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Influenza virus neuraminidase: Structure, antibodies, and inhibitors

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Abstract

The determination of the 3-dimensional structure of the influenza virus neuraminidase in 1983 has served as a platform for understanding interactions between antibodies and protein antigens, for investigating antigenic variation in influenza viruses, and for devising new inhibitors of the enzyme. That work is reviewed here, together with more recent developments that have resulted in one of the inhibitors entering clinical trials as an anti-influenza virus drug.

Keywords: antigen–antibody interactions; antigenic variation; drug design; enzyme inhibition

Influenza viruses continue to cause epidemics in man. Vaccination is moderately successful, but antigenic variation of the virus limits the protection afforded by this approach to homologous strains of the virus. Only 1 class of drug is approved for therapy, and its use rapidly results in the emergence of resistant strains. Protein crystallography has led to a description of the 3-dimensional structures of the 2 surface glycoprotein antigens of the virus. Studies of one of them, the neuraminidase, are reviewed here, with respect firstly to the understanding that has developed about antigenic variation and about antibody–antigen interactions in general, and secondly to the identification and characterization of new inhibitors of the protein and their properties as antiviral agents.

The existence of an enzyme activity on the surface of influenza viruses was discovered by Hirst (1942). He incubated red cells with virus, observed the hemagglutination reaction, and noticed that with the passage of time the reaction was not sustained. One of the 2 surface glycoproteins of the virus, the hemagglutinin (reviewed by Wharton et al., 1989), binds to red cells via sialic acid-containing glycoconjugates on the red cell surface. The other surface glycoprotein, the neuraminidase (reviewed by Colman, 1989), specifically liberates sialic acid (*N*-acetyl neuraminic acid, Neu5Ac, Fig. 1) from those glycoconjugates, thereby destroying the receptors for the virus. Neuraminidase (sialidase, EC 3.2.1.18) cleaves the α -ketosidic linkage between terminal Neu5Ac and a neighboring saccharide, usually galactose (Fig. 1). The viral enzyme shows some preference for α 2–3

linkages, but linkage and aglycon specificity is weak (Corfield et al., 1982, 1983; Paulson et al., 1982).

The role of a receptor-destroying enzyme in the viral life cycle is subtle and may not be completely understood. It has been suggested that the enzyme activity enables the virus to penetrate mucosal secretions that are rich in sialic acid, and thereby to reach target cells of the respiratory epithelium (Burnet et al., 1947; Burnet, 1948; Gottschalk, 1958, 1972; Allen, 1983). More direct experimental evidence is available to support a role for the enzyme in facilitating release of progeny virions from the surface of infected cells, where they would otherwise aggregate as a result of interactions between the hemagglutinin and sialic acid on the surface of the infected cell and on the progeny virion envelope (Palese et al., 1974; Palese & Compans, 1976; Griffin & Compans, 1979; Griffin et al., 1983).

Variation among influenza viruses has been reviewed by Smith and Palese (1989). Like hemagglutinin, neuraminidase is an important antigen of the virus. Two types of antigenic variation are observed among type A influenza viruses. Incremental changes in the amino acid sequences of the antigens are occurring continuously as a result of the selection pressure of antibodies in an immune host population. Occasionally, as a result of genetic reassortment, a virus emerges bearing an antigen sequence, which is dramatically (ca. 50%) different from the sequences of the antigens of circulating strains. Such changes in the subtype of either the hemagglutinin (H) or the neuraminidase (N) occurred most recently in human influenza in 1957 (when H1N1 viruses shifted to H2N2 viruses), in 1968 (H2N2 to H3N2), and in 1977 (H3N2 to H1N1, although H3N2 viruses continue to co-circulate in man), and they are responsible for the most recent pandemics of influenza in man. The different

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Fig. 1. A: α -2-3 Sialyl galactose, showing the bond cleaved by the action of neuraminidase. B: α Sialic acid (Neu5Ac). C: Neu5Ac2en. D: Proposed transition state structure. E: 4-Amino-Neu5Ac2en. F: 4-Guanidino-Neu5Ac2en.

subtypes of the antigens are defined by the failure of antisera to cross-react between them. In sequence terms, they differ by 50% or more, whereas within subtypes sequence differences range from a few percent up to ca. 30%. Type B influenza viruses do not undergo this subtype shifting of the glycoprotein antigens, but continual antigenic drift is observed as for type A viruses.

Antibodies to the hemagglutinin have been shown to be neutralizing in the sense that they can block infectivity. Anti-neuraminidase antibodies predispose the host to less severe

infection and illness (Kilbourne et al., 1968; Schulman, 1975) but do not prevent infection, consistent with the role described above for the neuraminidase in the infectious cycle of the virus.

Structure

Neuraminidase is a mushroom-shaped tetrameric protein, anchored to the viral membrane by a single hydrophobic sequence of some 29 amino acids near the N-terminus. Treatment of virions with pronase liberates a 200-kDa protein containing 4 identical and glycosylated polypeptides that have all of the antigenic and enzymatic properties of membrane-bound neuraminidase (Laver, 1978). This protein has been crystallized and its structure determined by X-ray diffraction (Varghese et al., 1983; Varghese & Colman, 1991). The 3-dimensional structures of influenza virus neuraminidases from 2 N2 strains (Varghese et al., 1983), 2 N9 strains (Tulip et al., 1991, 1992a), and 1 type B strain (Burmeister et al., 1992) are now known, together with structures for a number of antibody-selected variants of these (see below).

The structure displays a symmetrical folding pattern of six 4-stranded anti-parallel β -sheets arranged like the blades of a propeller (Fig. 2; Varghese et al., 1983). The central and first strand of each sheet is parallel to the propeller axis and the outermost strand is nearly perpendicular to it, giving each sheet the characteristic twist. The outermost strand of 1 sheet is then connected to the central strand of the following sheet. These connecting loops, on the upper surface of the enzyme with respect to its orientation on the viral membrane, contain many of the antigenically and enzymatically important amino acids (Colman et al., 1983; Varghese & Colman, 1991; Varghese et al., 1992).

The 4 identical subunits are arranged with circular 4-fold symmetry (Fig. 3), and the enzyme active site is located centrally on each subunit directly over the 6 central β -strands of each sheet. The site is a deep pocket, and amino acids which line and surround the walls of the pocket are invariant in all strains of influenza virus characterized so far (Fig. 3). These active site-associated, strain-invariant amino acids are of 2 types. Some of them are functional in the sense that they are in direct contact with bound substrate, and some provide only a structural framework for the functional residues (Colman et al., 1993). This division of the active site into functional and structural residues is not particularly precise but can be extended more sensibly by comparison of the active-site residues of the influenza virus neuraminidase with the neuraminidase from a bacterium, *Salmonella typhimurium* (Crennell et al., 1993). The active site is described in more detail below (see Inhibitors).

In 1 crystal form of the enzyme, carbohydrate plays an important and remarkable role in the crystallization. The oligosaccharide attached to Asn 146 has an unusual composition (Ward et al., 1983), and it is likely to be sulfated (Wagh & Bahl, 1981; Varghese & Colman, 1991). In crystals of the A/Tokyo/3/67 strain of neuraminidase, tetramers of the enzyme face each other across a center with point group symmetry 422. The closest approach of amino acids of the proteins in a pair of such related tetramers is approximately 40 Å. Crystallization is facilitated by symmetry-related interactions between the 8 oligosaccharide chains covalently attached to Asn 146 (Fig. 4). Most examples of oligosaccharide structure on glycoproteins show the saccharide units in close contact with the protein surface. In many cases, no structure is seen at all because of mobility or crystal-

Fig. 2. MOLSCRIPT (Kraulis, 1991) drawing of the β -sheet propeller fold of influenza virus neuraminidase. Color coding is progressively red to purple, highlighting the 6 β -sheets. The N-terminus is residue 82, to the rear of the figure and near the pro-nase cleavage site, which liberates soluble enzyme from the viral membrane.

line disorder of the sugar. The structure observed in this case points to the possibility of ordered, antenna-like structures of oligosaccharides on proteins.

Antibodies

Antibodies that bind exclusively to the conserved enzyme active-site structure should be protective against all strains of influenza virus. Apparently such antibodies are rare and several possible reasons for this were advanced when the structure was first described (Colman et al., 1983). One explanation was that antibody-protein antigen interactions should be like other protein-protein interactions in requiring at least 600 Å² of buried surface area on each side of the interface, and the small size of the enzyme active site precluded the possibility of any antibody binding only to that site.

The 3-dimensional structures of 2 monoclonal antineuraminidase antibodies complexed with neuraminidase have now been determined (Fig. 5; Colman et al., 1987; Tulip et al., 1992a; Malby et al., 1994). Both illustrate the importance of large and complementary buried surfaces in the formation of complexes between protein antigen and antibody. These 2 structures are part of a growing database of protein antigen-antibody complexes that all show complementary buried surfaces of between ca. 600 and 900 Å². We have introduced a quantitative measure of shape complementarity (Lawrence & Colman, 1993) that illustrates that, in protein-protein interactions, generally there is marginally better shape matching across the interface than in antibody-antigen interactions (Tulip et al., 1992a). Antibody-antigen interfaces typically involve about a dozen hydrogen bonds and contacts between about 15 amino acids from both the antibody and the antigen (Davies & Chacko, 1993).

Fig. 3. Structure of the neuraminidase tetramer. The subunit boundaries run north-south and east-west through the center of the tetramer, and the subunit in Figure 2 is the northwest member. Color coding is light blue for oligosaccharide chains, yellow for strain variable residues, and red, green, and dark blue for invariant acidic, neutral, and basic amino acids.

Fig. 4. Two neuraminidase tetramers face each other across a center of 422 symmetry in crystals of the A/Tokyo/3/67 strain of neuraminidase, and all of the intertetramer interactions are through the oligosaccharide attached at Asn 146 (Varghese & Colman, 1991). Neuraminidase is in yellow and white, carbohydrate in green and red. The crystal and molecular 4-fold axis is vertical, through the 2 tetramers.

These observations accord with the known 3-dimensional structures of 2 different neuraminidase subtypes, N2 and N9 (Tulip et al., 1991). An examination of these 2 structures shows that the largest contiguous conserved surface structure between them is the enzyme active site. The absence of common surface structures of sufficient surface area for antibody binding explains the failure of antisera to cross-react between these, and presumably other, subtypes.

On the other hand, despite the involvement of large surface areas and many contacting amino acids in antibody-antigen interfaces, many experiments with monoclonal antibodies have demonstrated that single amino acid sequence changes in one of the viral antigens can effectively abolish the binding of a particular antibody to that antigen (e.g., Webster et al., 1982). The 3-dimensional structures of a number of these so-called escape mutants have been determined both for the hemagglutinin (Knossow et al., 1984) and the neuraminidase (Varghese et al., 1988; Tulip et al., 1991). In all cases studied so far, there is only local change to the structure, i.e., confined to the site of the mutation and its immediate neighbors. How should this be rationalized against the participation of about 15 amino acids of the antigen in contacting the antibody? An antibody-antigen complex with a dissociation constant on the order of 10^{-8} M can be severely compromised by the loss of a single interaction whose contribution is on the order of 4 kcal/mol, for example, a hydrogen bond. The corresponding increase in the dissociation constant of nearly 3 orders of magnitude places the interaction in the class of nonspecific complexes. Furthermore, the require-

Fig. 5. Structures of the complexes of 2 different anti-neuraminidase antibodies with neuraminidase, showing only a single sub-unit of the enzyme and the Fv region of the antibodies. The binding sites of the 2 antibodies overlap by 80%. The NC41 complex is on the left (Tulip et al., 1992a) and the NC10 complex is on the right (Malby et al., 1994). Neuraminidase is in green; antibody heavy chains are in red, light chains in blue. Yellow and white segments indicate contacting regions on antibody and antigen.

ment for shape complementarity, which seems to be absolute for all complexes studied to date (Lawrence & Colman, 1993), jeopardizes the interaction in the case of volume-altering amino acid sequence substitutions.

The picture of antigenic variation that emerges from a study of escape variants is that very subtle changes in the antigen, e.g., Ser to Ala or Lys to Arg, can result in abrogation of binding. However, there are also data that illustrate that in some settings more dramatic structural changes can be tolerated. For example, an Ile to Arg substitution within the interface of one of the neuraminidase-antibody complexes is tolerated with a loss of a factor of 1–2 orders of magnitude in the association constant (Webster et al., 1987; Gruen et al., 1994). In this case, the structure of the mutant complex shows that amino acid side chains on the mutant antigen have different conformation in the free and complexed structures, and that side chains of the antibody have different conformation in the wild-type and mutant complexes (Tulip et al., 1992b). Thus, even in the case of this mutant, where chemistry, shape, and volume are all significantly altered, the structural context of the changed amino acid is such as to permit a relaxation of the neighboring structure to accommodate the change.

Differences in conformation of antigens and antibodies in their free and uncomplexed forms can occur at the level of amino acid side chains or of the polypeptide backbone. In this regard, they resemble other protein-protein interactions. Additionally, antibodies have the capacity to modulate the interface between the V_L and V_H domains, allowing for some repositioning of the complementarity determining regions of the heavy chain with respect to those of the light chain (Colman et al., 1987; Colman, 1988). The V_L - V_H domain interface has unique architectural characteristics (Chothia et al., 1985), and the hypothesis that it may play a role in immune recognition (Colman, 1988) has been

demonstrated now in several cases (Bhat et al., 1990; Herron et al., 1991; Stanfield et al., 1993).

One of the antineuraminidase antibodies has now been engineered into a single-chain Fv fragment and crystallized in complex with neuraminidase (Malby et al., 1993). The structure has been determined and illustrates that the oligopeptide linker, a 15-mer connecting V_H to V_L domains, has no significant effect on the domain pairing or on the structure of the complex formed with antigen (Kortt et al., 1994).

The information obtained from these and other studies of antibody-antigen interactions is beginning to find an application in medicine. There are many clinical situations in which antibodies are either the cause of the condition or the means of its relief. Therapeutic uses of antibodies may benefit from the discovery that the complementarity determining regions of antibodies are the main means of attachment to antigen. The repeated use of antibodies of murine origin in man can lead to adverse immune responses to the constant regions of the antibody. Human antibodies can now be grafted with complementarity determining regions of murine antibodies raised against the therapeutic target, enabling repeated administration of the antibody in therapy (reviewed by Winter & Milstein, 1991). However, antigenic variation of influenza viruses remains unpredictable and, despite our improved understanding of the requirements for antibody binding to viral antigens, there are no obvious applications to improve vaccination strategies for the control of influenza epidemics.

Inhibitors

A screening program for neuraminidase inhibitors was undertaken by Edmond et al. (1966) in the belief that such compounds could have antiviral activity. No potent compounds were iden-

tified in that study, the best being *N*-phenyloxamic acid and some of its derivatives.

Neu5Ac is the product of the reaction catalyzed by neuraminidase. It exists in solution as a mixture of the α - and β -anomers in the ratio of approximately 1:20, and only the α -anomer is bound by the enzyme. The K_i for α -Neu5Ac is ca. 50 μ M, and the preference for the β -anomer in solution has been proposed as one of the mechanisms by which the reaction product is released from its binding pocket in the active site of the enzyme (Varghese et al., 1992).

The first mechanism-based inhibitor of neuraminidase was synthesized by Meindl and Tuppy (1969). The compound, Neu5Ac2en (Fig. 1), was proposed to mimic the transition state of the reaction, at least insofar as the C₂ atom was trigonal. Neu5Ac2en has a K_i of 1 μ M (von Itzstein et al., 1993), a modest improvement only over that of Neu5Ac. A number of analogues of Neu5Ac2en were synthesized during the 1970s, and the most potent of these as a neuraminidase inhibitor was the trifluoroacetyl derivative with a K_i of 0.8 μ M (Meindl et al., 1974). This compound was used to demonstrate the role of neuraminidase in facilitating release of progeny particles from the surface of infected cells in tissue culture (Palese & Compans, 1976), but preliminary experiments in search of antiviral activity in animals were not successful (Palese & Schulman, 1977).

Thus, attempts to discover inhibitors of neuraminidase based either on screening or on consideration of the enzyme mechanism failed to result in compounds with sufficient inhibitory activity to be interesting as potential drugs. Can the 3-dimensional structure of the enzyme be used to design inhibitors with increased potency?

The active site of the enzyme contains approximately 18 strain-invariant residues, some of which, as discussed above, bind directly to substrate and participate in catalysis, and others which simply provide a structural framework. Comparison of the influenza virus neuraminidase structure with that of *S. typhimurium* (Crennell et al., 1993) and of *Vibrio cholerae* (Crennell

et al., 1994) illustrates that the structural shell of residues is quite different in the viral and bacterial enzymes and, furthermore, that a number of the residues directly in contact with substrate are also different. The pattern of neuraminidase active-site invariance that emerges (i.e., as opposed to influenza virus neuraminidase active-site invariance) is illustrated in Figure 6. It is striking that the invariant amino acids are primarily at the carboxylate end of the molecule and therefore also near the scissile bond. This partitioning is even more pronounced if one considers additionally a proposed model for the HN (hemagglutinin-neuraminidase) protein of paramyxoviruses, which lacks a counterpart of Trp 178 (Colman et al., 1993). Some of these differences in the sialic acid binding site may result from requirements for different kinetic properties of the enzymes in different microorganisms (Crennell et al., 1993).

Crystallographic studies of Neu5Ac and Neu5Ac2en bound to neuraminidase established that the enzyme bound the α -anomer of Neu5Ac in a half-chair configuration, with the carboxylate group at C₂ displaced from its low-energy axial position to an equatorial orientation (Figs. 6, 7; Burmeister et al., 1992; Varghese et al., 1992). The pyranose ring binds to the floor of the active-site cavity through its hydrophobic face, and the distorted half-chair geometry of the ring moves (1) the carboxylate from the floor of the site to its equatorial position surrounded by 3 arginyl residues (Arg 118, Arg 292, and Arg 371) and (2) the glycosidic oxygen from its equatorial position to an axial location, pointing out of the site and near one of the carboxylate oxygens of Asp 151, a strain-invariant active-site residue. The C₄ hydroxyl group is accommodated in a large pocket that is bounded in part by the conserved residues Glu 119 and Glu 227. The *N*-acetyl moiety attached at C₅ makes hydrogen bonds to the protein through both its amide nitrogen and carbonyl oxygen atoms, and the methyl group is situated against a hydrophobic surface patch. The glycerol side chain of the sugar makes hydrophobic contacts with the hydrocarbon chain of Arg 224 and the C₈ and C₉ hydroxyls interact with Glu 276. The C₇ hydroxyl

Fig. 6. Stereo image of the structure of sialic acid (green) bound in the active site of influenza virus neuraminidase. Amino acids conserved in all strains of influenza virus neuraminidase are colored yellow and red, and the subset of those that are conserved between known viral and bacterial neuraminidase structures are colored yellow (R118, D151, W178, E277, R292, R391, Y406, E425). W178 is not present in the proposed model for the parainfluenza virus HN protein. Of the red residues (E119, R152, R156, S179, D198, I222, R224, E227, E226, N294), D198 is Asn in N7 and N9 subtypes. The figure was generated with the HYDRASTER program, written by S. Watowich and L. Gross, based on work by D. Bacon and W. Anderson (RASTER3D) and R. Hubbard (HYDRA).

Fig. 7. Space-filling model of the active site, in a similar orientation to Figure 6, empty (left side) and filled with sialic acid (right side), illustrating again that the 4-hydroxyl binding pocket lies deep beneath the overhang of Asp 151.

makes no interaction with the enzyme. Identical interactions are observed for Neu5Ac2en, except that there is no glycosidic oxygen atom in that case (Varghese et al., 1992).

There are few similarities between the binding interactions of Neu5Ac with the viral hemagglutinin (Weis et al., 1988) and neuraminidase. In both cases, the same face of the sugar is directed toward the floor of the binding site and the *N*-acetyl group is located in a hydrophobic pocket. Other interactions are quite different. In hemagglutinin, Neu5Ac is bound with chair geometry, i.e., the carboxylate group is axial toward the floor of the binding site, and there is no charge compensation for the carboxylate by the protein.

The mechanism of the reaction has been studied by a variety of approaches, the results of which are largely consistent with the role of a general acid, probably a water molecule activated by Asp 151, attacking the glycosidic linkage and forming a carboxonium ion intermediate (Chong et al., 1992). The enzyme can also convert Neu5Ac into Neu5Ac2en (Burmeister et al., 1993). The group responsible for abstracting a proton from C₃ of the transition state in that case has not been formally identified.

The search for new inhibitors of the enzyme, based on this structural information, centered largely around the approach recommended by Goodford (1985), whose software, GRID, was used to evaluate likely binding sites within the enzyme active site for different chemical probes. Three examples of the results of this analysis are shown (Fig. 8), illustrating as contours of negative energy the preferred binding locations of (1) a carboxylate oxygen atom, (2) a methyl carbon atom, and (3) an amino (NH₂) nitrogen atom. The striking correspondence between theory and experiment in the first case is probably due to the tri-arginyl cluster that surrounds the binding pocket for the carboxylate group of the sugar. In the second case, the methyl carbon of the acetyl group is among the lowest energy sites. In addition, this calculation shows some space beyond the C₄ hydroxyl between Neu5Ac and the enzyme. This is further illustrated in the third case, which suggests that this space be filled by basic substituents to pick up favorable interactions between

the 2 conserved glutamyl residues referred to above, residue numbers 119 and 227.

The compounds targeted for synthesis as a result of this analysis were 4-amino-Neu5Ac2en and 4-guanidino-Neu5Ac2en (Fig. 1; von Itzstein, 1993). The *K_i*'s of these compounds against the neuraminidase of influenza virus A/Tokyo/3/67, the strain from which the design work was carried out, are 50 nM and 0.2 nM, respectively, and similarly tight, subnanomolar binding is observed for 4-guanidino-Neu5Ac2en with heterologous strains of different neuraminidase subtype (Table 1; von Itzstein et al., 1993). The binding affinity of 4-guanidino-Neu5Ac2en for the neuraminidase of A/Tokyo/3/67 influenza virus has now been reevaluated and a *K_i* of 0.03 nM is reported (Pegg & von Itzstein, 1994). No wild strain of the virus has yet been found to have a neuraminidase resistant to the inhibitors. Crystallographic studies of influenza virus neuraminidase complexed with 4-amino- and 4-guanidino-Neu5Ac2en show that both compounds bind in the active site of the enzyme as predicted (von Itzstein et al., 1993).

Table 1. Inhibition constants (*K_i*) or 50% inhibition levels (*IC*₅₀) for Neu5Ac2en and its 4-guanidino derivative against neuraminidases of different microbial and animal origins^a

Neuraminidase	Neu5Ac2en	4-Guanidino-Neu5Ac2en
Influenza N2 subtype (<i>K_i</i>)	1 × 10 ⁻⁶	2 × 10 ⁻¹⁰
Influenza N1 subtype (<i>K_i</i>)	—	7 × 10 ⁻¹¹
Influenza type B (<i>K_i</i>)	2 × 10 ⁻⁵	7 × 10 ⁻¹⁰
Parainfluenza (<i>K_i</i>)	1 × 10 ⁻⁵	8 × 10 ⁻⁴
<i>Vibrio cholerae</i> (<i>K_i</i>)	3 × 10 ⁻⁶	6 × 10 ⁻⁵
Ovine liver (<i>K_i</i>)	8 × 10 ⁻⁶	3 × 10 ⁻⁴
Human placenta (<i>IC</i> ₅₀)	1 × 10 ⁻⁵	1 × 10 ⁻³

^a Expressed in molar concentrations. Data from von Itzstein et al. (1993) and Holzer et al. (1993).

Fig. 8. GRID maps for (A) carboxylate oxygen, contoured at -6 kcal/mol; (B) methyl carbon, contoured at -3.5 kcal/mol; and (C) amino nitrogen (NH₂), contoured at -5.5 kcal/mol. Overlaid on the maps are the enzyme structure in red and the observed binding site of sialic acid (yellow).

The next level of assay is in tissue culture, where the capacity of the inhibitors to stop multicycle replication of virus can be measured by observing the effect of the compound on plaque formation. In nearly all cases studied, the reduction of plaques corresponds quantitatively with the improved enzyme inhibitory properties of the compounds assayed (von Itzstein et al., 1993).

Some strains of virus, in particular A/Stockholm/24/90, appear less sensitive to the inhibitors in this tissue-culture assay, although their neuraminidases are inactivated by the compounds (Woods et al., 1993).

Ferrets are the preferred animal model for influenza infection and were used in 1933 when the virus was first isolated. In challenge studies involving prophylactic administration of the compound, $50 \mu\text{g/kg}$, twice daily, 1 day before infection with virus and for the succeeding 6 days, 4-guanidino-Neu5Ac2en was shown to substantially reduce viral titers in nasal washings and to abolish the pyrexia usually observed on days 3–5 after infection (von Itzstein et al., 1993). The compound is also active in animal studies when the A/Stockholm/24/90 variant is used as the challenge virus (Woods et al., 1993). The genotype responsible for the resistance of this particular virus to the compound in tissue culture is not known.

The 4-substituted derivatives of Neu5Ac2en described above show poor inhibitory activity when assayed against neuraminidase from paramyxoviruses, bacteria, or mammals (Table 1; Holzer et al., 1993; von Itzstein, 1993). Neu5Ac2en, however, has a K_i of approximately $1 \mu\text{M}$ against all enzymes, consistent with the existence of a similar enzyme-reaction mechanism in each case. It might be concluded on this basis that the detailed structure of the active site in the vicinity of the 4-hydroxyl binding pocket is different in the enzyme from these other sources. Both the model for the paramyxovirus HN protein (Colman et al., 1993) and the experimentally determined structure of neuraminidase from *S. typhimurium* (Crennell et al., 1993) and *V. cholerae* (Crennell et al., 1994) are consistent with this conclusion. Neither of the 2 glutamyl residues that are conserved in influenza strains, Glu 118 and Glu 227, and that are located in the 4-hydroxyl binding pocket are found in the predicted HN structure or the bacterial neuraminidase structure.

There is a reasonable expectation that the use of any antibiotic will lead to the appearance of resistant strains. Two classes of drug are presently in use for treatment of influenza infection. Amantadine and rimantadine are related adamantanes with a similar mechanism of action against influenza viruses. At high concentrations ($>50 \mu\text{g/mL}$), amantadine is able to buffer the pH of endosomes and to deny virus the acidic environment needed for the hemagglutinin to fuse the viral membrane with that of the endosome (White et al., 1983). Resistant mutants show amino acid sequence changes in the hemagglutinin that can be generally interpreted as decreasing the stability of the hemagglutinin trimer (Daniels et al., 1985). Influenza viruses are also susceptible to low concentrations of amantadine ($<1 \mu\text{g/mL}$) that have no buffering capacity on endosomes. In this treatment regime, resistance maps to the transmembrane regions of the ion channel protein M₂ (Lubeck et al., 1978; Hay et al., 1985; Pinto et al., 1992). Although wild strains of influenza type A are sensitive to amantadine, resistance is rapidly selected in vitro, with mutations arising in either the hemagglutinin or M₂ as described above. Furthermore, drug-resistant virus can be isolated from humans as early as 2 days from the beginning of treatment with rimantadine (Hayden et al., 1991). Despite the rapid appearance of resistance, the drug displays a positive therapeutic benefit compared to placebo. The second class of anti-influenza drug, though not yet approved for use in man, is the guanosine analogue ribavirin, an inhibitor of viral replication (reviewed by Hayden, 1993). Resistance of influenza viruses to ribavirin has not yet been reported.

In the case of neuraminidase inhibitors, resistance might take 1 of 2 forms. On the one hand, any viral variants with a reduced dependency on the neuraminidase for elution from infected cells will be advantaged under the pressure of the inhibitors. On the other hand, neuraminidase variants that can bind and process substrate and not be inhibited by the compounds will also flourish in the presence of the compounds. The structural requirements for achieving this second form of resistance are apparent from the discussion above. The residues of the active site might conceivably mutate toward a structure more like that seen in the bacterial enzyme or the HN protein. In neither of those cases is 4-guanidino-Neu5Ac2en an inhibitor of the enzyme. Selection experiments are in progress to determine the frequency of occurrence and the nature of resistant strains. These experiments are mostly carried out in tissue culture, but the experience with the Stockholm viruses reported above points to the weakness in that approach. It will also be necessary to monitor the generation of resistant strains in animals being treated with the compound. The principal question to address in these studies is not so much whether or not resistance can develop to the neuraminidase inhibitors, but whether or not resistance will be clinically manageable.

In December 1993, the U.S. Food and Drug Administration approved an IND application for clinical studies of 4-guanidino-Neu5Ac2en. Challenge studies on human volunteers, as well as studies on natural infection, began in 1994.

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