

## Design and antiviral properties of influenza virus neuraminidase inhibitors

P. M. Colman

Biomolecular Research Institute, 343 Royal Parade, Parkville, Victoria 3052, Australia.

**Abstract:** During the infection cycle of influenza virus, progeny virions bud from the plasma membrane of infected cells. At that point they are potentially immobilised because of interactions between the viral haemagglutinin and sialic acid which is found in glycoconjugates on the cell surface and on glycoproteins of the virus itself. Neuraminidase inhibitors might therefore be expected to have an antiviral action by slowing release and subsequently reducing the viral burden in infected hosts. The three-dimensional structure of the influenza virus neuraminidase has been determined by X-ray crystallography to high resolution. The structure demonstrates that strain variation has not yet been observed to include active site residues. Studies of the structure of complexes between the substrate and substrate analogues with the enzyme reveal the method of enzyme-substrate interaction. The structure of the enzyme active site has been used to direct the design of new inhibitors of the enzyme. These inhibitors block multi-cycle replication of virus in tissue culture and have antiviral properties in animal models.

Influenza viruses remain a threat to public health because of their capacity to vary. Immunity to a particular strain, which develops during the course of an infection, is limited to that strain and its very close relatives. The development of drugs which are effective against the virus has proven difficult, partly because of the rapid emergence of resistant strains. Resistance to both drugs and antibodies has as its basis the high error frequency of the viral polymerase. This causes variants to be continuously generated, and when their viability is enhanced, either through the use of drugs or the presence of antibodies in the host, they dominate the population. This paper reviews studies of one of the membrane glycoproteins of the virus which have led to the discovery of a new class of antiviral agent which appears to have broad spectrum activity against type A and B influenza viruses.

Influenza is an enveloped virus and two glycoproteins are displayed on the viral envelope (reviewed in (1)), a haemagglutinin and a neuraminidase (sialidase). The receptor for influenza viruses is carbohydrate. Sialic acid (*N*-acetyl neuraminic acid, Neu5Ac, Fig. 1) is the critical sugar residue which interacts weakly with the viral haemagglutinin to cause attachment of virus to target cells. After infection and replication, progeny virions bud at the plasma membrane of the infected cell. A role of the neuraminidase is to facilitate their release to infect other cells.

The enzymatic activity of neuraminidase is illustrated in Fig. 1. The enzyme cleaves an  $\alpha$ 2,3 linkage between Neu5Ac and a neighbouring sugar, usually galactose (2-4). An  $\alpha$ 2,6 linkage is also cleaved by the viral enzyme, although less efficiently, and specificity for the aglycon is weak. This activity destroys the receptors for the viral haemagglutinin which are present on the surface of the infected cell and also on the oligosaccharide side chains of the newly synthesised haemagglutinin and neuraminidase polypeptides within the progeny virion envelope (5,6).

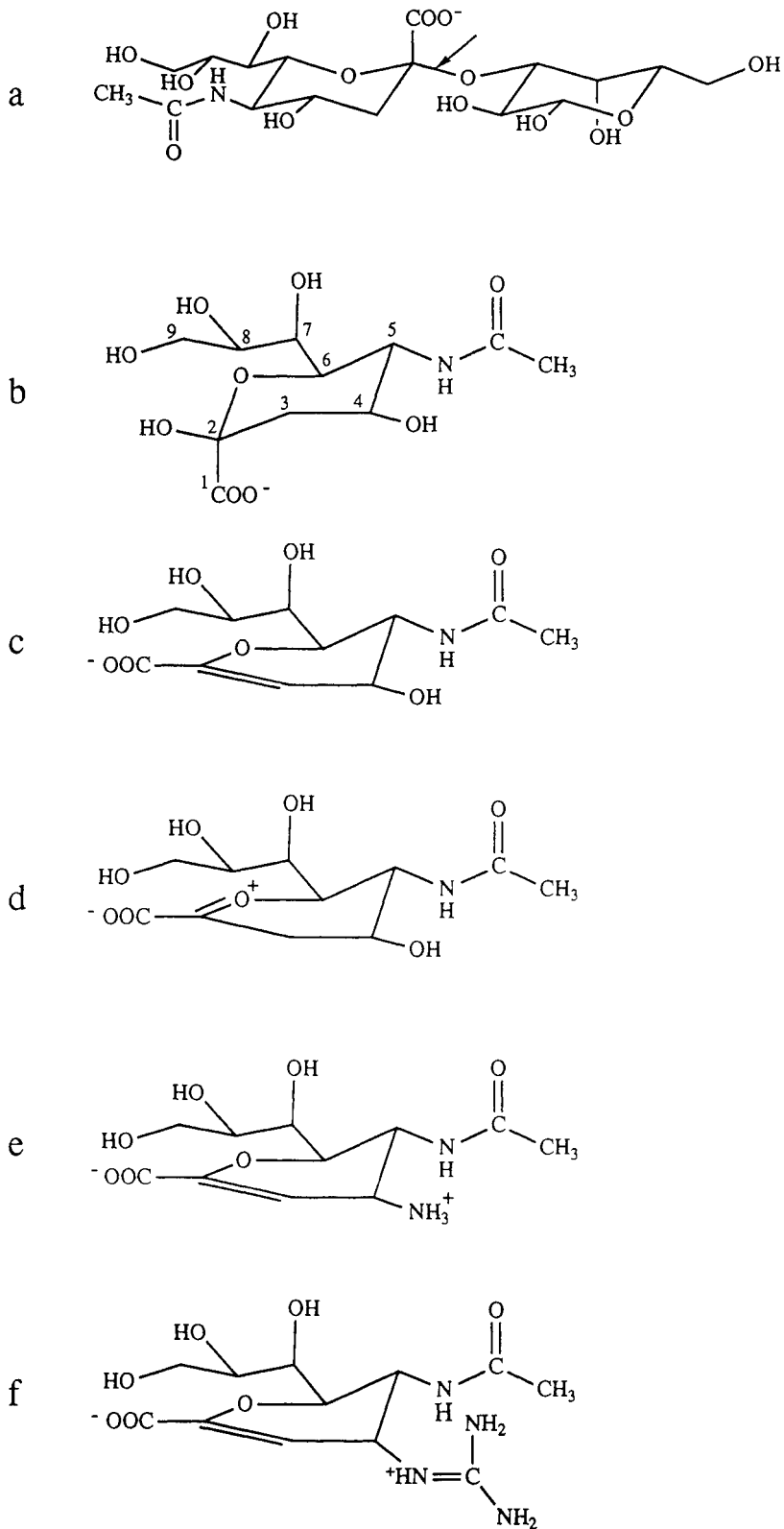


Fig. 1. (a)  $\alpha$  2-3 sialyl galactose, showing the bond cleaved by the action of neuraminidase; (b)  $\alpha$  sialic acid (Neu5Ac); (c) Neu5Ac2en; (d) proposed transition state structure; (e) 4-amino-Neu5Ac2en; (f) 4-guanidino-Neu5Ac2en

A screen of compounds for neuraminidase inhibitors was unsuccessful in 1966 (7). The first mechanism-based inhibitor of the enzyme was synthesised in 1969 (8). Neu5Ac2en (Fig. 1) was proposed to be a transition state analogue and it has a  $K_i$  of *circa*  $1\mu\text{M}$ . Subsequent derivatisation of Neu5Ac2en led to only marginal improvements in binding (9). These compounds are not only neuraminidase inhibitors, but also anti-virals in the sense that they stop multicycle replication of influenza in tissue culture (10). However, they were inactive as antivirals in an animal model (11). This result led to the view that neuraminidase was not a good target for antiviral prophylaxis or therapy, possibly because its role is late in the infectious cycle. The determination of the three-dimensional structure of neuraminidase in 1983 has seen renewed interest in the exploitation of the enzyme activity to make new antiviral compounds.

Neuraminidase (reviewed in (12)) is a tetramer of identical, 60KDa, glycosylated polypeptides, and it can be liberated from the viral membrane by digestion with proteases. The morphology of the tetramer is mushroom-like, and proteases cut the stalk of the tetramer, near residue 80, without cleaving the chain within the head domain. The soluble tetrameric particles are enzymatically and antigenically intact, and they can be crystallised. X-ray diffraction studies (13,14) revealed a polypeptide fold of  $\beta$  sheets, six in total, arranged like the blades of a propeller. Each sheet is composed of four antiparallel strands and has the topology of the letter 'W' (Fig. 2).

When the amino acid sequences of neuraminidases from different strains of virus are mapped onto the three-dimensional structure, it is seen that almost the entire surface of the molecule is variable. The only contiguous patch of surface structure which is invariant in all strains characterised so far is that associated with the enzyme active site. The location of that site has been verified independently by soaking substrates and inhibitors into the enzyme crystals and determining their location (15,16). The active site is centrally located on the neuraminidase subunit and lies on the propeller axis at the N-terminal ends of the first  $\beta$  strand of each sheet.

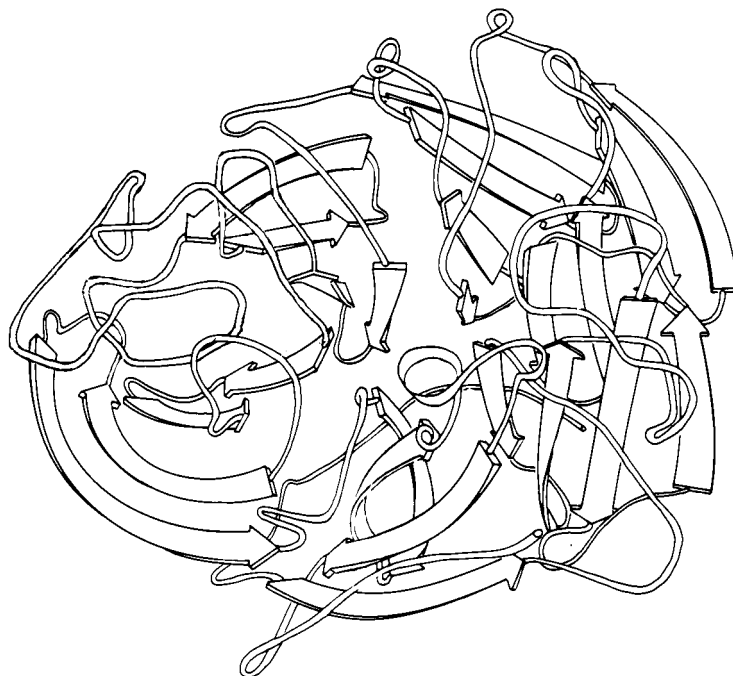


Fig. 2 Drawing of the polypeptide chain folding in a single subunit of influenza virus neuraminidase. The other three subunits of the tetrameric enzyme are related to this one by a four fold rotation axis, normal to the page in the lower right corner of the figure.

When Neu5Ac binds to neuraminidase it is distorted from a chair conformation towards a boat conformation (16). The carboxylate group is raised from its axial orientation towards the floor of the binding site to lie equatorially to the pyranose ring (Fig. 3), surrounded by three invariant arginyl residues (Arg118, Arg292, Arg371) on the protein. The glycosidic oxygen is raised from its equatorial location to point vertically out of the active site and to lie close to one of the carboxylate oxygen atoms of Asp151 on the enzyme. That particular oxygen atom is unusual in the sense that it does not participate in the complex and extensive network of hydrogen bonds which encircle the Neu5Ac binding site. In the proposed catalytic mechanism a proton attacks the glycosidic oxygen to form a sialosyl cation intermediate (17). Asp151 may be involved in this step of the reaction, possibly via a water molecule. The 4-hydroxyl group is directed towards a sub-cavity within the active site, the walls of which are lined by Glu119 and Glu227 (16). The N-acetyl group is located in a hydrophobic pocket, and the glycerol side chain is hydrogen bonded to Glu276 through the C<sub>8</sub> and C<sub>9</sub> hydroxyls. Identical interactions are observed for the binding of Neu5Ac2en to the enzyme, apart from the missing glycosidic oxygen in that case. One explanation for the increased binding affinity of Neu5Ac2en to neuraminidase, compared with Neu5Ac, is that the carboxylate is pre-positioned equatorially in the unsaturated sugar. Another is that the unsaturated compound may be somewhat more hydrophobic.

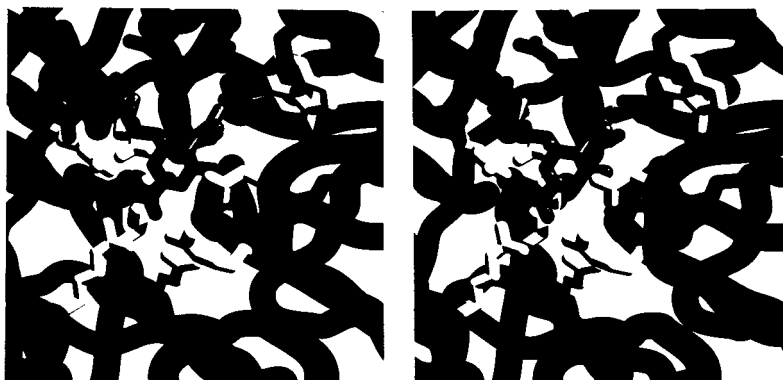


Fig. 3. Stereo image of sialic acid binding in the active site of the enzyme. Only strain invariant residues of the enzyme are shown. Amino acids of the protein which are also conserved in bacterial neuraminidases are shown in white.

How might this information be used to design more potent inhibitors of the enzyme?

Apart from manually inspecting the interactions between Neu5Ac2en and the enzyme and looking for places where more binding energy could be built into the interaction, the approach of Goodford (18) has been found to be useful. Calculations of the interaction energy between different chemical probes and the enzyme active site have pointed to the availability of space between Neu5Ac2en and the enzyme in the vicinity of the 4-hydroxyl binding pocket, and to the desirability of filling that space with basic substituents on the sugar (19). Based on this information, the compounds targeted for synthesis were 4-amino-Neu5Ac2en and 4-guanidino-Neu5Ac2en (Fig. 1). The  $K_i$  of these compounds are 20 and 5000 times respectively improved over that for Neu5Ac2en when assayed against the neuraminidase of the viral isolate from which the structure had been determined. Furthermore, 4-guanidino-Neu5Ac2en is a sub-nanomolar inhibitor of neuraminidases from all strains of influenza tested so far as shown in Table 1 (19), consistent with the observation that only strain-invariant residues are engaged in its binding to the enzyme.

TABLE 1 Inhibition constants (Ki) or 50% inhibition levels (IC<sub>50</sub>) expressed in Molar concentrations for Neu5Ac2en and its 4-guanidino derivative against neuraminidases of different microbial and animal origin. Data from von Itzstein et al., (1993) and Holzer et al., (1993).

Neuraminidase	Neu5Ac2en	4-guanidino-Neu5Ac2en
Influenza N2 subtype (Ki)	1x10 <sup>-6</sup>	2x10 <sup>-10</sup>
Influenza N1 subtype (Ki)	-	7x10 <sup>-11</sup>
Influenza type B (Ki)	2x10 <sup>-5</sup>	7x10 <sup>-10</sup>
Parainfluenza (Ki)	1x10 <sup>-5</sup>	8x10 <sup>-4</sup>
<i>vibrio Cholerae</i> (Ki)	3x10 <sup>-6</sup>	6x10 <sup>-5</sup>
ovine liver (Ki)	8x10 <sup>-6</sup>	3x10 <sup>-4</sup>
Human placenta (IC <sub>50</sub> )	1x10 <sup>-5</sup>	1x10 <sup>-3</sup>

These compounds have also been tested for their capacity to inhibit influenza virus replication in tissue culture, and against a wide range of field strain viruses they show activity in this assay proportional to their enzyme inhibitory activity (20). However, there are some naturally occurring strains of virus for which this is not so. For example, two separate viral isolates from Stockholm in 1990 and 1991 show 10 to 100 times reduced sensitivity to 4-guanidino-Neu5Ac2en in this assay compared to their expected levels of sensitivity based on inhibition of their enzyme activities (20).

Both mice and ferrets have been used to demonstrate the efficacy of the compounds as antiviral agents in animal models of influenza. In ferrets, prophylactic administration of the compound reduces viral titres in nasal washings and abrogates the fever associated with the infection (19).

The compound 4-guanidino-Neu5Ac2en is selective for influenza virus neuraminidases and shows poor inhibitory activity against neuraminidases of other microbial and animal origin (Table 1, and (19,21)). Apparently these neuraminidases have rather different structures at least in the vicinity of the 4-hydroxyl binding pocket (see below).

Resistance to existing drugs for influenza is well documented. Amantadine and rimantadine exert their antiviral action through interfering either with the haemagglutinin-mediated entry of virus into cells through endosomes or with the proton pumping activity of the viral protein M<sub>2</sub>. Variants of both the haemagglutinin (22) and the M<sub>2</sub> protein (23) have been selected *in vitro* by growing virus in the presence of these compounds. Furthermore, resistant strains are found in man within two days of treating influenza infection with rimantadine (24).

Experiments are now in progress to determine the nature and frequency of resistance to the neuraminidase inhibitor 4-guanidino-Neu5Ac2en. Resistance could take one of two forms. Viruses either with decreased dependency on the neuraminidase for elution from cells or with a neuraminidase which was not inhibited by the compound would be advantaged by growth in the presence of the inhibitor. The latter phenotype can be considered on the basis of the structural requirements for the enzyme to be able to process substrate but not be bound by the inhibitor. A model for the paramyxovirus neuraminidase (25) and an experimentally determined structure of a bacterial neuraminidase (26) illustrate how this condition may arise. Although the bacterial enzyme has the same three-dimensional fold as the viral enzyme, its active site lacks a number of the strain invariant features of the influenza enzyme. A subset of these features, including the triarginyl cluster and the catalytic aspartic acid, are present, but others, in particular the two glutamyl residues in the 4-hydroxyl binding pocket, are not (Fig. 3). That pocket in the bacterial enzyme is both smaller and of different chemical character. It is not clear yet to what extent the viral enzyme can progressively or otherwise alter its structure to close off this pocket to the 4-guanidino group.

The compound 4-guanidino-Neu5Ac2en is now in clinical studies in man.

## Acknowledgements

I acknowledge the contributions of my colleagues Jose Varghese, Mike Lawrence and Paul Davis, to the work described here.

## References

1. R.M. Krug (Ed.), *The Influenza Viruses*, Plenum, New York (1989).
2. A.P. Corfield et al. *Eur. J. Biochem.* **124**:521-525 (1982).
3. A.P. Corfield et al. *Biochim. Biophys. Acta* **744**:121-126(1983).
4. J.C. Paulson et al. *J. Biol. Chem.* **257**:12734-12738 (1982).
5. J.A. Griffin and R.W. Compans. *J. Exp. Med.* **150**:379-391 (1979).
6. J.A. Griffin et al. *Virology* **125**:324-334 (1983).
7. J.D. Edmond et al. *Br.J.Pharmac.Chemother.* **27**:415-426 (1966).
8. P. Meindl P and H. Tuppy. *Monatsh. Chem.* **100**:1295-1306 (1969).
9. P. Meindl et al. *Virology* **58**:457-463 (1974).
10. P. Palese et al. *Virology* **61**:397-410 (1974).
11. P. Palese and J.L. Schulman, In: *Chemophrophylaxis and Virus Infections of the Upper Respiratory Tract*, **1**, (J.S.Oxford, Ed.), CRC Press 189-205, (1977).
12. P.M. Colman, In: *The Influenza Viruses*, (R.M.Krug, Ed.), Plenum, New York. 175-218 (1989).
13. J.N. Varghese, et al., *Nature* **303**, 35-40 (1983).
14. J.N. Varghese and P.M. Colman. *J. Mol. Biol.* **221**, 473-486 (1991).
15. P.M. Colman et al. *Nature*, **303**, 41-44 (1983).
16. J.N. Varghese et al. *Proteins: Structure, Function, and Genetics*, **14**, 327-332 (1992).
17. N. R. Taylor and M. von Itzstein. *J.Med.Chem.* **37**, 616-624 (1994).
18. P.J. Goodford. *J.Med.Chem.* **28**:849-857 (1985).
19. M. von Itzstein et al. *Nature*, **363**, 6428, 418-423 (1993).
20. JM Woods et al. *Antimicrobial Agents and Chemotherapy* **37**:1473-1479 (1993).
21. C.T. Holzer et al. *Glycoconjugate J* **10**:40-44 (1993).
22. R.S. Daniels et al. *Cell* **40**:431-439 (1985).
23. A.S. Hay et al. *EMBO J.* **4**:3021-3024 (1985).
24. F.G. Hayden et al. *Antimicrobial Agents and Chemotherapy.* **35**:1741-1747 (1991).
25. P.M. Colman et al. *J.Virol.*, **67**, 6, 2972-2980 (1993).
26. S.J. Crennell et al. *Proc. Natl. Acad. Sci. USA* **90**:9852-9856 (1993).