

## Review

# Neuraminidase inhibitors as anti-influenza virus agents

Choung U Kim\*, Xiaowu Chen and Dirk B Mendel

Gilead Sciences, 333 Lakeside Drive, Foster City, CA 94404, USA

\*Corresponding author: Tel: +1 650 522 5616; Fax: +1 650 522 5899; E-mail: Choung\_Kim@gilead.com

**Influenza virus neuraminidase (NA) catalyses the cleavage of sialic acid residues terminally linked to glycoproteins and glycolipids and plays an important role in the replication of the virus. Recently, several potent NA inhibitors have been synthesized based on the rational design of mimicking the transition state of the sialic acid cleavage. Zanamivir and oseltamivir (GS 4104, the prodrug**

**of GS 4071) have emerged as promising influenza NA inhibitors for the treatment and prophylaxis of human influenza virus infection. This review describes the recent work toward the discovery and development of influenza NA inhibitors.**

**Keywords: Influenza; neuraminidase; inhibitors; GS 4104; zanamivir**

## Introduction

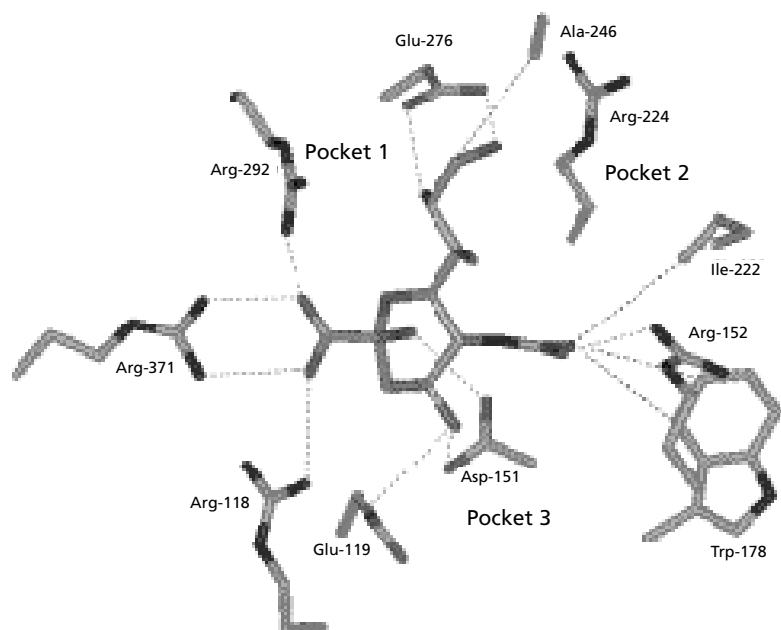
Influenza continues to be a serious health concern with yearly epidemics causing significant morbidity and mortality, even in nations with the most advanced health care systems. Attempts to control this disease through immunization have been hampered by the rapidity with which the virus mutates (Couch *et al.*, 1996). Thus, there has been a long-standing interest in the development of effective and safe antiviral agents with which to treat infected individuals. To date, amantadine and rimantadine are the only antiviral agents approved for the prevention and treatment of influenza virus infections. However, the effectiveness of these two related compounds is limited by adverse side effects, their inability to work against influenza B viruses (Hay *et al.*, 1985) and by the fact that resistant viruses that remain transmissible and pathogenic emerge readily during clinical use (Hayden & Couch, 1992; Monto & Arden, 1992).

Much of the effort to identify other suitable molecular targets for antiviral intervention has focused on the influenza virus neuraminidase (NA), one of two major glycoproteins located on the virus surface (Dowle & Howes, 1998; Meanwell & Krystal, 1996). This enzyme, which cleaves terminal sialic acid residues from glycoconjugates, is essential for virus replication and infectivity (Palese *et al.*, 1974; Palese & Compans, 1976; Klenk & Rott, 1988; Liu *et al.*, 1995). Early attempts to identify inhibitors of this enzyme were reasonably successful, with the most potent inhibitors able to inhibit enzyme activity and virus replication in the low micromolar range *in vitro* (Meindl *et al.*, 1974). However, these compounds were not specific, exhibited comparable activity against other viral, bacterial and mammalian NAs and they had no activity in animal models of influenza virus infection.

In the early nineties, the high-resolution crystal structures of sialic acid and the transition state analogue Neu5Ac2en bound to influenza A and B virus NAs were solved (Burmeister *et al.*, 1992; Varghese *et al.*, 1992; von Itzstein *et al.*, 1993). These structures provided an opportunity to use a rational approach to design more potent inhibitors of the influenza virus NAs. To date, this approach has led to the development of several potent and specific inhibitors of the influenza virus NA. Two of these, oseltamivir (GS 4104) (Kim *et al.*, 1997; Li *et al.*, 1998) and zanamivir (von Itzstein *et al.*, 1993), are currently in the late stages of clinical development for the treatment of influenza virus infection. In this article we will review the design of NA inhibitors, as well as the *in vivo* activity of oseltamivir (GS 4104) and zanamivir (GG167).

## Neuraminidase crystal structures

A number of high-resolution crystal structures of influenza NA and its complex with various small molecule inhibitors have been determined and are available from Brookhaven Protein Databank. Analysis of these structures has revealed some common structural features among all influenza NAs: (1) the NA active site contains some well-formed, large and relatively rigid pockets; (2) all residues making direct contact with the substrate are strictly conserved and interact in a similar fashion with both substrate and inhibitor molecules; and (3) unlike the active site of most other enzymes, the NA active site contains an unusually large number of polar or charged residues, suggesting that electrostatic interactions might play a critical role for any successful inhibitors. This is not

**Figure 1.** Key interactions between NA active site residues (only side chains are shown) and sialic acid

Atoms are coloured as follows: red for oxygen; blue for nitrogen; green for NA carbon and brown for sialic acid carbon. Dashes indicate polar (red) and hydrophobic (black) interactions.

unexpected because the NAs carbohydrate substrates are quite polar. Indeed, as discussed later, electrostatic interactions do play a significant role in the binding of NA inhibitors reviewed here.

Complex structures of NA and sialic acid (Burmeister *et al.*, 1992; Varghese *et al.*, 1992) and a number of inhibitors (Varghese *et al.*, 1998; Kim *et al.*, 1997) reveal several common key interactions between NA and its inhibitors (Figure 1). The negatively charged carboxylate group of sialic acid makes strong charge–charge interactions with a cluster of positively charged side chains of the Arg triad (Arg 118, 292 and in particular 371) of NA and the N-acetyl group of sialic acid, opposite to the carboxylate group, making both polar and hydrophobic interactions with NA. These two interactions help to anchor the scaffold of sialic acid, thus providing a structural frame for introducing additional interactions that would lead to more potent inhibitors.

The NA active site can be further divided into three major binding pockets based on the crystal structures (Figures 1 and 2), which could be explored for improving inhibitor binding. Pocket 1 is formed by Glu-276, Glu-277, Arg-292 and Asn-294. This pocket interacts with the glycerol moiety of sialic acid. As to be discussed later in this review, even though this pocket contains three charged and one highly polar residue, novel non-polar interactions have been discovered in this pocket, which prove to be the key to the binding of potent cyclohexene-based NA inhibitors (Kim *et al.*, 1997).

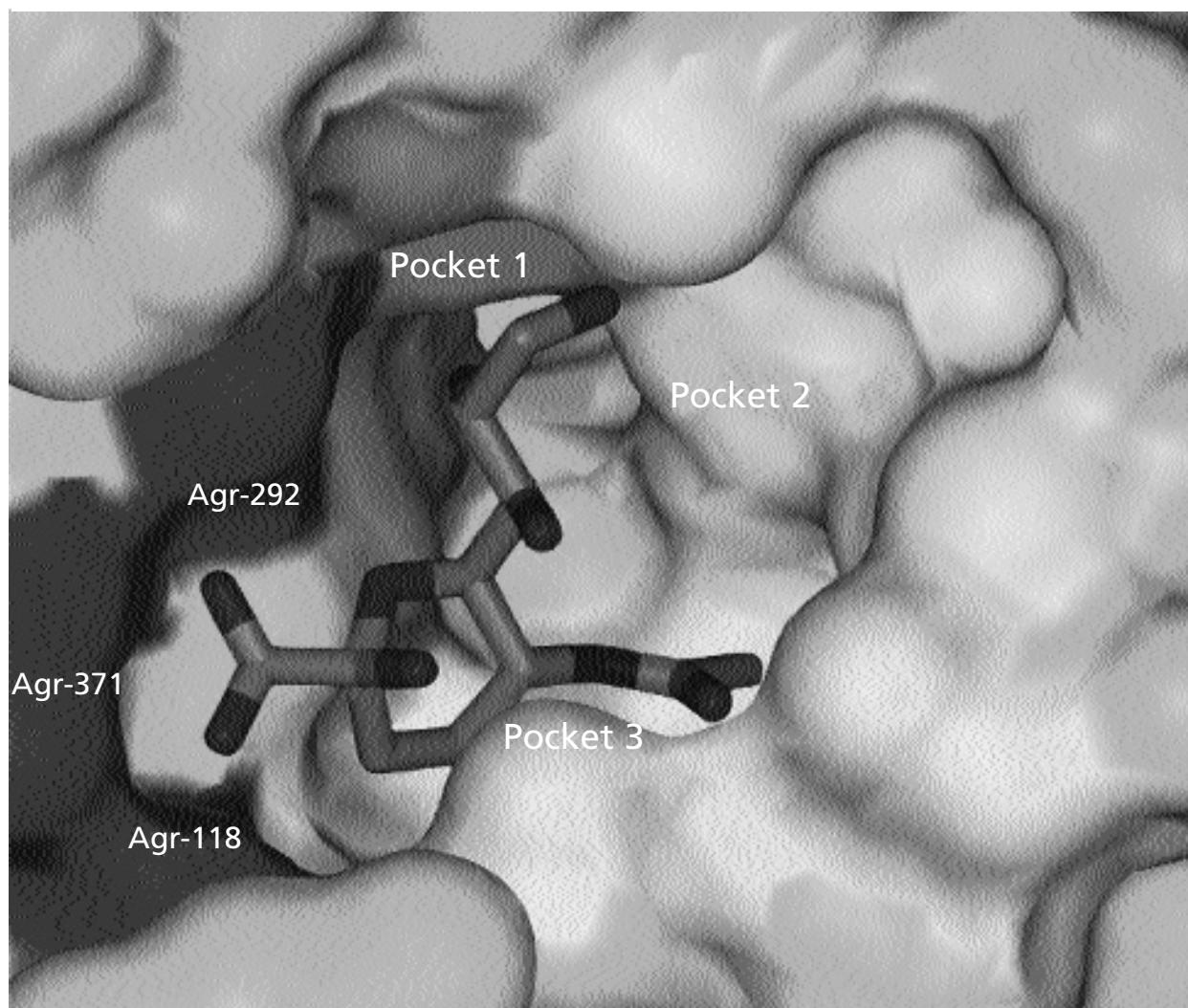
Close inspection of NA structures also reveals the pres-

ence of a well-formed hydrophobic pocket that is not utilized by sialic acid for binding. This pocket (pocket 2) is surrounded on one side by Ala-246, on the other side by Ile-222 and an Arg-224 side-chain forms the bottom. All three residues are highly conserved and could provide hydrophobic interactions with potential inhibitors. Indeed, the cyclohexene series of inhibitors do bind in this pocket and form favorable hydrophobic interactions with the residues present (Kim *et al.*, 1997).

Glu-119, Asp-151, Arg-152, Trp-178, Ser-179, Ile-222 and Glu-227 of NA form pocket 3 and interact with both the C-4 hydroxyl and N-acetyl groups of sialic acid. This pocket is very large, becomes deeply buried upon inhibitor binding and is not fully utilized by sialic acid (Figures 1 and 2). In particular, there is a cluster of negatively charged residues (Glu-119, Glu-227 and Asp-151) near the C-4 hydroxyl group that could be further explored for possible charge–charge interactions. As will be described later, introducing such interactions does help to improve inhibitor binding significantly.

### Drug design of neuraminidase inhibitors

The high-resolution crystallographic structure of sialic acid complexed with NA revealed that sialic acid binds the enzyme in a considerably deformed conformation owing to the strong ionic interactions of the carboxylic acid moiety in sialic acid with three arginines, 118, 292 and 371, in the active site. The binding mode is very similar to that found

**Figure 2.** Complex structure of NA (shown in surface representation) and sialic acid (in stick representation)

Arg triads are highlighted in blue, pocket 1 residues in brown, pocket 2 residues in cyan and pocket 3 in yellow.

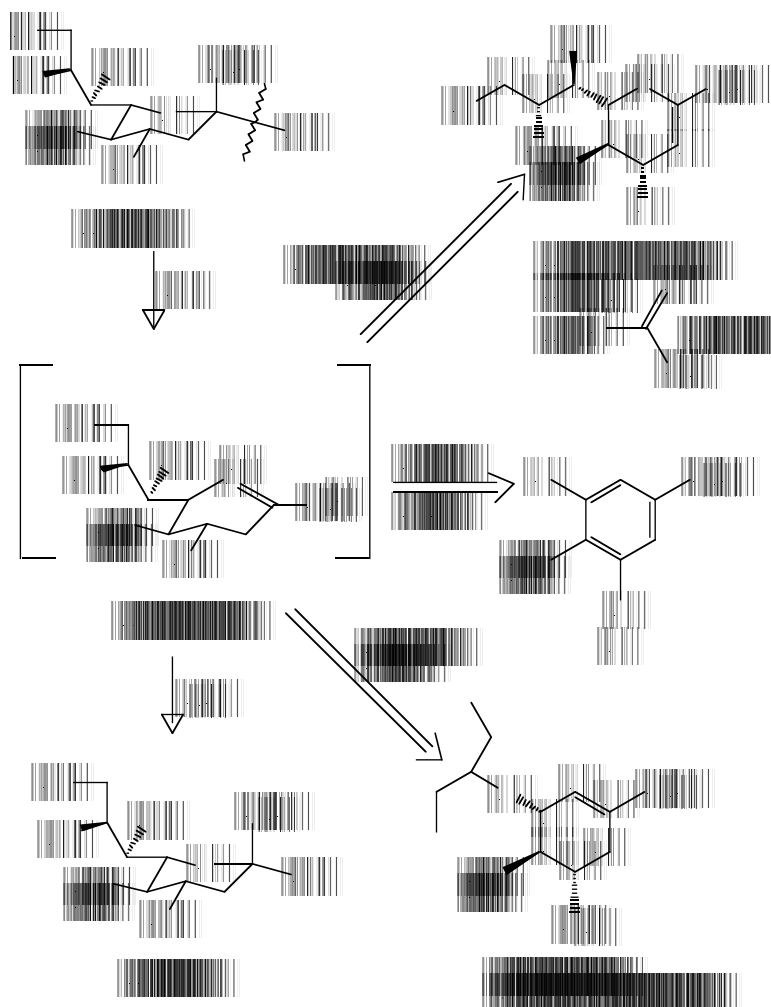
in the X-ray crystal structure of Neu5Ac2en (**4**) bound to NA. In this case, the double bond of **4** constrains the pyranose ring into a planar structure around the ring oxygen. On the basis of this structural information and biochemical mechanistic studies, it has been proposed that the catalytic mechanism for the cleavage of sialic acid from glycoconjugate (**1**) by NA proceeds via the carbonium cation transition state **2**. Taking into consideration that transition state **2** forms the rather flat oxonium cation, several transition state mimics have been devised including the dihydropyran, benzene and cyclohexene scaffolds (Figure 3).

### Sialic acid-based neuraminidase inhibitors

As described above, influenza NA cleaves terminal sialic acid from glycoconjugates to produce free sialic acid (**3**),

which itself is a weak NA inhibitor with an  $IC_{50}$  in the low mM range. The first lead compound in sialic acid-based NA inhibitors was the dehydro analogue of **3** (compound **4**) with a  $K_i$  of 4  $\mu$ M when tested against influenza NA (Figure 4) (Holzer *et al.*, 1993). However, compound **4** was a non-selective NA inhibitor, and did not exhibit *in vivo* efficacy in animals infected with influenza virus. Recently, based on the X-ray crystal structure of **4** complexed with NA, rationally designed NA inhibitors **5** and **6** were prepared (Taylor & von Itzstein, 1994; von Itzstein *et al.*, 1993). In comparison to **4**, both **5** and **6** are more potent NA inhibitors with  $K_i$  values of  $10^{-8}$  M and  $10^{-10}$  M, respectively. X-ray crystal structures revealed that the amino group in **5** and the guanidino group in **6** both form a strong salt bridge with Glu-119 in the NA active site. The guanidino group also adds a strong charge-charge interaction with Glu-227 (Taylor & von Itzstein, 1994). Consistent

**Figure 3.** The catalytic mechanism of the cleavage of sialic acid from **1** and the transition states including the three transition state mimics



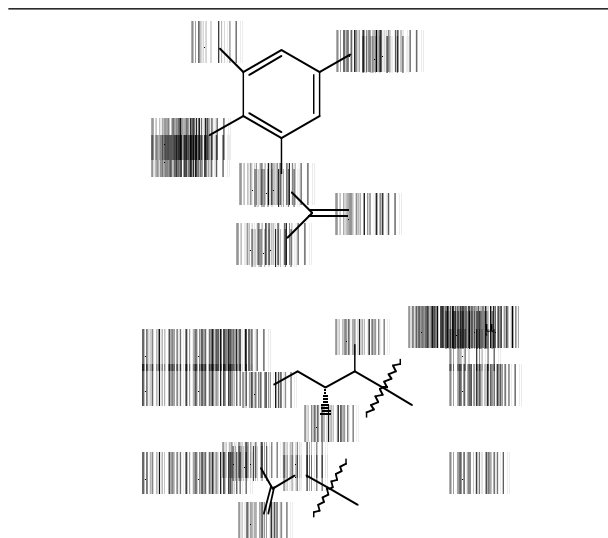
**Figure 4.** After the first lead compound in sialic acid-based NA inhibitors, the dehydro analogues of **3** (compound **4**) were discovered, the other compounds were designed rationally



with its potent activity *in vitro*, **6** (zanamivir) also exhibited potent antiviral activity against a variety of influenza A and B virus strains in the cell culture assay and exhibited *in vivo* efficacy in influenza virus-infected animal models via intranasal application (von Itzstein *et al.*, 1993). Zanamivir is currently being evaluated in human clinical trials, and has shown efficacy in Phase III studies in both prophylaxis and treatment of influenza virus infection. However, poor oral bioavailability and rapid excretion precluded zanamivir as a potential oral agent against influenza infection. Therefore, zanamivir has been administered by either intranasal or inhaled routes in clinical trials.

The importance of the glycerol side chain in **5** has been demonstrated by the decreased NA inhibitory activity of analogues with truncated glycerol side chains (compounds **11** and **12**) (Bamford *et al.*, 1995). The terminal diol of the glycerol side chain interacts with Glu-276 in a bidentate mode and contributes considerable binding to NA.

**Figure 5.** Benzoic acid-based NA inhibitors and their IC<sub>50</sub> values



However, replacement of the glycerol side chain with more lipophilic amides (compounds **13** and **14**) led to very potent NA inhibitors (Sollis *et al.*, 1996). Interestingly, this class of compounds exhibited more potent influenza A NA inhibitory activity compared to that of influenza B. The detailed X-ray crystallographic analysis of **13** bound to NA illustrates a different binding mode of compound **13** against influenza A virus NA versus influenza B virus NA (Taylor *et al.*, 1998). The large alkyl amide appears to be a less suitable fit in the influenza B virus NA activity site because of its smaller and more rigid active site pocket compared to that of the influenza A virus NA.

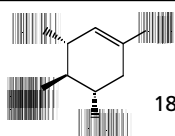
### Benzoic acid-based neuraminidase inhibitors

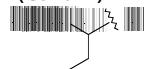
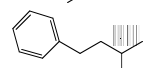
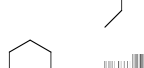
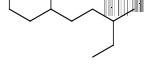
Recently, benzoic acid analogues that closely mimic sialic acid analogues have been reported (Tedrzejnas *et al.*, 1995; Chand *et al.*, 1997). The approach of using the benzene ring as a template was based on the observation that in Neu5Ac2en (**4**)-NA crystal structure, all substituents attached to the dihydropyran ring are positioned in an equatorial orientation (Bossart-Whitaker *et al.*, 1993). Therefore, replacement of the dihydropyran ring with the benzene ring was predicted to generate minimal disturbance for the overall binding mode. However, the zanamivir analogue with the benzene template (compound **16**) did not exhibit NA inhibitory activity (Williams *et al.*, 1995) (Figure 5). Interestingly, the benzoic acid analogue **15** without the glycerol side chain showed similar NA inhibitory activity comparable to that of Neu5Ac2en (**4**). The X-ray crystal structure of **15** bound to NA revealed that the guanidino group in **15** oriented into the glycerol binding pocket

(Tedrzejnas *et al.*, 1995). Addition of another guanidino group (compound **17**) resulted in much reduced NA inhibitory activity. Clearly, the phenyl ring template had limited ability of attaining the right orientation for all substituents for the optimal binding to the NA active site.

### Inhibitor design based on a cyclohexene ring

As described above, zanamivir is administered via the inhaled route because of its poor oral bioavailability. In the search for orally active NA inhibitors, a new approach based on the cyclohexene scaffold was investigated (Kim *et al.*, 1997, 1998). The double bond of the cyclohexene ring was considered as an isostere of the flat oxonium cation shown in the transition state **2** (Figure 3), keeping the conformational change to a minimum. The cyclohexene ring could be expected to be chemically and enzymatically stable. This carbocyclic system also allowed easier chemical manipulation of side chains attached to the cyclohexene ring for optimizing the biological activity. Crystallographic studies of sialic acid and its analogues bound to NA revealed that the C-7 hydroxyl of the glycerol side chain does not interact with any amino acids of the NA active site (Colman, 1994). Therefore, in the design of the carbocyclic NA inhibitors, the C-7 hydroxy methylene was replaced with the oxygen atom linker. The choice of the oxygen atom was based on the synthetic practicality to generate a variety of R groups, as shown in structure **18** (Table 1). Two terminal hydroxyls of a glycerol side chain in the sialic acid crystal structure bound to NA to form a bidentate interaction with Glu-276. However, it is also noted that the C-8 of the glycerol side chain makes hydrophobic contacts with the hydrocarbon chain of Arg-224 (Colman, 1994). Therefore, optimization of this hydrophobic interaction was systematically investigated. In designing orally active drugs, we feel that balancing lipophilicity and water solubility is as critical as the size of the drug molecules for oral absorption from the intestinal tract (Lipinski *et al.*, 1997). Since the carbocyclic structure **18** already possesses highly polar functional groups (C-1-carboxylate, C-4-acetamido and C-5-amino groups) for the optimal NA activity, it was crucial to increase lipophilicity of the molecule by adding the alkyl group (**R** in **18**) for oral bioavailability. As shown in Table 1, the length, size of branching and geometry of the alkyl group in **18** profoundly influenced the NA activity. The over 20-fold increase in the NA activity of the *n*-propyl analogue **22** compared to that of the methyl analogue **20** implicated a significant hydrophobic interaction of the propyl group in the glycerol portion of the NA binding pocket. Further extension of the alkyl chain beyond the *n*-propyl length did not improve the influenza A activity, but greatly reduced influenza B activity.

**Table 1.** Activity of carbocyclic influenza virus NA inhibitors


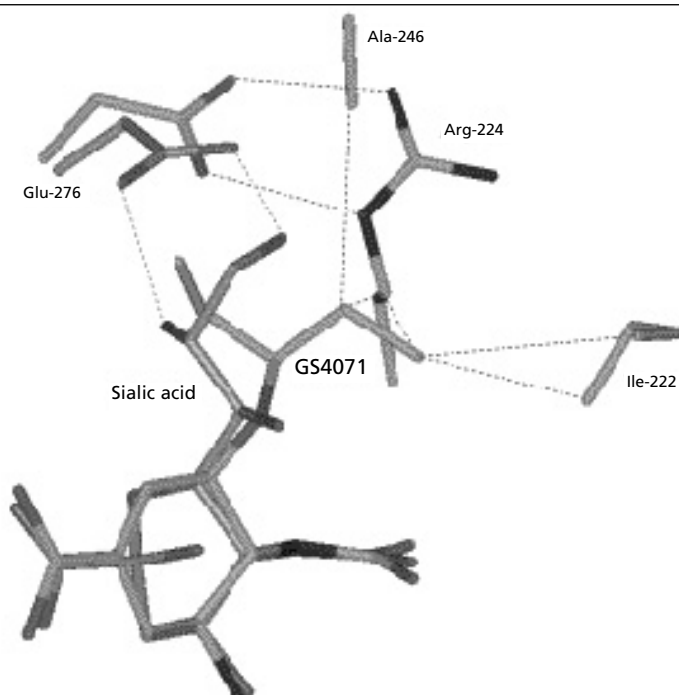
	R	IC <sub>50</sub> (nM)	
		Influenza A	Influenza B
19	H-	6300	-
20	CH <sub>3</sub> -	3700	-
21	CH <sub>3</sub> CH <sub>2</sub> -	2000	5-
22	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>2</sub> -	180	-
23	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>3</sub> -	300	215
24	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>5</sub> -	150	1450
8	(CH <sub>3</sub> CH <sub>2</sub> ) <sub>2</sub> CH (GS4071)	1	3
25		1	4
26 (S)		0.3	70
27 (R)		12	35
28		1	21500
6	(Zanamivir)	1	3

The C-7 hydroxy methylene was replaced with the oxygen atom linker. The generation of a variety of R groups was based on synthetic practicality.

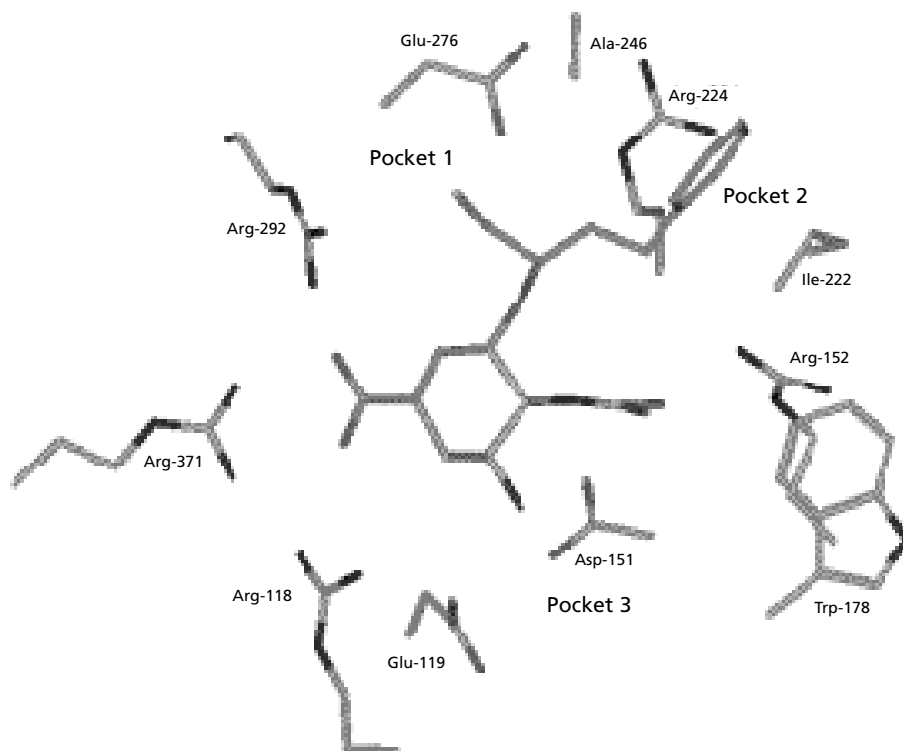
Branching at the  $\beta$ -carbon of the *n*-propyl group led to the significant increase in the NA activity, as demonstrated by the 3-pentyl analogue **8** (GS 4071). This result strongly suggested that there may be two hydrophobic pockets oriented in two different directions for the binding of the **R** group in **18**.

The crystal structure of **8** (GS 4071) complexed with NA (Figure 6) (Kim *et al.*, 1997) indicates that this series of inhibitors adopts a binding mode similar to that of sialic acid because the carboxylate and N-acetyl moieties of inhibitors interact with NA in a fashion similar to that with sialic acid. Such interactions anchor the scaffold in the NA active site. Replacing the C-4 hydroxyl group with a positively charged amino group further strengthens the binding by introducing additional electrostatic interactions with the negatively charged residues in pocket 3. However, the crystal structure does reveal hydrophobic interactions not previously observed between NA and sialic acid. One branch of the 3-pentyl side chain of **8** make several favourable contacts with residues Ile-222, Arg-224 and Ala-246, resulting in a significant increase in binding affinity (Figure 6). This is not unexpected because hydrophobic interactions tend to be the driving force for inhibitor binding.

A truly surprising discovery from this structure is the ability of the other branch of the 3-pentyl group of **8** to bind in an apparently highly polar pocket 1. Such a bind-

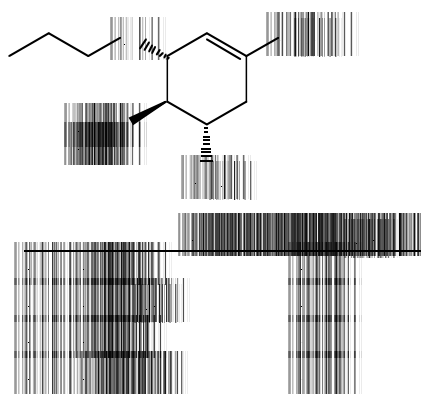
**Figure 6.** Neuraminidase Glu-276 side chain can adopt alternative conformations upon binding of sialic acid or GS4071

Atoms are coloured as follows: blue for nitrogen; red for oxygen; brown for carbon in sialic acid complex and green for carbon in GS4071 complex.

**Figure 7.** Complex structure of neuraminidase and **26**

Atoms are coloured as follows: red for oxygen; blue for nitrogen; green for NA carbon and brown for compound 26 carbon.

ing mode becomes possible because the Glu-276 side chain can adopt two different conformations, depending upon the nature of the inhibitor moiety bound in pocket 1 (Figure 6) (Kim *et al.*, 1997). Upon the binding of sialic acid, the Glu-276 side chain adopts a conformation that is facilitated by a bidentate H-bonding interaction with the hydrophilic glycerol moiety of sialic acid. However, on the binding of **8**, the Glu-276 side chain moves away from the middle of pocket 1 and adopts an alternative conformation, which is stabilized by a strong charge–charge interaction

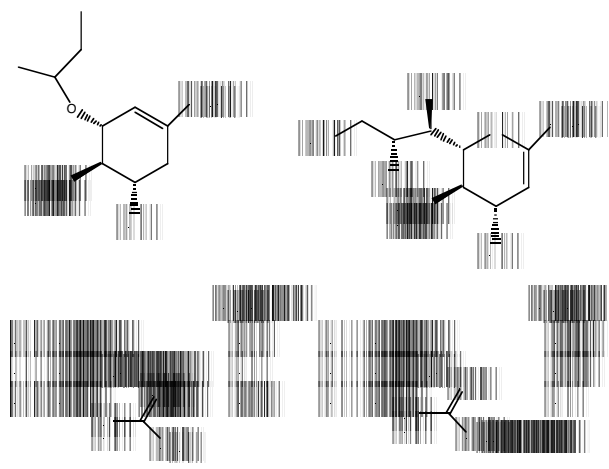
**Figure 8.** Structure of the *n*-propyl analogues (compounds **30–32**)

with the nearby Arg-224 side chain. Such a conformational change effectively makes pocket 1 larger and creates a much less polar environment, making it possible for this pocket to accommodate the hydrophobic moiety of an inhibitor molecule.

Interestingly, extension of one of the alkyl chains in **8** (compound **25**) did not influence the ability to inhibit influenza A and B virus NAs (Kim *et al.*, 1998). Two diastereomers with the terminal phenyl ring (compounds **26** and **27**) exhibited potent influenza A virus NA inhibitory activity, but the influenza B virus activities of **26** and **27** were significantly reduced compared to that of GS 4071 (**8**). As expected, the crystal structure of **26** shows that the larger phenethyl chain binds in pocket 2, whereas the smaller ethyl group occupies pocket 1 (Figure 7) (Kim *et al.*, 1998).

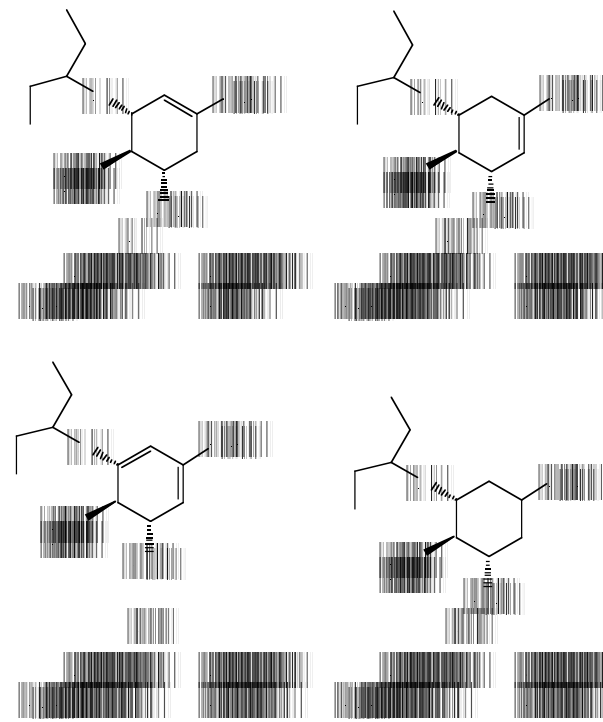
The cyclohexyl analogue **28** retained the potent influenza A virus NA activity, but the influenza B NA activity was reduced over 1000-fold. This result suggests that the binding affinity of inhibitors to influenza B virus NA may be influenced significantly by increasing steric bulkiness of the structure **18**.

As mentioned above, the oxygen linker atom at the C<sub>3</sub> position was chosen based on practical synthetic consideration. For structure–activity relationship (SAR) studies, the

**Figure 9.** Comparative activity of carbocyclic versus dihydropyran-based neuraminidase inhibitors

*n*-propyl analogues with different linker atoms (compounds **30–32**) (Figure 8) were prepared (Lew *et al.*, 1997). They exhibited similar NA inhibitory activity to the oxygen counterpart **22** (Lew *et al.*, 1997). This result confirms that these linker atoms may not be part of the inhibitor interaction with the NA active site. In the series of the sialic acid-based inhibitors, the conversion of the C-4 amino group in **5** to the guanidino functionality (**6**, zanamivir) imparted a 100-fold increased potency in NA inhibitory activity (von Itzstein *et al.*, 1993). However, as shown in Figure 9, the guanidino analogue of GS 4071 (compound **34**) had only modestly improved NA inhibitory activity, implying that the SAR of the amino versus the guanidino groups may not be extrapolated from one series to another. As shown in Figure 9, the carbocyclic series is over 100-fold more active than the corresponding dihydropyrane analogues in the case of X = OH (**33** versus **4**) and NH<sub>2</sub> (**8** versus **5**). The most important result derived from this comparison is that the 3-pentyl group in the carbocyclic series imparts considerable binding energy through the network of the hydrophobic interaction.

The importance of the double bond position in the carbocyclic series was investigated in detail. In the GS 4071 series (Figure 10), the olefinic isomer **35** exhibited significantly reduced NA inhibitory activity, especially against influenza B virus (Williams MA, Lew W & Kim CU, unpublished results). The crystal structures of two isomers, **8** and **35**, provided no clear clue on the binding mode difference to NA. Further SAR information was provided by the diene analogue **36** and the saturated analogue **37**. Much reduced NA inhibitory activity of these analogues strongly suggested that structure **8** is the best transition state mimic among the olefinic analogues. The fact that the cyclohexane analogue **37** displayed significantly reduced NA inhibition to the parent compound **8** rein-

**Figure 10.** Comparative activity of carbocyclic versus dihydropyran-based neuraminidase inhibitors

forced the validity of the transition state mimic in the enzyme inhibitor design.

Although GS 4071 was designed with the intent of developing an orally active influenza NA inhibitor, pharmacokinetic experiments revealed that the orally bioavailability of GS 4071 in rats was only approximately 5%, which is similar to that of zanamivir (Ryan *et al.*, 1994; Li *et al.*, 1998). However, the bioavailability of GS 4071 following oral administration of GS 4104, the ethyl ester pro-drug of GS 4071, was found to be more than fivefold higher than that of the parent compound in rats. Importantly, oral administration of GS 4104 also effectively delivers GS 4071 to respiratory secretions (Eisenberg *et al.*, 1997). A high bioavailability of GS 4071 following oral administration of GS 4104 was also found in mice (approximately 30%) and dogs (approximately 70%) (Li *et al.*, 1998) and in humans (approximately 80%) (H Wiltshire, personal communication). A similar approach of generating the ester of zanamivir did not increase its bioavailability following oral administration (Li *et al.*, 1998).

### Resistance to neuraminidase inhibitors

Several groups have isolated influenza variants with decreased susceptibility to NA inhibitors following serial passage of virus in cell culture in the presence of inhibitor

(Blick *et al.*, 1995; Staschke *et al.*, 1995; Gubareva *et al.*, 1996, 1997; McKimm-Breschkin *et al.*, 1996a, 1998; Tai, 1998). Genotypic analysis of these variants has indicated that mutations in the haemagglutinin (HA) as well as the NA gene can cause reduced susceptibility to NA inhibitors in antiviral assays, with the HA mutants being easier to generate.

### Neuraminidase mutants

As described above, most of the interactions between zanamivir or GS 4071 and conserved residues in the NA enzyme active site are similar to those for the natural substrate. However, both compounds also rely on interactions with conserved residues that differ from the interactions with the natural substrate. In the case of zanamivir, the C-4 guanidino group of the inhibitor interacts strongly with the conserved Glu-119 and Glu-227 residues in the enzyme active site, which do not interact with the natural substrate (von Itzstein *et al.*, 1993). In the case of GS 4071, it is the reorientation of the conserved Glu-276 that normally interacts with hydroxyl groups on the glycerol side chain of the natural substrate, which differs from the binding of the natural substrate (Kim *et al.*, 1997). Not surprisingly, mutations in the NA enzyme active site that confer decreased sensitivity to each of the NA inhibitors involve residues that contribute to the binding interactions, which differ from those of the natural substrate.

In the case of zanamivir, the most commonly identified NA mutation involves a Gly substitution for the conserved Glu-119 residue. This mutation results in a 100-fold decrease in sensitivity to zanamivir at the enzyme level (Staschke *et al.*, 1995; McKimm-Breschkin *et al.*, 1996a), with little or no change in sensitivity to GS 4071 (Hayden & Rollins, 1997). Comparison of the wild-type and mutant enzymes indicates that this mutation does not change the affinity for the substrate or the activity of the enzyme (McKimm-Breschkin *et al.*, 1996a). It does, however, cause the enzyme tetramer to become unstable (McKimm-Breschkin *et al.*, 1996b; Colacino *et al.*, 1997) and results in lower apparent enzymatic activity when assayed *in vitro* (Staschke *et al.*, 1995). Mutations of Glu-227, which also interact with the guanidino side chain of zanamivir have not been reported, presumably because mutations to this conserved residue severely compromise enzyme activity (Goto *et al.*, 1997).

A second NA mutation, in which a Lys is substituted for the conserved Arg at residue 292 (R292K), has also been identified in influenza virus following *in vitro* passage in the presence of zanamivir (Gubareva *et al.*, 1997). However, this mutation only causes a minor (approximately 10-fold) change in sensitivity to zanamivir (Gubareva *et al.*, 1997; McKimm-Breschkin *et al.*, 1998; Tai *et al.*, 1998) owing to alterations in the interaction with the carboxylate

of the inhibitor (Varghese *et al.*, 1998). Since the interaction between Arg-292 and the carboxylate of the substrate is also important, this mutation causes a reduction in the activity of the enzyme (Gubareva *et al.*, 1997; McKimm-Breschkin *et al.*, 1998; Tai *et al.*, 1998).

The same R292K NA mutation has also been identified in influenza A virus passaged *in vitro* in the presence of GS 4071 (Tai *et al.*, 1998). In contrast with what is observed with zanamivir, this mutation causes a 5000–30000-fold reduction in sensitivity to GS 4071 (McKimm-Breschkin *et al.*, 1998; Tai *et al.*, 1998). The large effect of this mutation on the affinity for GS 4071 is due to its ability to block the reorientation of Glu-276 that is necessary to form the hydrophobic pocket to accommodate the inhibitor (Varghese *et al.*, 1998; Tai *et al.*, 1998).

Although viruses containing mutant NAs with decreased sensitivity to zanamivir and GS 4071 have been isolated following passage *in vitro*, mutants have not been reported following passage of virus in mice under conditions that would be expected to cause resistance to amantadine (von Itzstein *et al.*, 1993; Mendel & Sidwell, 1998). This is likely because alteration to Glu-119 or Arg-292 of NA compromise the ability of the virus to replicate in mice. Gubareva and co-workers have shown that reassortant virus containing NA with mutations to Glu-119 or Arg-292 are fourfold or 600-fold less infectious, respectively, than the wild-type virus in mice (Gubareva *et al.*, 1997). Similar results have been obtained using a different R292K NA mutant, although this mutant was more than 10000-fold less infectious than the wild-type virus from which it was derived (Tai *et al.*, 1998). Thus NA mutants, although they can be generated *in vitro*, are likely to be compromised in the natural setting.

### Haemagglutinin mutants

Influenza virus variants containing mutations in the sialic acid binding site of the HA have been isolated following passage *in vitro* in the presence of NA inhibitors. These mutations cause reduced affinity for the cellular receptors (McKimm-Breschkin *et al.*, 1996a; Sahasrabudhe *et al.*, 1996), making the viruses less dependent on NA activity to release from cells after infection. Consequently, these variants exhibit a large decrease in susceptibility to all NA inhibitors in antiviral assays. In addition to decreased susceptibility to the NA inhibitors, several of these mutants have been reported to have a drug-dependent phenotype in which the variants grow better in the presence of inhibitor than in its absence (McKimm-Breschkin *et al.*, 1996a; Sahasrabudhe *et al.*, 1996). The drug-dependent phenotype is thought to occur because of the interdependence of the HA and NA functions during the infection process, such that in the presence of normal NA function a virus with a weakly binding HA would exhibit reduced ability to infect

cells. Thus, in the presence of sufficient inhibitor to block NA function, the mutant would more effectively infect cells and replicate.

Although HA mutations can be readily selected *in vitro*, the *in vivo* consequence of this type of mutation is not clear. Penn and co-workers have investigated the zanamivir responsiveness in mice of an influenza A/Singapore/2/57 (H2N2) variant containing an HA mutation that confers a >1000-fold decrease in susceptibility to zanamivir in culture. The results of this study indicate that the variant replicated well in the mice and exhibited a normal response to zanamivir treatment even though the virus did not revert to the wild-type genotype (Penn *et al.*, 1996). Thus, this particular mutation, which causes reduced affinity for the receptor present on MDCK cells, does not appear to affect its affinity for the receptors present in the mouse respiratory tract.

Although this mutant does not exhibit a change in sensitivity to zanamivir *in vivo*, Gubareva and co-workers have recently identified a zanamivir-resistant clinical isolate containing an HA mutation that causes reduced affinity for the type of cellular receptors present in the human respiratory tract (Gubareva *et al.*, 1998). However, it is important to note that this mutant was isolated from a severely immunocompromised bone marrow transplant recipient with a persistent viral infection. It is unclear whether such a mutant could develop in an individual with a normal immune system capable of curtailing virus replication in a reasonable amount of time.

### ***In vivo* activity of neuraminidase inhibitors**

#### **Animal studies**

The only NA inhibitors for which animal studies of efficacy have been reported are GS 4071 and zanamivir. Because of its poor oral bioavailability, animal studies of the *in vivo* activity of zanamivir have used topical administration of the compound to the respiratory tract using intranasal drops. The efficacy of GS 4071 in animal models has largely been conducted using oral administration of the prodrug GS 4104.

In mice infected with influenza A or B virus, intranasal administration of zanamivir could inhibit virus replication, lung consolidation and mortality. In mice infected with influenza A/Singapore/1/57 (H2N2) virus, intranasal zanamivir was found to be 100-fold and 1000-fold more potent an inhibitor of virus replication than amantadine or ribavirin, respectively, administered via the same route (von Itzstein *et al.*, 1993). When administered prior to inoculation with influenza A virus, twice daily intranasal doses of 0.1 mg/kg of zanamivir were sufficient to cause a significant reduction in lung consolidation and mortality (Ryan *et al.*, 1994). Twice daily doses of 0.4 mg/kg of zanamivir to

mice infected with influenza A or B viruses were sufficient to cause a significant reduction in lung virus titres as well (Ryan *et al.*, 1994). A higher dose of zanamivir was required to achieve reductions in virus titres if treatment was initiated 3 h after inoculation (Ryan *et al.*, 1994). In one study (Sidwell *et al.*, 1998), the relative potencies of GS 4071 and zanamivir were compared following intranasal administration. In this study, GS 4071 was found to be approximately 10-fold more potent than zanamivir at protecting mice against the lethal effect of infection with influenza A NWS/33 (H1N1) virus, with a minimal effective dose of 0.01 mg/kg/day for GS 4071.

Oral administration of GS 4104 has also been shown to provide protection against the lethal effects of influenza A and B virus infection in mice (Sidwell *et al.*, 1998; Mendel *et al.*, 1998). In mice infected with an influenza A/H1N1 virus, a 1 mg/kg/day dose of GS 4104 administered for 5 days beginning 4 h prior to infection was sufficient to provide significant reduction in mortality. Increases in survival time and arterial oxygen saturation levels were observed in mice given a 0.1 mg/kg/day dose of GS 4104. Tenfold higher oral doses of GS 4071 and zanamivir were required to achieve comparable results for each of the end points tested. Higher oral doses of GS 4104 were required to provide significant protection against mortality in mice infected with influenza A/H3N2 virus (1 to 10 mg/kg/day) or influenza B virus (3.2 mg/kg/day).

In an experiment designed to determine the effect of delaying treatment with oral GS 4104 until after infection, a 10 mg/kg/day dosage of GS 4104 was administered for 5 days. Commencement as late as 60 h after infection was able to protect the mice from death following infection with sufficient influenza A/NWS/33 (H1N1) virus to kill 85% of untreated animals. Using a 10-fold higher inoculum of this virus, treatment could be delayed for up to 36 h after infection.

GS 4104 and zanamivir have also been tested for efficacy using the ferret model of influenza virus infection. Ferrets infected with influenza virus develop a disease state of limited duration composed of symptoms such as fever and lethargy that are similar to those seen in humans. Thus ferrets provide a useful experimental animal model in which to measure symptomatic as well as virological responses to treatment with the NA inhibitors.

Intranasal administration of 0.04–0.75 mg/kg zanamivir twice daily for 5 days, beginning 1 day prior to infection, caused significant reductions in the febrile response and the total amount of virus shed from the nasal cavity of animals infected with influenza A or B virus (Ryan *et al.*, 1995). For each of the viruses tested, zanamivir was approximately 100-fold more potent than ribavirin. In this model, delaying treatment with zanamivir until as much as 24 h after infection did not eliminate its therapeutic effect and

zanamivir did not inhibit the immune response to the infection (Ryan *et al.*, 1995).

Twice daily oral administration of GS 4104 for 5 days beginning 4 h after infection was also found to be effective in the ferret influenza model (Mendel *et al.*, 1998). A 5 and 25 mg/kg/dose treatment with GS 4104 was sufficient to reduce the febrile response by 58% and 93%, respectively, in ferrets infected with a high dose of an influenza A/H3N2 virus. Both doses prevented the infected animals from becoming lethargic and from developing nasal signs (sneezing, runny nose, mouth breathing) and significantly reduced the immune response at the site of infection. Both doses of GS 4104 reduced peak virus titres detected in nasal wash samples, but did not reduce the overall amount of virus shed from the nasal cavity during the course of the infection (Mendel *et al.*, 1998).

Previous studies had demonstrated that after oral dosing of the prodrug to rats, GS 4071 levels in the bronchoalveolar lining fluid reached maximum concentrations similar to that in plasma, and showed longer persistence (Eisenberg *et al.*, 1997). This result indicated that oral administration of GS 4104 effectively delivers active drug to the respiratory tract in animals. A similar conclusion was reached based on the results of a study in which ferrets were given an oral dose of [<sup>14</sup>C]GS 4104. Whole-body autoradiography of these animals demonstrated systemic exposure to the label, with good penetration to the various sites of viral replication, including the middle ear and nasal mucosa, as well as the lower respiratory tract. In light of these results and the demonstration that reasonable plasma levels of GS 4071 were achieved in ferrets given oral GS 4104 (Li *et al.*, 1998), the apparent inability of GS 4104 treatment to cause a more pronounced antiviral effect in the ferrets was surprising.

A possible explanation for the inability of GS 4104 to significantly reduce the total amount of virus shedding in nasal wash samples from this experiment is because the animals were inoculated with a large dose of virus (10<sup>6</sup> egg infectious doses per animal). For comparison, despite the size difference, 10-fold less virus (10<sup>5</sup> TCID<sub>50</sub> per subject) has been used to experimentally infect human volunteers with influenza A/Texas/36/91 (H1N1) virus (Hayden *et al.*, 1998b). In support of this hypothesis, significant reductions in both peak nasal virus titres and total amount of virus shedding have been detected for both the 5 and 25 mg/kg doses of GS 4104 in ferrets infected with a lower dose of influenza A virus (Mendel & Roberts, 1998).

### Clinical studies

Both oral GS 4104 and intranasal zanamivir are in the late stages of clinical development and have been tested for efficacy in humans using blinded, placebo-controlled trials. In

prophylaxis models, both oral GS 4104 (Hayden FG *et al.*, 1998c) and intranasal zanamivir (Hayden *et al.*, 1998b) were able to prevent experimental influenza virus infection in healthy volunteers when treatment began prior to a virus challenge. Both compounds also significantly reduced the amount of virus shedding, symptom severity and symptom duration when treatment was initiated a day after infection with influenza virus in healthy volunteers (Hayden *et al.*, 1998b; Hayden FG, Lobo M, Treanor JJ, Miller M & Mills RG; Efficacy and tolerability of oral GS 4104 for early treatment of experimental influenza in humans. *11th Interscience Conference of Antimicrobial Agents and Chemotherapy*, 28 September–1 October 1997; Toronto, Ontario, Canada, Late Breaker Session). Importantly, oral administration of GS 4104 also reduced the levels of inflammatory mediators (cytokines and chemokines) produced in response to the experimental viral infection when treatment was initiated a day after infection (Hayden FG, Fritz RS, Lobo M, Kinnersley N, Mills RG & Straus SE; Effects of the oral neuraminidase inhibitor GS 4104 on cytokine responses during experimental human influenza A virus infection. *11th Conference on Antiviral Research*, 5–11 April 1998, San Diego, California, USA, Late Breaker Session).

GS 4104 and zanamivir have also demonstrated efficacy in the treatment (Hayden *et al.*, 1997; Silagy CA, Campion CJ & Keene O; The efficacy and safety of Zanamivir in the treatment of influenza in otherwise healthy and "high risk" individuals. *38th Interscience Conference of Antimicrobial Agents and Chemotherapy*, 24–27 September 1998, San Diego, California, USA, Abstract H-56); Aoki F, Oershaus A, Rimmelzwaan G, Kinnersley N & Ward P; Oral GS 4104 successfully reduces duration and severity of naturally acquired influenza. *38th Interscience Conference of Antimicrobial Agents and Chemotherapy*, San Diego, California, USA, 24–27 September 1998, Abstract pp. 24–27; Hayden *et al.*, 1998c) and prevention (Monto AS, Robinson DP, Herlocher L, Hinson JM, Elliott M & Keene O; Efficacy and safety of Zanamivir in prevention of influenza among healthy adults. *38th Interscience Conference of Antimicrobial Agents and Chemotherapy*, 24–27 September 1998, San Diego, California, USA, Late Breaker Session; Hayden *et al.*, 1998a) of naturally acquired influenza virus infection. In these multicentre trials, both compounds reduced the duration and severity of clinical symptoms when a 5 day treatment regimen was started after the onset of influenza symptoms. An important finding from these studies is that oral GS 4104 treatment caused a reduction in the incidence of secondary complications due to influenza infection even though this study was carried out in otherwise healthy volunteers. Zanamivir treatment caused a reduc-

tion in the incidence of secondary complications in patients considered to be at high risk of developing complications (Silagy CA, Campion CJ & Keene O; The efficacy and safety of Zanamivir in the treatment of influenza in otherwise healthy and "high risk" individuals. *38th Interscience Conference of Antimicrobial Agents and Chemotherapy*, 24–27 September 1998, San Diego, California, USA, Abstract H-56). In separate studies, the two compounds were 67–84% effective at preventing influenza virus infection during trial periods in which susceptible subjects took once or twice daily oral doses of 75 mg GS 4104 for 6 weeks or once daily doses of 10 mg zanamivir by inhalation for 4 weeks.

## Conclusion

At present, there are no effective anti-influenza virus agents available for the treatment and prevention of human influenza infection. Influenza virus NA has proved to be a valid target for the development of anti-influenza virus drugs, as demonstrated by efficacy of zanamivir and GS 4104 in human clinical trials. Although it remains to be further investigated, the resistance development from treatment with NA inhibitors appears to not be clinically significant. Thus, in the future, it is likely that influenza NA inhibitors being developed as inhaled or oral agents will find wide use for the treatment and prophylaxis of human influenza virus infection.

## References

- Bamford MJ, Pichel JC, Husman W, Patel B, Storer R & Weir NG (1995) Synthesis of 6-, 7- and 8- carbon sugar analogues of potent anti-influenza 2, 3-dihydro-2, 3-dideoxy-N-acetylneuramic acid derivatives. *Journal of the Chemical Society, Perkin Transcripts I*: 1181–1187.
- Blick TJ, Tiong T, Sahasrabudhe A, Varghese JN, Colman PM, Hart GJ, Bethell RC & McKimm-Breschkin JL (1995) Generation and characterization of an influenza virus neuraminidase variant with decreased sensitivity to the neuraminidase-specific inhibitor 4-guanidino-Neu5Ac2En. *Virology* **214**:475–484.
- Bossart-Whitaker P, Carson M, Babu YS, Smith CD, Laver WG & Air GM (1993) Three-dimensional structure of influenza A N9 neuraminidase and its complex with the inhibitor 2-deoxy-2,3-dehydro-N-acetyl neuraminic acid. *Journal of Molecular Biology* **232**:1069–1083.
- Burmeister WP, Ruigrok RW & Cusack S (1992) The 2.2 Å resolution crystal structure of influenza B neuraminidase and its complex with sialic acid. *EMBO Journal* **11**:49–56.
- Chand P, Babu YS, Bantia S, Chu N, Cole B, Kotian PL, Laver WG, Montgomery JA, Pathak VP, Petty SL, Shrout DP, Walsh DA & Walsh GM (1997) Design and synthesis of benzoic acid derivatives as influenza neuraminidase inhibitors using structure-based drug design. *Journal of Medicinal Chemistry* **40**:4030–4052.
- Colacino JM, Chirgadze NY, Garman E, Murti KG, Loncharich RJ, Baxter AJ, Staschke KA & Laver WG (1997) A single sequence change destabilizes the influenza virus neuraminidase tetramer. *Virology* **236**:66–75.
- Colman PM (1994) Influenza virus neuraminidase: structure, antibodies and inhibitors. *Protein Science* **3**:1687–1696.
- Couch RB, Keitel W, Cate TR, Quarles JA, Taber LA & Glezen WP (1996) Prevention of influenza virus infections by current inactivated influenza virus vaccines. In *Options for the Control of Influenza III*, pp. 97–106. Edited by LE Brown, AW Hampson & RG Webster. Amsterdam: Elsevier.
- Dowle MD & Howes PD (1998) Recent advances in sialidase inhibitors. *Expert Opinions on Therapeutic Patents* **8**:1461–1478.
- Eisenberg EJ, Bidgood A & Cundy KC (1997) Penetration of GS 4071, a novel influenza inhibitor, into rat bronchoalveolar lining fluid following oral administration of the prodrug, GS 4104. *Antimicrobial Agents and Chemotherapy* **41**: 1949–1952.
- Goto H, Bethell RC & Kawaoka Y (1997) Mutations affecting the sensitivity of the influenza virus neuraminidase to 4-guanidino-2,4-dideoxy-2,3-dehydro-N-acetylneuraminic acid. *Virology* **238**:265–272.
- Gubareva LV, Bethell R, Hart GJ, Murti KG, Penn CR & Webster RG (1996) Characterization of mutants of influenza A virus selected with the neuraminidase inhibitor 4-guanidino-Neu5Ac2En. *Journal of Virology* **70**:1818–1827.
- Gubareva LV, Robinson MJ, Bethell RC & Webster RG (1997) Catalytic and framework mutations in the neuraminidase active site of influenza viruses that are resistant to 4-guanidino-Neu5Ac2En. *Journal of Virology* **71**:3385–3390.
- Gubareva LV, Matrosovich MN, Brenner MK, Bethell RC & Webster RG (1998) Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. *Journal of Infectious Diseases* **178**:1257–1262.
- Hay AJ, Wolstenholme AJ, Skehel JJ & Smith MH (1985) The molecular basis of the specific anti-influenza action of amantadine. *EMBO Journal* **4**:3021–3024.
- Hayden FG & Couch RB (1992) Clinical and epidemiological importance of influenza A viruses resistant to amantadine and rimantadine. *Reviews in Medical Virology* **2**:89–96.
- Hayden FG & Rollins BS (1997) In vitro activity of the neuraminidase inhibitor GS4071 against influenza viruses. *Antiviral Research* **34**:A86, Abstract 159
- Hayden FG, Osterhaus AD, Treanor JJ, Fleming DM, Aoki FY, Nicholson KG, Bohnen AM, Hirst HM, Keene O & Wightman K (1997) Efficacy and safety of the neuraminidase inhibitor zanamivir in the treatment of influenza virus infections. *New England Journal of Medicine* **337**:874–880.
- Hayden FG, Atmar R, Schilling M, Johnson C, Portez D, Parr D, Huson L, Wand P and Mills R (1998a) Safety and efficacy of oral GS 4104 in longterm prophylaxis of natural influenza. *Journal of the American Medical Association* **281**: In press
- Hayden FG, Treanor JJ, Betts RF, Lobo M, Esinhart JD & Hussey EK (1998b) Safety and efficacy of the neuraminidase inhibitor GG167 in experimental human influenza. *Journal of the American Medical Association* **276**:295–299.
- Hayden FG, Treanor JJ, Fritz RS, Lobo M, Betts RF, Miller M, Kinnersley N, Mills RG, Ward P & Straus SE (1998c) Use of the oral neuraminidase inhibitor oseltamivir in experimental human influenza: Randomized, controlled trials for prevention and treatment. *Journal of the American Medical Association* **281**: In press
- Holzer CT, von Itzstein M, Jin B, Pett MS, Stewart WP & Wu WY (1993) Inhibition of sialidase from viral, bacterial and mammalian sources by analogues of 2-deoxy-2, 3-didehydro-N-acetylneuramic acid modified at the C-4 position. *Glycoconjugates Journal* **10**:40–44.
- Kim CU, Lew W, Williams MA, Liu H, Zhang L, Swaminathan S, Bischofberger N, Chen MS, Mendel DB, Tai CY, Laver WG & Stevens RC (1997) Influenza neuraminidase inhibitors possessing a novel hydrophobic interaction in the enzyme active site: design,

- synthesis and structural analysis of carbocyclic sialic acid analogues with potent anti-influenza activity. *Journal of the American Chemical Society* **119**:681–690.
- Kim CU, Lew W, Williams MA, Wu H, Zhang L, Chen X, Escarpe PA, Mendel DB, Laver WG & Stevens RC (1998) Structure-activity relationship studies of novel carbocyclic influenza neuraminidase inhibitors. *Journal of Medicinal Chemistry* **41**:2451–2460.
- Klenk H-D & Rott R (1988) The molecular biology of influenza virus pathogenicity. *Advances in Virus Research* **34**:247–280.
- Lew W, Williams MA, Mendel DB, Escarpe PA & Kim CU (1997) C<sub>3</sub>-Thia and C<sub>3</sub>-Carba isosteres of a carbocyclic influenza neuraminidase inhibitor (3R, 4R, 5S)-4-acetamido-5-amino-3-propoxy-1-cyclohexene-1-carboxylic acid. *Bioorganic and Medicinal Chemistry Letters* **7**:1843–1846.
- Lew W, Wu H, Mendel DB, Escarpe PA, Chen X, Laver WG, Graves B & Kim CU (1998) A new series of C<sub>3</sub>-Aza carbocyclic influenza neuraminidase inhibitors: synthesis and inhibitory activity. *Bioorganic and Medicinal Chemistry Letters* **8**:3321–3324.
- Li W-X, Escarpe PA, Eisenberg EJ, Cundy KC, Sweet C, Jakeman KJ, Merson J, Lew W, Williams M, Zhang L, Kim CU, Bischofberger N, Chen MS & Mendel DB (1998) Identification of GS 4104 as an orally bioavailable prodrug of the influenza virus neuraminidase inhibitor GS 4071. *Antimicrobial Agents and Chemotherapy* **42**:647–653.
- Lipinski CA, Lombardo F, Dominy BW & Feeney PJ (1997) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews* **23**:3–25.
- Liu C, Eichelberger MC, Compans RW & Air GM (1995) Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly or budding. *Journal of Virology* **69**:1099–1106.
- McKimm-Breschkin JL, Blick TJ, Sahasrabudhe A, Tiong T, Marshall D, Hart GJ, Bethell RC & Penn CR (1996a) Generation and characterization of variants of NWS/G70C influenza virus after in vitro passage in 4-amino-Neu5Ac2En and 4-guanidino-Neu5Ac2En. *Antimicrobial Agents and Chemotherapy* **40**:40–46.
- McKimm-Breschkin JL, McDonald M, Blick TJ & Colman PM (1996b) Mutation in the influenza virus neuraminidase gene resulting in decreased sensitivity to the neuraminidase inhibitor 4-guanidino-Neu5Ac2en leads to instability of the enzyme. *Virology* **225**:240–242.
- McKimm-Breschkin JL, Sahasrabudhe A, Blick TJ, McDonald M, Colman PM, Hart GJ, Bethell RC & Varghese JN (1998) Mutations in a conserved residue in the influenza virus neuraminidase active site decreases sensitivity to Neu5Ac2en-derived inhibitors. *Journal of Virology* **72**:2456–2462.
- Meanwell NA & Krystal M (1996) Taking aim at a moving target - inhibitors of influenza virus. Part 2: Viral replication, packaging and release. *Drug Discovery Today* **1**:388–397.
- Meindl P, Bodo G, Palese P, Schulman J & Tuppy H (1974) Inhibition of neuraminidase activity by derivatives of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid. *Virology* **58**:457–463.
- Mendel DB & Roberts NA (1998) In vitro and in vivo efficacy of influenza neuraminidase inhibitors. *Current Opinion in Infectious Diseases* **11**:727–732.
- Mendel DB & Sidwell RW (1998) Influenza virus resistance to neuraminidase inhibitors. *Drug Resistance Updates* **1**:184–189.
- Mendel DB, Tai CY, Escarpe PA, Li W, Sidwell RW, Huffman JH, Sweet C, Jakeman KJ, Merson J, Lacy SA, Lew W, Williams MA, Zhang L, Chen MS, Bischofberger N, Kim CU (1998) Oral administration of a prodrug of the influenza neuraminidase inhibitor GS 4071 protects mice and ferrets against influenza infection. *Antimicrobial Agents and Chemotherapy* **42**:640–646.
- Monto AS & Arden NH (1992) Implications of viral resistance to amantadine in control of influenza A. *Clinical Infectious Diseases* **15**:362–367.
- Palese P, Tobita K, Ueda M & Compans RW (1974) Characterization of temperature sensitive influenza virus mutants. *Virology* **61**:397–410.
- Palese P & Compans RW (1976) Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA): mechanism of action. *Journal of General Virology* **33**:159–163.
- Penn CR, Barnett JM, Bethell RC, Fenton R, Gearing KL, Healy N & Jowett AJ (1996) Selection of influenza virus with reduced sensitivity *in vitro* to the neuraminidase inhibitor GG167 (4-guanidino-Neu5Ac2en): changes in haemagglutinin may compensate for loss of neuraminidase activity. In *Options for the Control of Influenza III*, pp. 735–740. Edited by LE Brown, AW Hampson & RG Webster. Elsevier: Amsterdam, The Netherlands.
- Ryan DM, Ticehurst J, Dempsey MH & Penn CR (1994) Inhibition of influenza virus replication in mice by GG167 (4-guanidino-2,4-dideoxy-2,3-dehydro-N-acetylneuraminic acid) is consistent with extracellular activity of viral neuraminidase (sialidase). *Antimicrobial Agents and Chemotherapy* **38**:2270–2275.
- Ryan DM, Ticehurst J & Dempsey MH (1995) GG167 (4-guanidino-2,4-dideoxy-2,3-dehydro-N-acetylneuraminic acid) is a potent inhibitor of influenza virus in ferrets. *Antimicrobial Agents and Chemotherapy* **39**:2583–2584.
- Sahasrabudhe A, Blick T & McKimm-Breschkin J (1996) Influenza virus variants resistant to GG167 with mutations in the haemagglutinin. In *Options for the Control of Influenza III*, pp. 748–752. Edited by LE Brown, AW Hampson & RG Webster. Australia: Elsevier Science B.V.
- Sidwell RW, Huffman JH, Barnard DL, Bailey KW, Wong M-H, Morrison A, Syndergaard T & Kim CU (1998) Inhibition of influenza virus infections in mice by GS4104, an orally effective influenza neuraminidase inhibitor. *Antiviral Research* **37**:107–120.
- Sollis SL, Smith PW, Howes PD, Cherry PC & Bethell RC (1996) Novel inhibitors of influenza sialidase related to GG167. *Bioorganic and Medicinal Chemistry Letters* **6**:1805–1808.
- Staschke KA, Colacino JM, Baxter AJ, Air GM, Bansal A, Hornback WJ, Munroe JE & Laver WG (1995) Molecular basis for the resistance of influenza viruses to 4-guanidino-Neu5Ac2En. *Virology* **214**:642–646.
- Tai CY, Escarpe PA, Sidwell RW, Williams MA, Lew W, Wu H, Kim CU & Mendel DB (1998) Characterization of human influenza virus variants selected in vitro in the presence of the neuraminidase inhibitor GS 4071. *Antimicrobial Agents and Chemotherapy* **42**:3234–3241.
- Taylor NR & von Itzstein M (1994) Molecular modeling studies on ligand binding to sialidase from influenza virus and the mechanism of catalysis. *Journal of Medicinal Chemistry* **37**:616–624.
- Taylor, NR, Cleasby A, Singh O, Skarzyski T, Wonacott AJ, Smith PW, Sollis SL, Howes PD, Cherry PC, Bethell R, Colman P & Varghese J (1998) Dihydropyran carboxamides related to zanamivir: a new series of inhibitors of influenza virus sialidases. 2. crystallographic and molecular modeling study of complexes of 4-amino-4H-pyran-6-carboxamides and sialidase from influenza virus types A and B. *Journal of Medicinal Chemistry* **41**:798–807.
- Tedrzejas MJ, Singh S, Brouillette WJ, Laver GW, Air GM & Luo M (1995) Structure of aromatic inhibitors of influenza virus neuraminidase. *Biochemistry* **34**:3144–3151.
- Varghese JN, McKimm-Breschkin JL, Caldwell JB, Kortt AA & Colman PM (1992) The structure of the complex between influenza virus neuraminidase and sialic acid, the viral receptor. *Proteins* **14**:327–332.
- Varghese JN, Smith PW, Sollis SL, Blick TJ, Sahasrabudhe A,

McKimm-Breschkin JL & Colman PM (1998) Drug design against a shifting target: a structural basis for resistance to inhibitors in a variant of influenza virus neuraminidase. *Structure* **6**:735–746.

von Itzstein M, Wu WY, Kok GB, Pegg MS, Dyason JC, Jin B, Phan TV, Smyth ML, White HF, Oliver SW, Colman PM, Varghese JN, Ryan DM, Wood JM, Bethell RC, Hotham VJ,

Cameron JM & Penn CR (1993) Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature* **363**:418–423.

Williams MA, Bischofberger N, Swaminathan S & Kim, CU (1995) Synthesis and influenza neuraminidase inhibitory activity of aromatic analogues of sialic acid. *Bioorganic and Medicinal Chemistry Letters* **5**:2251–2254.

---

Received 11 March 1999; accepted 23 April 1999