it is clear that there is, in fact, a great deal of specificity for the active sites of sialidase from both A and B type influenza viruses, with the sheep enzyme possessing a 10^5-fold lower affinity (K_i 10^{-4} M) compared with the initial apparent K_i of 4-guanidino-Neu5Ac2en. This binding is 10^6-fold weaker than the slow, tight-bound interaction observed. Interestingly, the interaction is also very much weaker for the parainfluenza and bacterial enzymes, although the K_i's obtained range from 6×10^{-5} M for *V. cholerae* sialidase to $>10^{-2}$ M for sialidase from *A. ureafaciens*. However, it should be noted that the sialidase preparations from sheep liver, parainfluenza and influenza B are not pure and some caution needs to be exercised in interpreting these results. Nevertheless, there is no evidence of any differences in kinetic behaviour due to impurities for any of these enzymes. For example, we have observed little difference between the K_i value of Neu5Ac2en against pure influenza A, N2 sialidase compared with preparations of whole influenza A virus containing N2 sialidase. On the other hand, the dramatic differences between enzymes observed for the inhibitor 4-guanidino-Neu5Ac2en, warrant the inclusion of these results in this report.

4-Amino-Neu5Ac2en is also a strong inhibitor of N2 sialidase (K_i of 1×10^{-8} M), and the results obtained for this inhibitor with the other enzymes broadly follow the pattern observed for 4-guanidino-Neu5Ac2en. The result obtained with 4-amino-Neu5Ac2en in the case of *V. cholerae* sialidase (3×10^{-4} M) compares with the findings of Schreiner *et al.* [13], who determined a K_i of 1×10^{-3} M. Although there is a threefold difference in the results, this is not very significant when considering the effects of orders

Table 1. Inhibition of sialidases from various sources by analogues of Neu5Ac2en modified at the C-4 position.

	Sialidase-K_i (M)						
	Flu A	*Flu B*	*Paraflu*	*C. perf.*	*V. chol.*	*A.urea.*	*Sheep*
1[a]	4×10^{-6}	2×10^{-5}	1×10^{-5}	8×10^{-6}	3×10^{-6}	1×10^{-6}	8×10^{-6}
2	1×10^{-9}	1×10^{-8}	8×10^{-4}	$>10^{-4}$[b]	6×10^{-5}	$>10^{-2}$[c]	3×10^{-4}
3	4×10^{-8}	1×10^{-6}	3×10^{-4}	7×10^{-4}	3×10^{-4}	3×10^{-4}	1×10^{-4}
4	3×10^{-6}	1×10^{-6}	1×10^{-4}	3×10^{-4}	$>5 \times 10^{-4}$[b]	$>5 \times 10^{-4}$[b]	n.d.[d]
5	2×10^{-6}	1×10^{-6}	5×10^{-5}	3×10^{-3}	6×10^{-3}	3×10^{-3}	$>10^{-3}$[c]
6	2×10^{-4}	8×10^{-4}	4×10^{-5}	$>10^{-3}$[b]	$>5 \times 10^{-4}$[b]	$>10^{-3}$[b]	$>10^{-3}$[c]
7	6×10^{-6}	1×10^{-5}	2×10^{-5}	$>10^{-3}$[b]	3×10^{-3}	4×10^{-3}	$>10^{-3}$[b]
8	8×10^{-6}	9×10^{-5}	3×10^{-5}	5×10^{-5}	$>5 \times 10^{-5}$[b]	$>10^{-4}$[b]	$>10^{-3}$[c]
9	4×10^{-6}	1×10^{-6}	1×10^{-4}	7×10^{-3}	3×10^{-3}	$>5 \times 10^{-3}$[b]	$>10^{-3}$[c]

[a] Compound numbers as set out in Figure 1.
[b] Derived from a preliminary estimate of the order of magnitude of inhibition only. K_i estimates were not performed due to insufficient availability of compound.
[c] No significant inhibition observed at a concentration of 1 mM of compound.
[d] ND, not determined.

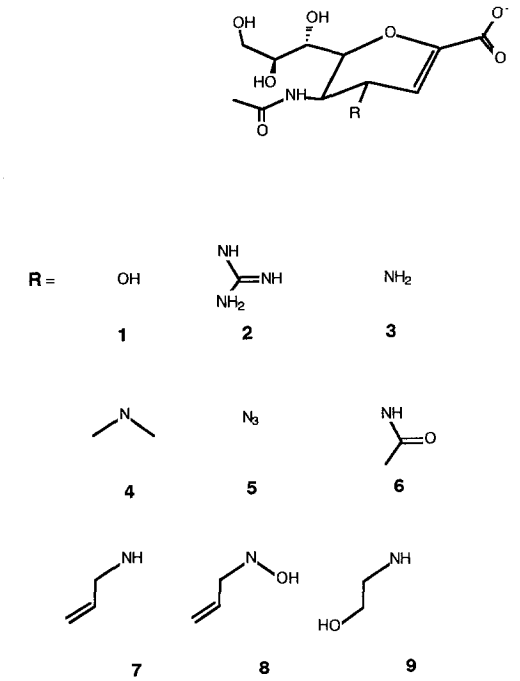

Figure 1. 2,3-Unsaturated sialic acid analogues used in this study. The compound numbers refer to those listed in Table 1 and throughout the text.

of magnitude. Furthermore, the buffer system used by these workers contained a much lower concentration of calcium (0.5 mM compared with 6 mM in our system). We have previously reported that minor changes in ionic strength, as well as calcium concentration, can have marked effects on kinetic parameters in the case of sialidase from influenza virus A [19], and a similar phenomenon might also contribute to the minor differences observed in the present case, particularly as the activity of sialidase from *V. cholerae* is calcium dependent [23].

From molecular modelling of the x-ray crystal structure of N2 sialidase [7], as well as kinetic investigations [9–11], the detailed interactions of Neu5Ac2en, and the guanidino and amino functionalities of the respective analogues, with the active site residues of N2 are well understood [11]. Thus, both functional groups are believed to form salt bridges with the conserved active site residue Glu 119 of N2, while the guanidino functionality also strongly interacts with Glu 227. The dramatic reduction in affinity of these inhibitors observed for sialidases from non-orthomyxoviridae sources provides strong evidence of major differences in this region of the active site. Specifically, it seems likely that there are no suitably positioned acid residues in this region of the active sites of the bacterial or sheep sialidases. The structure of the active sites of sialidases from these other sources has not yet been reported. A number of other 4-substituted analogues of Neu5Ac2en were therefore investigated in an attempt to shed further light on the nature of these differences.

Substitution of the amino group in **3** for the corresponding *N,N*-dimethyl moiety to produce **4** results in an increase in K_i for sialidase from influenza A to B to 10^{-6} M, emphasizing the role of the charge–charge interaction between the amino group of **3** and Glu 119 of the influenza sialidase active site. In contrast, however, the inhibition of sialidase from *C. perfringens* by **4** is comparable (3×10^{-4} M) when compared with **3**, suggesting the absence, in this active site, of an appropriately positioned acid group(s), such as the glutamic acids in influenza sialidase, precluding the formation of charge–charge interactions. This suggests that the C-4 position binding pocket within the *C. perfringens* sialidase active site is either more hydrophobic in nature or possibly more positively charged. Indeed, this trend is also consistent with the other bacterial enzymes as well as the parainfluenza enzyme. The K_i of **3** remains approximately the same order

of magnitude as for **4** for *V. cholerae*, *C. perfringens* and parainfluenza sialidase, suggesting once again that there is an absence of acid groups and a more hydrophobic or positively charged C-4 position binding pocket compared with the influenza virus sialidases.

Compounds **7**–**9** are analogues of **3** substituted with alkyl, alkenyl, and hydroxyl groups. The K_i values for these compounds across the viral sialidases were found to be virtually identical to the benchmark inhibitor **1**. The substantial loss of the potency of these inhibitors across the bacterial sialidases would appear to be from the increased steric bulk of these extensions. Noticeably, when more than three atoms are introduced at this position, inhibition of bacterial sialidases appears to be reduced generally. An exception occurs in the case of inhibition of sialidase from *C. perfringens* by **8**. In this case the hydroxyl group appears to restore the ability of the inhibitor to form a hydrogen bond and may reflect a specific difference of the active site of sialidase from *C. perfringens*. Nevertheless, all of the viral sialidases appear to be significantly more tolerant towards increase in steric bulk at this position. The reduction in influenza virus sialidase inhibition measured for the *N*-acetyl analogue **6** is arguably from the decrease in proton acceptor capability of the amide nitrogen in comparison with that of the amines **3**, **4**, **7**, **8**, and **9**. As was anticipated, the parainfluenza enzyme did not show this trend. This is consistent with our previous observation of parainfluenza sialidase possibly not having an acid group suitably positioned to participate in a charge–charge interaction as is seen in the influenza sialidase case.

Generally, substitution of an azide group at the 4-position of Neu5Ac2en, to produce compound **5**, resulted in strong inhibition across the viral sialidases. Interestingly, **5**, although inhibiting all of the bacterial sialidases, was approximately 1000-fold weaker than **1** across these enzymes. This compound was also shown by Schreiner *et al.* [13] to be a competitive inhibitor of sialidase from *V. cholerae* with a K_i of 8×10^{-5} M which is at variance with our present determination of 6×10^{-3} M. These differences might be due to differences in assay conditions, such as calcium concentration as previously mentioned, and/or to the differences between the compounds; in our case, the free acid was used to determine a K_i value, whereas in [13] the ammonium salt of **5** was used. The relative variations in K_i for the various enzymes also parallels that of **6** and **7**. These data support the notion that this position can accommodate at least three heavy atoms across the various sialidases.

The sialidase from sheep liver appears to have very little ability to bind compounds with any of the hydrophobic extensions tested. Moreover, the active site at this position, not unlike the bacterial sialidases, appears to be less tolerant of substitution compared with viral sialidases. This indicates either a small 4-position binding pocket or a somewhat more hydrophobic region in this pocket, although, as in the case of the bacterial sialidases, a positively charged environment in this pocket cannot be excluded.

Several workers have previously reported the inhibition of various sialidases by Neu5Ac2en analogues [13–16]. A number of analogues substituted at the C-4 position were shown to weaken the strength of inhibition to varying degrees for sialidase from *V. cholerae* [13], consistent with our observations. Other workers have investigated the effects of modifications of the C-7 to C-9 glycerol side chain and have also found varying effects with sialidase from *V. cholerae*, although all modifications reported weakened the degree of inhibition relative to Neu5Ac2en [14, 15], as was also the case for modifications of the ring heteroatom [15]. The current results extend this work to a range of other sialidases and for a number of novel inhibitors, and demonstrate significant differences between the susceptibility of these different sialidases to inhibition by C-4 substituted analogues of Neu5Ac2en.

In conclusion some trends are apparent. These results, together with our knowledge of the active sites of influenza A and B sialidases [7, 8, 10, 11], enable us to propose some hypotheses regarding the likely active site groups involved in binding this region of Neu5Ac2en analogues, in the case of the other viral, bacterial and mammalian sialidases studied. For example, it appears that the involvement of acid residues (Glu 119 and Glu 227) in the interactions around the 4 position of the sialyl moiety when bound in the active site is unique to the sialidases from influenza virus. All of the 4-*N* substituted inhibitors appear to participate in a charge–charge interaction with an acid residue(s) at this position in the active sites of the viral sialidases.

Much of our data concerning the bacterial and sheep sialidases leads us to conclude that the 4-binding position in these sialidases has a more hydrophobic or positively charged environment than the corresponding position in influenza sialidase. Moreover, the tolerance of these non-viral sialidases towards sterically larger groups at this site is not high. Unlike the viral sialidases, the bacterial and sheep sialidases appear not to possess comparable acid residues. At the pH used, all the C-4 *N*-substituted groups studied act as would be expected for positively charged groups in a hydrophobic or positively charged pocket.

It is also clear from our data that although parainfluenza sialidase can accommodate C-4 modified Neu5Ac2en analogues it, like the bacterial sialidases, does not have the same micro-environment about the C-4 position binding pocket with respect to the positioning of acid residues as do the influenza sialidases. However, as previously noted, the pocket appears to be larger than observed for the bacterial sialidases, being comparable in this regard to the influenza sialidases, and presumably can better accommodate a cation, possibly without solvation.

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