

Medicinal Chemistry Feature Molecule

Discovery and Development of GS 4104 (oseltamivir): An Orally Active Influenza Neuraminidase Inhibitor

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Abstract: Rational drug design utilizing available X-ray crystal structures of sialic acid analogues bound to the active site of influenza virus neuraminidase has led to the discovery of a series of potent carbocyclic influenza neuraminidase inhibitors. From this series, GS 4104 (oseltamivir, TAMIFLU™) has emerged as a promising antiviral for the treatment and prophylaxis of human influenza infection. This article will summarize the design, discovery, and development of oseltamivir as an oral therapeutic to treat influenza infection.

Introduction

Influenza remains a very serious respiratory illness with annual epidemics producing significant morbidity and mortality. At the present time, options for controlling influenza infection are limited. Protection through vaccination is limited due to the antigenic variation of the influenza virus. Amantadine and the chemically related rimantadine are two antiviral agents currently approved for the prevention and treatment of influenza A virus infections. However, these two drugs are limited by adverse side effects, their lack of effectiveness against influenza B viruses [1], and the rapid development of viral resistance. As a result, there is an urgent need for safe and effective antiviral agents for the treatment of influenza infected individuals.

The majority of research efforts aimed at identifying suitable molecular targets for antiviral intervention are focused on the influenza neuraminidase, one of two major glycoproteins located on the influenza virus membrane envelope [2, 3]. This enzyme is responsible for the cleavage of terminal sialic acid residues from glycoconjugates and is essential for virus replication and infectivity [4-7]. Early efforts to identify influenza neuraminidase inhibitors were met with reasonable success. The most potent inhibitors exhibited low μM activity for enzyme inhibition and virus replication *in vitro* [8]. However, these compounds were not specific and exhibited comparable activity against other viral,

bacterial, and mammalian neuraminidases. In addition, they did not exhibit inhibitory activity in animal models of influenza virus infection.

By utilizing rational drug design in conjunction with available high resolution X-ray crystal structures of sialic acid (**3**) and the transition state analog Neu5Ac2en (**4**) bound to influenza A and B neuraminidases [9-11], researchers were able to design potent inhibitors of the influenza neuraminidase enzyme. To date, this approach has led to the development of several potent and specific influenza neuraminidase inhibitors. Most notably, oseltamivir (**9**) (GS 4104, the oral prodrug of GS 4071 (**8**)) [12, 13] and zanamivir (**6**) (GG167) [11]. This paper will describe the design, discovery, and development of oseltamivir.

Influenza Neuraminidase X-Ray Crystal Structures

High resolution crystal structures of influenza neuraminidase and its complex with sialic acid (**3**) (neuraminidase's catalytic product) and other transition state analogues reveal several common structural features among all influenza neuraminidases. In particular, the neuraminidase active site contains several large well-defined pockets. All residues which make direct contact with the substrate are strictly conserved among both influenza A and B neuraminidases. These residues interact similarly with both substrate and inhibitor molecules, and, not surprisingly, the neuraminidase active site contains a rather large number of polar or charged residues since its carbohydrate substrates are quite polar.

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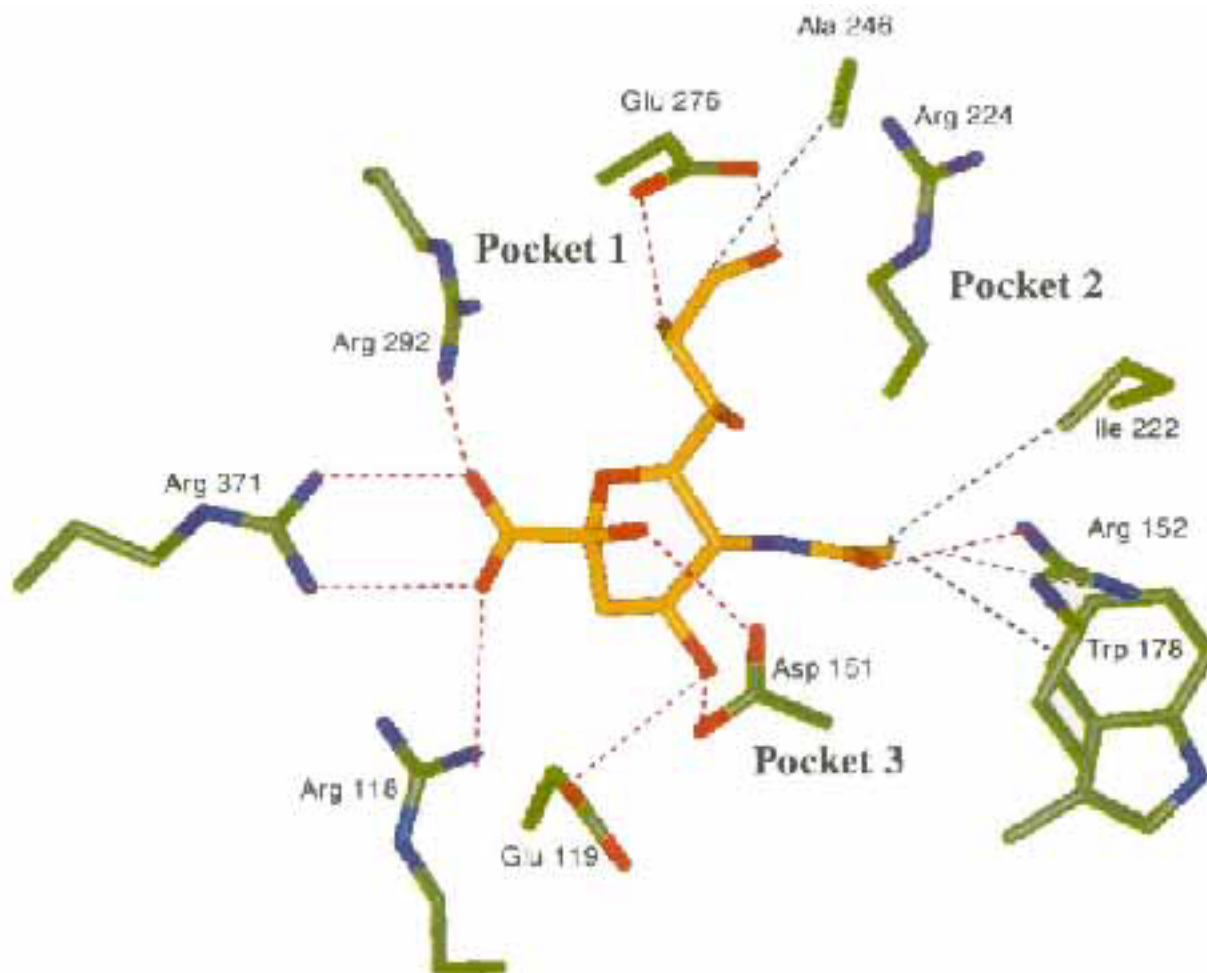


Fig. (1). Key interactions between neuraminidase active site residues (only side chains are shown) and sialic acid. Atoms are colored as following: red for oxygen, blue for nitrogen, green for NA carbon, brown for sialic acid carbon. Dashes indicate H-bonding (red) and hydrophobic (black) interactions. Some residues are omitted for clarity.

Detailed analysis of available X-ray crystal structures indicates several common critical interactions between neuraminidase and its inhibitors. For example, the negatively charged carboxylate group of sialic acid makes strong charge-charge interactions with the positively charged side chains of the Arg triad (Arg 118, 292, and in particular 371) of neuraminidase as shown in Fig. (1). Furthermore, the N-acetyl group of sialic acid makes both polar and nonpolar contacts with Arg152, Trp178, and Ile222. These two interactions help to anchor the scaffold of sialic acid in the active site of neuraminidase, thus laying a structural foundation for introducing additional interactions which could lead to more potent inhibitors.

The neuraminidase active site can be divided into three major binding pockets. The highly polar residues Glu 276, Glu 277, Arg 292, Asn 294, and hydrophobic Ala 246 form Pocket 1. Although this pocket appears to be highly polar in nature, as evidenced by its

interactions with the glycerol moiety of sialic acid, novel nonpolar interactions have been discovered and prove to be the key in achieving high binding affinity with cyclohexene based neuraminidase inhibitors [12]. The X-ray crystal structure also reveals the presence of a well-formed hydrophobic pocket which is not utilized by sialic acid for binding. This pocket (Pocket 2) consists of the highly conserved amino acid residues Ile 222, Arg 224, and Ala 246. The cyclohexene series of inhibitors takes advantage of this pocket and forms favorable hydrophobic interactions with residues in this pocket [12]. The third binding pocket (Pocket 3) is large and contains both hydrophobic and hydrophilic residues (Glu 119, Asp 151, Arg 152, Trp 178, Ser 179, Ile 222, and Glu 227). The C-4 hydroxyl and the C-5 N-acetyl groups of sialic acid bind in this pocket but do not fully interact with all of the residues in the binding pocket. In particular, there is a cluster of negatively charged residues (Glu 119, Glu 227, and Asp 151) near the C-4 hydroxyl group of sialic acid

which could be further explored for possible charge-charge interactions.

The Design of Influenza Neuraminidase Inhibitors

The X-ray crystal structure of the sialic acid/neuraminidase complex reveal that sialic acid binds to the enzyme in a considerably deformed conformation due to the strong ionic interactions of the sialic acid carboxyl moiety with the Arg 118, 292, and 371 triad. This binding mode is very similar to that found in the X-ray crystal structure of Neu5Ac2en (**4**) complexed to neuraminidase. Namely, the double bond of **4** constrains the pyranose ring into a planar structure around the ring oxygen. Based on structural information and biochemical mechanistic studies, it has been proposed that the catalytic mechanism for the neuraminidase cleavage of sialic acid from glycoconjugate **1** proceeds via transition-state **2** (Scheme 1). Efforts aimed towards mimicking transition-state **2** have resulted in the design and synthesis of a variety of transition-state analogues based on a dihydropyran ring (**4-6**), benzene ring (**7**) [14, 15], and cyclohexene ring (**8**).

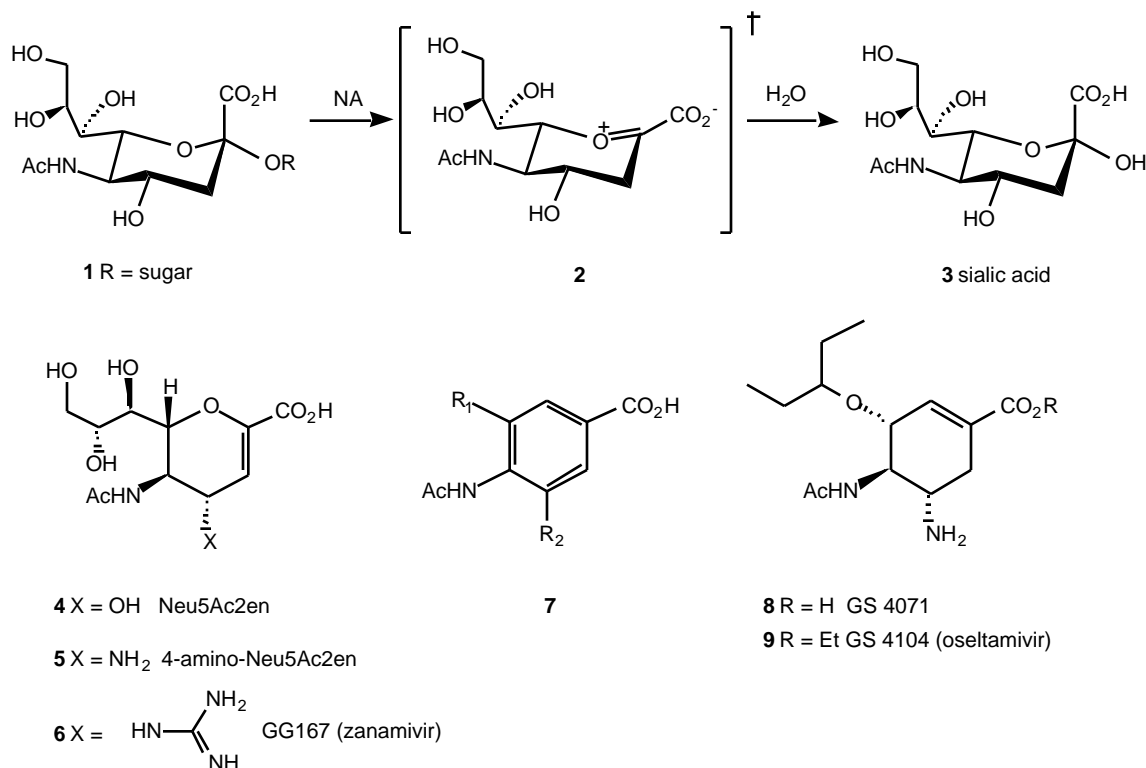
Sialic Acid-Based Influenza Neuraminidase Inhibitors

Based on information derived from the available X-ray crystal structures of sialic acid and its analogues

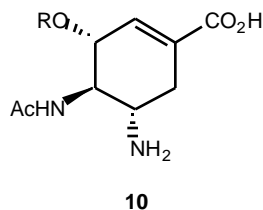
bound with neuraminidase, inhibitors **5** and **6** were rationally designed and synthesized [11, 16]. Compounds **5** and **6** exhibited potent neuraminidase inhibitory activity with K_i values of 10^{-8} M and 10^{-10} M, respectively. Consistent with its potent *in vitro* inhibitory activity, **6** (zanamivir) exhibited potent antiviral activity against a variety of influenza A and B strains in cell culture and demonstrated *in vivo* efficacy in the influenza infected animal models via intranasal administration [11]. Currently zanamivir, marketed as Relenza[®], is approved for the treatment of influenza infection in the U.S., Europe and Australia and is administered via inhalation.

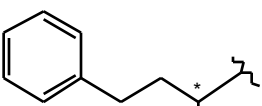
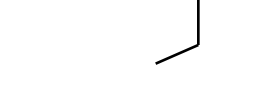
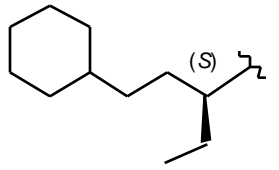
Inhibitor Design Based on a Cyclohexene Scaffold

In the search for potent, orally active neuraminidase inhibitors, an approach based on a cyclohexene scaffold was investigated [12, 17]. The double bond of the cyclohexene ring was considered as an isostere of the flat oxonium cation shown in transition-state **2** (Scheme 1). The cyclohexene ring is expected to be chemically and enzymatically stable and provide a stable template for chemical modifications of substituents for optimizing biological activity. X-ray crystallographic studies of sialic acid and related analogues bound to neuraminidase revealed that the C-7 hydroxyl of the glycerol side chain does not interact with amino acid residues in the neuraminidase active site [18]. Taking this into consideration, an oxygen



Scheme 1.

Table 1. Influenza Neuramindase Inhibition

Compound	R	Enzyme IC ₅₀ (nM)	
		flu A ^a	flu B ^b
11	H-	6300	ND ^c
12	CH ₃ -	300	ND ^c
13	CH ₃ CH ₂ -	2000	185
14	CH ₃ (CH ₂) ₂ -	180	15
15	CH ₃ (CH ₂) ₃ -	300	215
16	CH ₃ (CH ₂) ₅ -	150	1450
8	(CH ₃ CH ₂) ₂ CH-	1	3
17	(S) 	0.3	70
18	(R) 	12	35
19	(S) 	1	2150

^aA/PR/8/34 (H1N1); ^bB/Lee/40; ^cnot determined

atom was incorporated as a replacement for the C-7 hydroxy methylene unit. In addition, this would allow for the preparation of a variety of alkyl side chain analogues, as shown in structure **10** (Table 1). Although the C-8 and C-9 hydroxyl groups of the sialic acid glycerol side chain in the neuraminidase complex form a bidentate interaction with Glu 276, X-ray crystal structures indicate that the C-9 carbon of the sialic acid glycerol side chain makes hydrophobic contacts with the hydrocarbon chain of Arg 224. Therefore, optimization of this hydrophobic interaction was systematically investigated.

In the design of orally active antivirals, we feel that balancing lipophilicity and water solubility is as important as the size of the drug molecule for oral absorption from the intestinal tract [19]. Consequently, it was important to increase the overall lipophilicity of the molecule to

balance the effects of the polar functional groups present in the molecule (*i.e.*, the C-1-carboxylate acid, C-4-acetamide and C-5-amino groups which are essential for potent inhibitory activity). Namely, the addition of a lipophilic alkyl group (**R** in **10**) would potentially enhance overall oral bioavailability.

Discovery of GS 4071

As shown in Table 1, the length, substitution, and geometry of the C-3 alkyl side chain in **10** profoundly influenced neuraminidase inhibitory activity [12, 17]. A greater than 20-fold increase in inhibitory activity of *n*-propyl analogue **14** compared to that of methyl analogue **12** implicated a significant hydrophobic interaction of the *n*-propyl group with either Pocket 1 or 2. Further extension of the alkyl chain beyond *n*-propyl

did not improve flu A inhibitory activity but adversely affected flu B inhibitory activity. Branching of the *n*-propyl group adjacent to the ether oxygen led to a significant increase in potency, as demonstrated by the 3-pentyl analogue **8** (GS 4071).

The X-ray crystal structure of **8** bound to neuraminidase (Fig. (2)) [12] indicates that the binding mode for this series of carbocyclic inhibitors is similar to that of 4-amino-Neu5Ac2en (**5**). Namely, the corresponding C-1 carboxylate, C-4 acetyl, and C-5 amino groups of **8** and **5** interact with the same active site amino acid residues upon binding in the neuraminidase active site. More importantly, the X-ray crystal structure of **8** reveals that the 3-pentyl side chain binds in an extended conformation and forms hydrophobic interactions previously not observed in the sialic acid/neuraminidase complex. One of the ethyl groups of the 3-pentyl side chain of **8** makes several favorable hydrophobic contacts with amino acid residues Ile 222, Arg 224, and Ala 246, resulting in a significant increase in binding affinity. This is not

entirely unexpected since hydrophobic interactions play a vital role in inhibitor binding.

A truly surprising discovery from this structure is the ability of the other ethyl group of the 3-pentyl side chain to bind in an apparently highly polar region (Pocket 1). Such binding becomes possible because the Glu 276 side chain can adopt two different conformations [12]. When sialic acid binds in the neuraminidase active site, the Glu 276 side chain adopts a conformation in which a bidentate hydrogen bonding interaction with the glycerol side chain of sialic acid is achieved; Whereas upon binding of **8** in the neuraminidase active site, the Glu 276 side chain rotates away from the center of pocket 1 and adopts an alternate conformation which is stabilized by a strong charge-charge interaction with the nearby guanidino group of Arg 224. Such a conformational change effectively enlarges Pocket 1 and creates a much less polar environment which can accommodate a hydrophobic binding group such as the ethyl group of the 3-pentyl side chain of **8**.

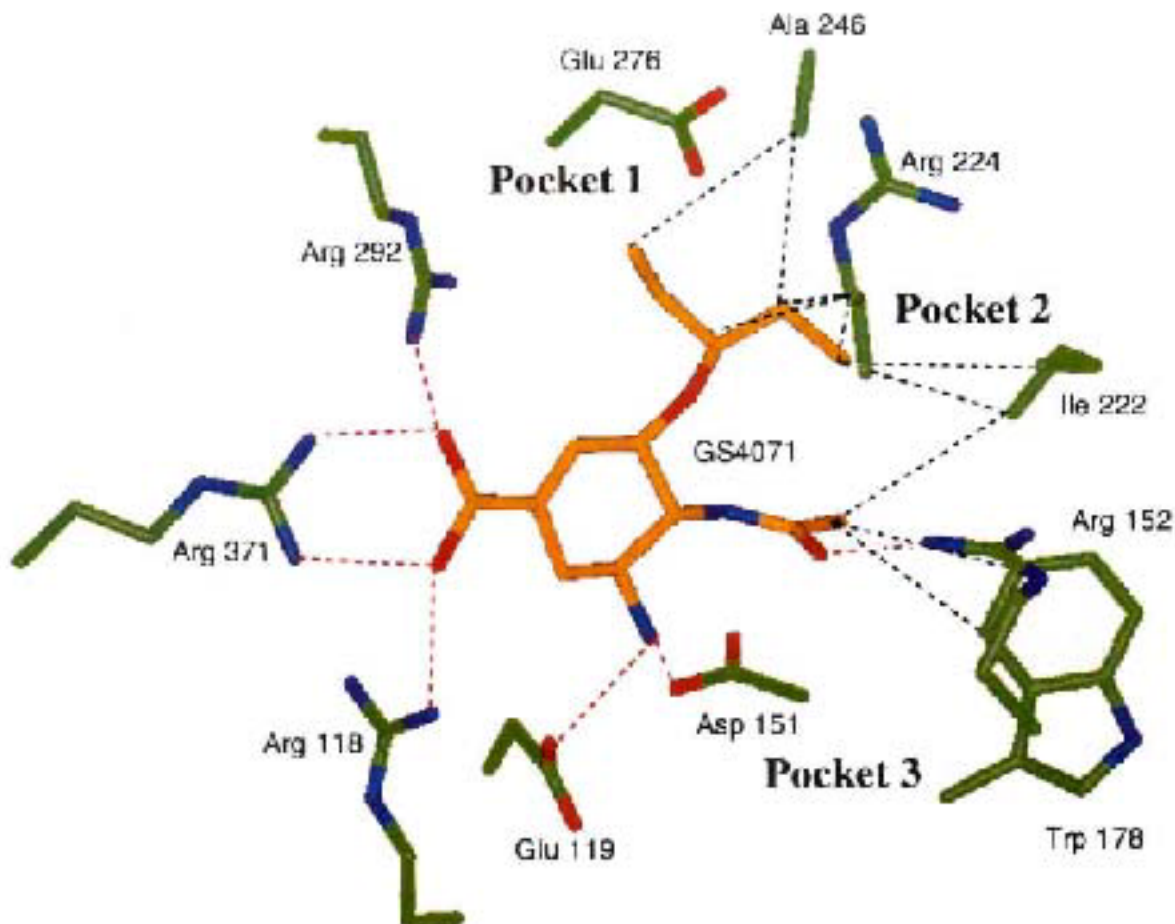


Fig. (2). GS 4071 (**8**) and neuraminidase X-ray crystal structure. Atoms are colored as following: red for oxygen, blue for nitrogen, green for NA carbon, brown for GS4071 carbon. Dashes indicate H-bonding (red) and hydrophobic (black) interactions, only side chains are shown, some active residues are omitted for clarity.

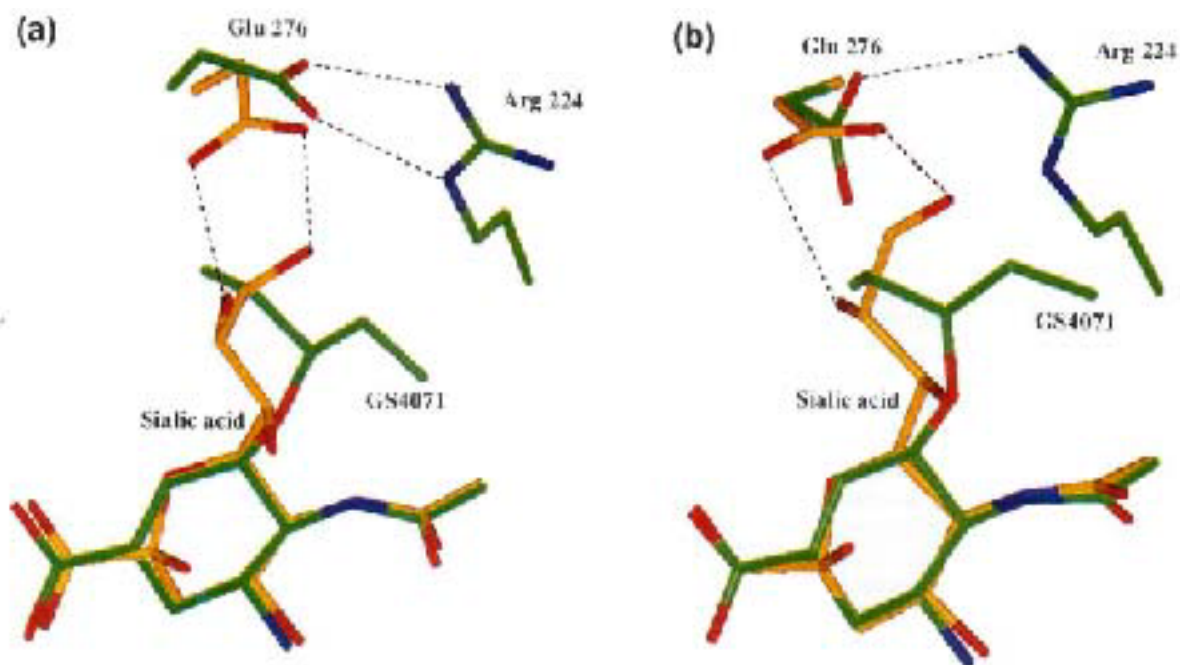


Fig. (3). Neuraminidase Glu 276 sidechain [(a) type A neuraminidase complex; (b) type B neuraminidase complex] can adopt alternative conformations upon binding of sialic acid or GS 4071. Dashes indicate H-bond. Atoms are colored as following: blue for nitrogen red for oxygen, brown for carbon in sialic acid complex, green for carbon in GS4071 complex.

Detailed comparison of the X-ray crystal structures of **8** complexed with type A neuraminidase and type B neuraminidase reveals some difference in how the inhibitor interacts with the neuraminidase enzyme. The Glu 276 of type B neuraminidase (using the numbering scheme for type A neuraminidase) undergoes a much smaller conformational change upon binding of **8** (Fig. 3b)). As a result, the size of the binding pocket does not change significantly. Consequently, its polar nature is not decreased sufficiently enough to allow optimum binding of one of the ethyl groups of the 3-pentyl side chain. This result suggests that type B neuraminidase depends more likely on Pocket 2 for achieving high binding affinity.

Structure Activity Relationship of Carbocyclic Inhibitors

Compounds **17** and **19** possess branched alkyl groups with essentially the same steric demands. The X-ray crystal structure of **17** (Fig. 4)) demonstrates that, given the (*S*) configuration of the C-3 side chain, the smaller ethyl group binds in Pocket 1 while the larger phenylethyl group occupies Pocket 2. Molecular modeling and analysis of X-ray crystal structures confirmed this observation, *i.e.*, Pocket 1 is smaller and cannot accommodate large binding groups like the phenyl ring, while Pocket 2 is larger and can accommodate larger binding groups. Therefore, it is likely that compound **19** shares the same binding

mode and places the larger group of the sidechain in Pocket 2. As the side chains of these inhibitors become bulkier, the binding affinity against type B influenza neuraminidase decreases significantly (Table 1). The cyclohexyl analogue (**19**) retained potent flu A inhibitory activity but exhibited a dramatic 1000-fold (30X compared to (**17**)) decrease in potency for flu B inhibition. These results suggest that Pocket 2 of influenza B neuraminidase is very sensitive to the size of inhibitor bound and binding affinity is significantly affected by the increased steric bulk of the C-3 side chain. Such an observation is consistent with our hypothesis that type B neuraminidase depends more on Pocket 2 interactions for binding.

The influence of the double bond position on neuraminidase enzyme inhibition is clearly demonstrated in the inhibitory activity of the series of analogues possessing the 3-pentyl side chain (Fig. 5)). The double bond isomer **20** exhibited a significant reduction in neuraminidase inhibitory activity, especially against flu B. A clear explanation as to the difference in binding modes of isomers **8** and **20** was not evident from molecular modeling and X-ray crystallographic analysis. Further structure-activity information was provided by diene analogue **21** and the saturated analogue **22** which exhibited dramatic decreases in inhibitory potencies for both flu A and B [20]. As suggested from these data, it appears that, in this series, isomer **8** is the ideal transition-state mimic.

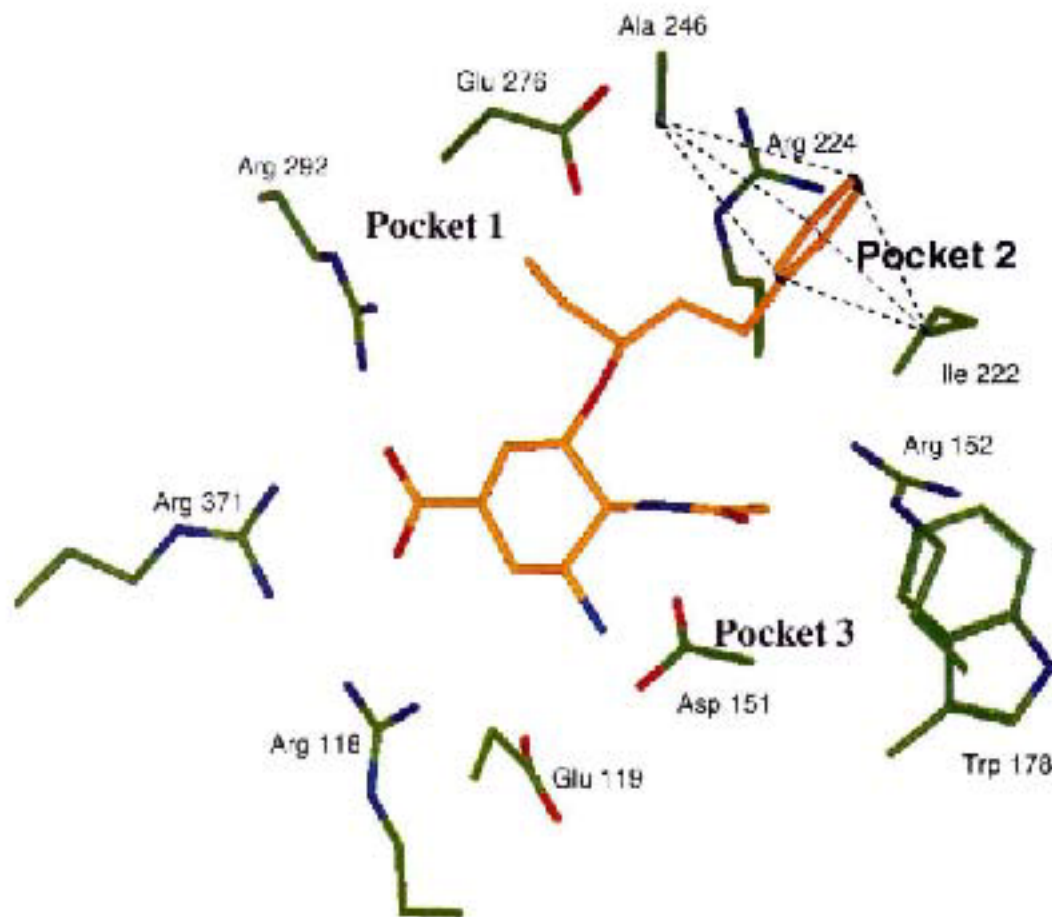


Fig. (4). Compound (17) complex with neuraminidase. Atoms are colored as following: red for oxygen, blue for nitrogen, green for NA carbon, brown for compound (17) carbon. Dashes indicate hydrophobic interactions in pocket 2.

As shown in Fig. (6), the guanidino analogue **24** of GS 4071 exhibited only a modest improvement in neuraminidase inhibitory activity. By comparison, the carbocyclic analogues are over 100-fold more active than the corresponding dihydropyran analogues in the series when X = OH (**23**) vs. **4** or NH₂ (**8**) vs. **5**. This result implies that the 3-pentyl group in the carbocyclic series contributes considerable binding energy via the network of hydrophobic interactions.

C-3- Isosteres of Carbocyclic Influenza Neuraminidase Inhibitors

Structure-activity relationship studies have demonstrated that the corresponding C-3-carba-, -thia-, and -aza isosteres (**25** - **27**), respectively, exhibit comparable influenza A inhibitory activity relative to the C-3-oxa analogue (Fig. (7)) [21]. In this series, the aza isostere was examined extensively [22] since it provided the opportunity to optimize binding interactions in Pockets 1 and 2 with different alkyl substituents on the C-3-nitrogen without the

introduction of an asymmetric center. The two compounds possessing a 3-pentyl side chain, **28** and **29**, are direct side chain analogues of GS 4071 and exhibit comparable influenza A neuraminidase inhibition to that of GS 4071 and reduced inhibitory activity against influenza B neuraminidase (Fig. (8)). Further investigation into the structure-activity relationship of this series of compounds is in progress and will be reported in due course.

In vivo Activity of GS 4104 (oseltamivir)

Although GS 4071 was designed with the intent of developing an orally active influenza neuraminidase inhibitor, pharmacokinetic experiments demonstrated that the oral bioavailability of GS 4071 was only ~5% in rats [13]. However, conversion of GS 4071 to the corresponding ethyl ester prodrug (**9**) (oseltamivir) resulted in a highly orally bioavailable form of GS 4071. The oral bioavailability in rats following oral administration of oseltamivir was found to be more than

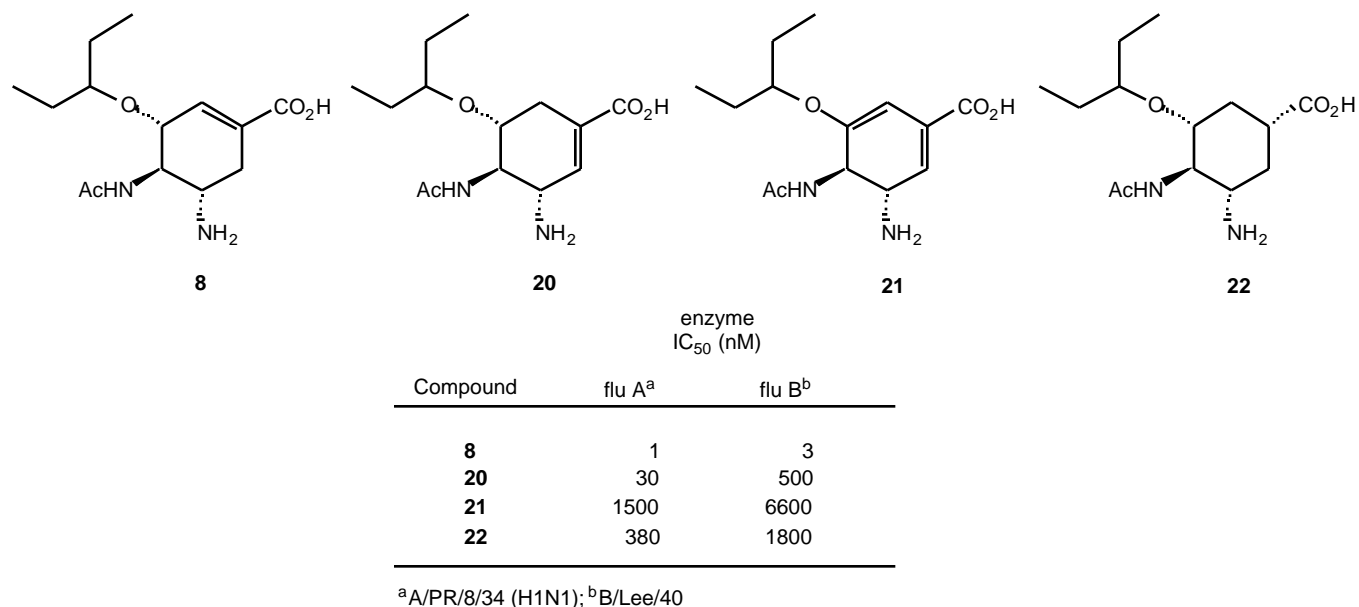


Fig. (5). Influenza neuraminidase inhibition of GS 4071 olefin isomers.

5-fold higher than that of the parent compound. In addition, a high concentration of GS 4071 in plasma following oral administration of oseltamivir was observed in mice (~30%), dogs (~70%) [13], and humans (~80%) [H. Wiltshire, Roche Products Limited; personal communication].

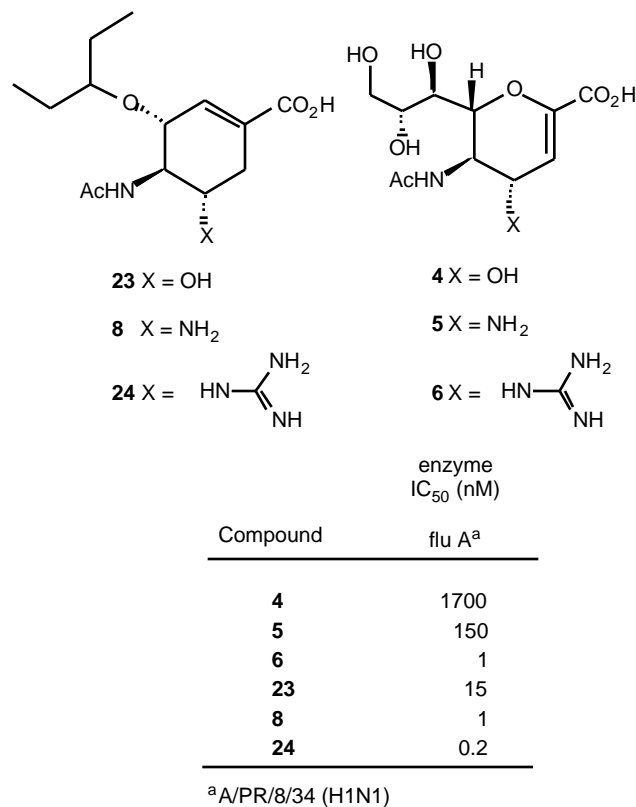


Fig. (6). Comparative influenza A neuraminidase inhibitory activity of cyclohexene and dihydropyran based scaffolds.

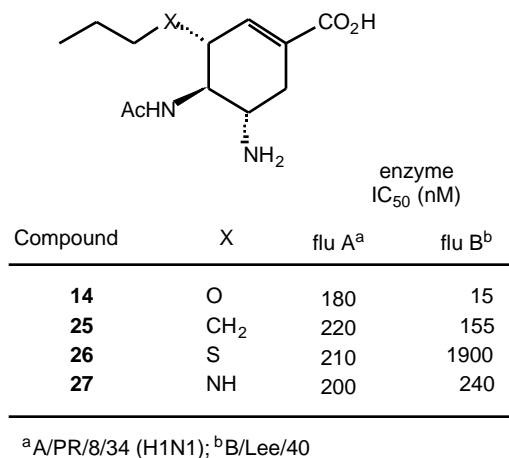


Fig. (7). Influenza neuraminidase inhibitory activity of C3-isosteres

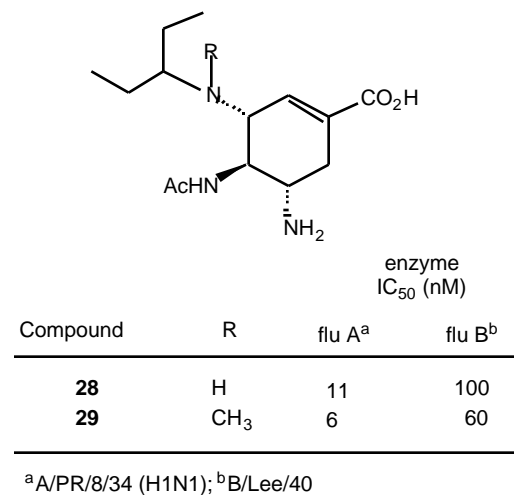


Fig. (8). Influenza neuraminidase inhibitory activity of C3-Aza analogues of GS 4071.

Animal Studies

Due to the poor oral bioavailability of GS 4071, *in vivo* efficacy in animal models has largely been conducted using oral administration of oseltamivir. Oral administration of oseltamivir has been shown to provide protection against the lethal effects of influenza A and B virus infection in mice [23, 24]. In addition to the mouse animal model, ferrets provide a useful experimental animal model in which to measure symptomatic and virological responses to treatment with neuraminidase inhibitors. When infected with the influenza virus, ferrets develop a disease state of limited duration similar to that seen in humans. Oral administration of oseltamivir twice a day for 5 days beginning 4 hours after infection was found to be effective in the ferret influenza model [24]. In addition, previous studies have demonstrated that after oral dosing of oseltamivir to rats, GS 4071 levels in the bronchoalveolar lining fluid reached maximum concentrations similar to that in plasma, and showed longer persistence [25]. This result indicated that oral administration of oseltamivir effectively delivers active drug to the respiratory tract in animals.

Clinical Studies

In prophylaxis models, oseltamivir [26] was effective in preventing experimental influenza virus infection in healthy volunteers when treatment began prior to a virus challenge. The amount of virus shedding, symptom severity, and symptom duration was significantly reduced when treatment of oseltamivir was initiated a day after infection with influenza virus in healthy volunteers [27]. In addition, oral administration of oseltamivir 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 [28].

Oseltamivir has also demonstrated efficacy in the treatment [26, 29, 30] and prevention [31] of naturally acquired influenza virus infection. In multi-center trials, oseltamivir reduced the duration and severity of clinical symptoms when a 5 day treatment regimen is started after the onset of influenza symptoms. An important finding from these studies is that oral treatment with oseltamivir caused a reduction in the incidence of secondary complications due to influenza infection even though this study was carried out in otherwise healthy volunteers. In separate prophylaxis studies, oseltamivir was 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 oseltamivir for 6 weeks.

Resistance to Influenza Neuraminidase Inhibitors

Several groups have isolated influenza variants with decreased susceptibility to influenza neuraminidase inhibitors following serial passage of virus in cell culture in the presence of inhibitor. Genotypic analysis of these variants has indicated that mutations in the hemagglutinin as well as the neuraminidase gene can cause reduced susceptibility to neuraminidase inhibitors in antiviral assays, with the hemagglutinin mutants being easier to generate. Although a number of studies indicate that neuraminidase mutants can be generated *in vitro*, they are likely to be compromised to replicate *in vivo*. For a thorough discussion of resistance to influenza neuraminidase inhibitors, refer to the cited references [32-38].

Conclusion

A rational approach to drug design in conjunction with available X-ray crystal structures was utilized in the discovery of potent inhibitors targeting influenza neuraminidase. In the past several years, influenza neuraminidase has been validated as a target for the development of anti-influenza drugs as demonstrated by the efficacy of zanamivir and oseltamivir. Although it remains to be further investigated, the resistance development from neuraminidase inhibitors appears to not be clinically significant. Oseltamivir (TAMIFLU™) was approved in October 1999 by the U.S. Food and Drug Administration for use as the first orally administered neuraminidase inhibitor for the treatment of influenza infection.

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