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Review Article

Recent developments in sialidase inhibitors for the treatment of influenza

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Introduction

Influenza is a serious respiratory illness producing significant morbidity and mortality (1-3). The familiar symptoms of the disease include cough, catarrh, headache, fever, chills and myalgia (muscle aches), and are characterized by their abrupt onset. The systemic nature of many of these symptoms distinguishes influenza from the “common cold” where effects are usually restricted to the upper airways. The disease is caused by a highly contagious airborne virus which replicates in the epithelial lining of the respiratory tract.

Over recent years increased understanding of the influenza virus replication cycle has led to the identification of several targets for therapeutic intervention (4-9). This review focusses exclusively on the viral sialidase, a glycohydrolase found on the surface of the virus which has emerged as a highly attractive target for drug design and chemotherapeutic intervention. The background to this area and the rational design of novel inhibitors have been described elsewhere in a series of recent articles (10-22) and will not be covered here. Following these efforts, two influenza virus sialidase inhibitors (zanamivir, Glaxo Wellcome/Biota and GS4104 Gilead/Roche) are now undergoing clinical evaluation. In this review we update on the progress in the development of these compounds and summarize recent work towards the discovery of new sialidase inhibitors which has occurred during 1997.

Clinical studies with zanamivir

Preclinical studies of zanamivir (GG167) demonstrating its efficacy against influenza viruses *in vitro* and *in vivo* have been reviewed recently in this journal (23). Pharmacokinetic studies in both animals and man have shown that the oral bioavailability of zanamivir is less than 10% in all species thus far studied, and the compound is therefore most active when administered directly to the sites of replication of the virus. The clinical trials of zanamivir have been designed to determine not only its safety and efficacy but also the most appropriate method to deliver compound to the respiratory tract.

Experimental influenza

The first reported studies of the safety and efficacy of zanamivir in man were conducted in volunteers who were experimentally inoculated with ~ 10^5 tissue culture infectious doses ($TCID_{50}$) of influenza A/Texas/91 (H1N1) using nasal drops (24). Unlike naturally acquired influenza, which commonly involves the lower respiratory tract, experimental infections caused by intranasal inoculation of virus are typically confined to the nasal passages. Zanamivir was therefore administered either by intranasal drops or by intranasal spray. In order to investigate the prophylactic efficacy of zanamivir, the drug was first administered 4 hours before infection with continuing administration for 5 days. When compared with patients receiving placebo, zanamivir was found to prevent viral shedding in 96% of patients (p <0.001) and prevent infection, as determined by a rise in hemagglutination-inhibition titers, in 82% of patients (p <0.001) across dosing regimens which varied between 16 mg 6 times daily and 3.6 mg twice daily. The level of efficacy was similar across all prophylactic regimens, although there was a trend toward lower efficacy in those patients who received the

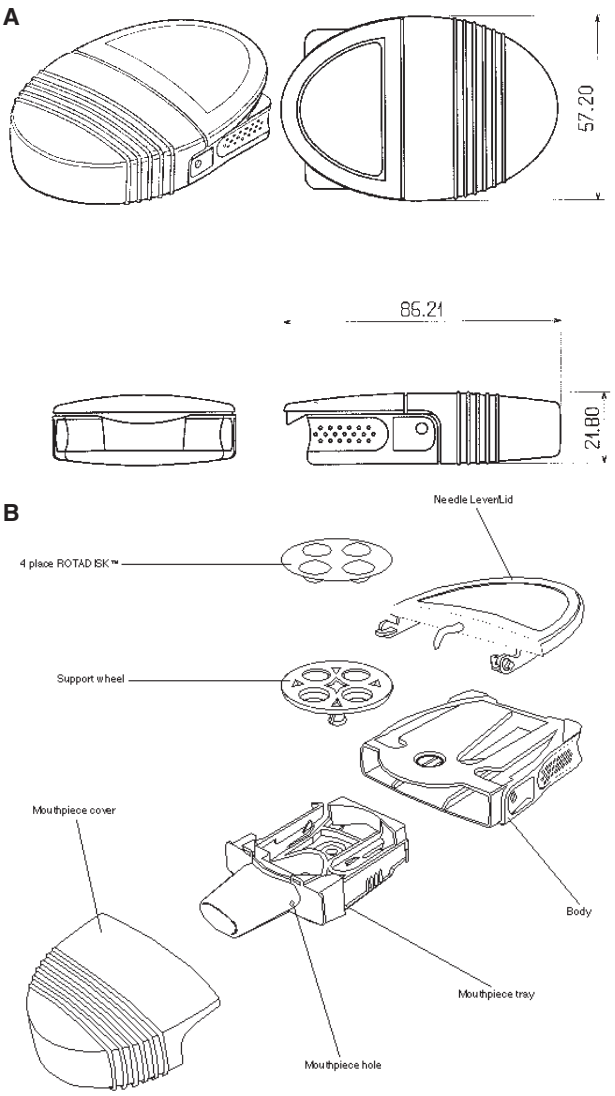


Fig. 1. A) The assembled Diskhaler (dimensions in mm); B) an exploded diagram of the Diskhaler.

drug by nasal spray rather than by drops. This trend was attributed to differences in distribution of the drug within the upper respiratory tract after the different methods of administration. Since the virus inoculum was administered to all patients in the form of drops, the trend toward higher efficacy when the drug was administered as drops may be the result of nasal drops affording better delivery to the sites of viral replication in the experimental infection model. Treatment dosing regimens, in which the first dose of zanamivir was administered after infection, were of 4 days duration, with patients receiving 16 mg zanamivir either 6 times daily or twice daily. Analysis was confined to those subjects with laboratory confirmed infection. Early treatment, defined as commencement of drug administration between 26 and 32 hours after infection, was associated with reductions in the duration of viral shedding of 3 days (p <0.001) and the viral titer AUC of 87% (p <0.001). In addition to an antiviral effect, early treatment led to a reduction in the occurrence of febrile illness of 84% (p <0.01) and to reductions in total symptom scores. Delayed treatment, defined as commencement of drug administration 50 hours after infection and when the majority of subjects were already ill, was also associated with statistically significant reductions in the duration and magnitude of viral shedding but without a statistically significant effect on symptoms. Zanamivir was well tolerated, with no adverse events or changes in laboratory measurements associated with the its use.

Zanamivir has also been shown to be effective against experimental human influenza B infection (25), as expected from its *in vitro* antiviral profile. Zanamivir (3.2 mg b.i.d. or 6.4 mg b.i.d.) administered by nasal spray resulted in statistically significant reductions in the numbers of virus-positive subjects (p <0.05) and in the number of subjects who developed an upper respiratory tract illness (p <0.05).

Delivery of zanamivir

In phase II and in the current phase III clinical trials zanamivir has been delivered as a dry powder formulation using a Diskhaler device (Fig. 1). Delivery by this route has the advantages of rapid delivery of high local concentrations of the drug to the site of viral replication. The device is also very simple to use. Direct delivery to the respiratory tract also has the additional advantages of reducing the overall dosage level of drug required for efficacy, and minimizing systemic exposure, which thus minimizes the risk of unwanted side effects.

The Diskhaler is an all plastic construction which is assembled from five separate components. The body, rotadisk support wheel, mouthpiece cover and needle lever/lid are molded in acetal and the mouthpiece is molded in polypropylene.

Naturally acquired influenza

The first major study of the efficacy and safety of zanamivir for the treatment of naturally acquired acute influenza, in which involvement of the lower respiratory tract is common, compared both inhaled zanamivir, and zanamivir administered by both the intranasal and inhaled routes, with placebo (26). 417 patients in Europe and North America presenting with influenza-like illness within 48 hours of symptom onset received either inhaled zanamivir (10 mg b.i.d.) and intranasal placebo, or inhaled zanamivir (10 mg b.i.d.) and intranasal zanamivir (6.4 mg b.i.d. nasal spray) or inhaled placebo and intranasal placebo. The duration of dosing was 5 days and the primary clinical endpoint was the time to alleviation of all major symptoms of influenza: feverishness, headache, myalgia, cough and sore throat. In the 262 patients who were subsequently confirmed to have been infected with influenza, either by laboratory isolation or

rise in hemagglutination-inhibition antibody titers, the administration of zanamivir was found to result in a reduction in the median time to symptom alleviation of 1 day, a reduction of 20% relative to patients who received placebo. The magnitude of the benefit relative to placebo was the same in influenza-positive patients who received inhaled zanamivir alone (p = 0.053) and those who received both inhaled and intranasal zanamivir (p = 0.025), and there was no apparent difference between the two treatment groups. There was no treatment benefit observed in patients who were not infected with influenza. Statistically significant improvements in the rate of influenza symptom alleviation have also been observed with the same zanamivir dosing regimens in a smaller Japanese study (27).

A more detailed analysis of these data showed that zanamivir was more effective in alleviating the symptoms of influenza if treatment was begun early after symptom onset. In this group, the median time to the alleviation of all major symptoms was 3 days shorter in both treatment groups than in the placebo group ($p \leq 0.001$). Similarly, zanamivir reduced the median time to symptom alleviation by 3 days, a 40% reduction relative to patients receiving placebo (p = 0.010, placebo *vs.* inhaled zanamivir; p = 0.001, placebo *vs.* intranasal and inhaled zanamivir) who were febrile at entry into the study. As expected from the antiviral profile of zanamivir in both preclinical and early clinical studies, but unlike the clinical profiles of amantadine and rimantadine (12), there was no evidence for a difference in the treatment effect when zanamivir was administered to patients who were infected with influenza A or influenza B viruses. Zanamivir was found to be both safe and well tolerated, as was previously observed in the studies of the drug in experimental influenza infection in man.

Virological analysis was performed on nasal wash samples from influenza-infected patients. There was a reduction in virus titer in these samples of 2.1 $\log_{10}(TCID_{50}/ml)$ on day 2 of treatment and a reduction of 1.5 $\log_{10}(TCID_{50}/ml)$ on day 4 in the group that received both intranasal and inhaled zanamivir. No reductions in nasal wash virus titers were observed in patients who received zanamivir by the inhaled route alone, which was expected since the inhaled route of administration is not expected to result in the deposition of drug in the nasal passages. There were no apparent differences in the humoral immune response to infection between the patient groups receiving zanamivir and the group that received placebo.

GS4104

A novel series of cyclohexene carboxylates has recently been described as inhibitors of influenza virus sialidases (28). The most active of these compounds, GS4071, has a K_i value for the inhibition of influenza A sialidase of 0.45 nM and a K_i value for the inhibition of influenza B sialidase of 4.2 nM (29). This compound has also been shown to be selective for influenza virus sialidases relative to human lysosomal sialidase (28). The antiviral activity of GS4071 in influenza virus plaque reduction assays is similar to that of zanamivir (30).

The ethyl ester of this compound, GS4104, has been shown to be orally bioavailable when administered to mice, rats, ferrets and dogs (31). Analysis of plasma levels of drug has demonstrated that the oral bioavailability of GS4071 in these species lies in the range of 11-73% after oral administration of this prodrug. In addition to demonstrating that the active sialidase inhibitor is found in the plasma, distribution studies have also been conducted by bronchoalveolar lavage in rats in order to determine drug levels within the respiratory tract, which is the site of replication of influenza viruses in humans (32). Comparison of the levels of GS4071 in bronchoalveolar lining fluid (BALF) and plasma revealed that the levels of GS4071 were similar in both fluid compartments, and that the elimination half-life of GS4071 from the BALF was longer than in plasma.

As a result of the oral bioavailability of GS4071 after the oral administration of GS4104, the latter has been shown to be effective in experimental influenza virus infections in animals. Oral administration of GS4104 has been shown to result in an increased survival of mice which had been infected with quantities of virus that were approximately equivalent to an LD_{90} (33). The viruses used in this study included H1N1, H3N2 strains of influenza A and also influenza B/Hong Kong/5/72. GS4104 also reduced the level of lung consolidation and increased the level of arterial oxygen saturation, demonstrating that the compound had an effect on the lung pathology that results from the influenza virus infection. In view of the very low oral bioavailability of zanamivir and reports that it did not reduce lung virus titers even when high doses were administered parenterally, it was therefore surprising that in this model of influenza virus infection zanamivir was found to be active after oral administration in a number of experiments in which it had been included as a control (33). While GS4104 was more active than zanamivir when administered orally, which is to be expected given its greater oral bioavailability, the implication is that a very low plasma concentration of a sialidase inhibitor is sufficient in order achieve a therapeutic effect in the Utah State mouse model of influenza virus infection.

GS4104 has also been assessed in the ferret model of influenza virus infection. When administered at either 5 or 25 mg/kg b.i.d., commencing 2 hours postinfection with influenza virus reassortant 7a, drug administration resulted in a significant decrease in the number of inflammatory cells in nasal washing obtained from the infected animals, as well as a dose-dependent decrease in the pyrexic response to infection (30). However, neither dose resulted in a significant decrease in the overall level of virus shedding, although the higher dose did result in an 8-fold reduction in the peak nasal wash virus titers. The weak antiviral activity of orally administered GS4104 in the ferret model is in marked contrast with the high level

of activity of intranasally administered zanamivir. In this same animal model, intranasal doses of 50 mcg/kg have been shown to result in a statistically significant decrease in the overall level of virus shedding as assessed by a reduction in the area under the nasal wash virus titer curve (34). The levels of virus titer reductions that can be achieved by oral administration of GS4104 in the ferret model of infection are therefore substantially lower than are achieved by the topical (*i.e.*, intranasal) administration of zanamivir. The superiority of intranasal administration of GS4071 and zanamivir relative to the oral administration of either GS4104 or zanamivir has also been reported in the mouse model of influenza virus infection (33).

GS4104 is presently undergoing clinical trials. A preliminary report has indicated that the early oral administration of GS4104 results in significant clinical efficacy in experimental influenza A virus infections in human volunteers (35).

New inhibitors

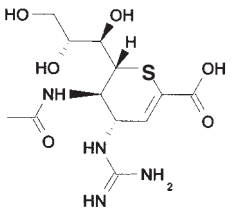
Zanamivir analogs

Much of this area has been recently reviewed (10) and this section will cover only very recent developments during 1997.

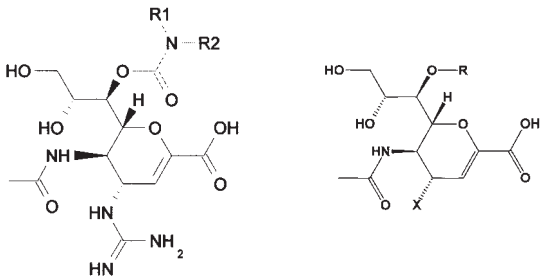
A total synthesis of the analog of zanamivir in which the ring oxygen is replaced with sulfur has been reported (36) [I]. The compound showed slightly reduced activity against influenza A sialidase but was significantly less active against influenza B.

1) 7-Carbamates

X-ray crystallography of the complexes formed between zanamivir and influenza A and B sialidases revealed that the 7-hydroxyl group of the inhibitor makes no interactions with the enzyme but rather projects into bulk solvent. It was therefore concluded that derivatization of this hydroxyl group should be possible without loss of inhibitor binding. A series of 7-carbamates were synthesized to test this hypothesis and some of these compounds were, indeed, found to be potent inhibitors of influenza sialidases (37-39) [II]. In particular, simple alkyl carbamates retaining a 4-guanidino group displayed excellent activity against both influenza A and B.



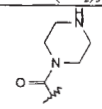
[I]



[II]

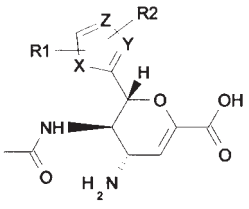
However, compounds bearing bulky and highly lipophilic carbamate substituents in general showed poorer activity than those bearing polar or ionizable substituents and the 4-amino analogs were >100-fold less active than the 4-guanidino derivatives (similar to the difference observed between zanamivir and its 4-amino analog). The inhibitory activity of many carbamates was found to be significantly greater against influenza A sialidase. Compounds of this type have potential in the development of novel influenza diagnostics, and patents for this use have been filed (38, 39) (Table I).

Table I: Inhibitory activity of 7-carbamates.

	X = NH_2		*X = guanidino*	
R	*Flu A (μM)*	*Flu B (μM)*	*Flu A (μM)*	*Flu B (μM)*
-$CONH(CH_2)_6Me$	6.0	1.95	0.004	0.05
-$CONMe(CH_2)_5Me$	2.9	1.3	0.014	0.33
	-	-	0.038	0.033
-$CON(iPr)_2$	48	24	0.064	1.0
H	0.32	0.41	0.005	0.004

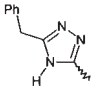
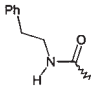
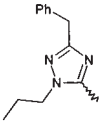
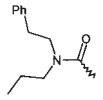
2) Nonhydroxylated side chains

Oxidative cleavage of the glycerol side chain of zanamivir and replacement with an ethyl or propyl tertiary carboxamide led to a series of potent inhibitors which show ~1000-fold selectivity for influenza A sialidase (40-43). The *N*-ethyl or propyl group on the carboxamide is oriented *cis*-relative to the dihydropyran ring in the inhibitor complex and occupies a hydrophobic pocket created by a conformational change which occurs within the enzyme. This change appears to be energetically much more favorable for the influenza A enzyme and so the compounds are highly selective. In contrast to the glycerol derivatives, similar excellent inhibitory activity is observed in this series with both 4-amino and 4-guanidino analogs. A new related series of heterocyclic analogs has recently been described (44) in which the heterocyclic ring appears to behave as a bioisostere of the carboxamide group [III]. The observed activity of the heterocyclic analogs parallels closely the previously observed pattern of carboxamide activity depending on the



[III]

Table II: Inhibitory activity of heterocyclic analogs.

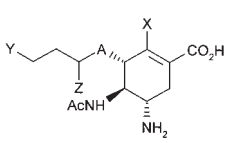
Side chain	Flu A (μM)	Flu B (μM)
	87	130
	12	67
	0.07	56
	0.003	2

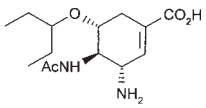
positional arrangement of substituents about the ring (Table II).

Analogs of GS4071/4104

A number of papers have been published recently describing analogs of the cyclohexene, GS4071 (45-49). The position of the alkene bond appears to be critical for optimum inhibitory activity in this series with the corresponding olefin isomers showing significantly lower inhibitory activity (28, 50). Substitution at the 2-position of the double-bond reduced activity to an even greater extent (47). Modification of the ether substituent has been extensively explored and a number of analogs with comparable activity to GS4071 have been identified (45, 50). A branched alkyl chain is essential for good inhibitory activity. Hydrophobic contacts are formed between the ether side chain and the hydrophobic surface created by the hydrocarbon chains of Glu^{276}, Ala^{26}, Ile^{222} and Arg^{224}. Simple extension of the pentyl chain is tolerated, but in some cases small modifications to the pentyl ether can also reduce activity significantly (45). The synthesis of highly lipophilic cyclohexene analogs has been described recently. Among these compounds, the (*R*)- and (*S*)-2-phenethylpropoxy ethers were described with the *S*-isomer found to be significantly more active (48, 49). The ether oxygen atom through which the lipophilic side chain is attached has also been replaced by a sulfur or methylene group without significant loss of activity, at least for simple alkyl analogs (46) (Table III).

Table III: Inhibitory activity of analogs of GS 4071.





IC_{50} 30 nM

	A	X	Y	Z	IC_{50} (nM)
GS4071	O	H	H	Et	1.4
	O	Me	H	Et	2300
	O	Cl	H	Et	3100
	O	SMe	H	Et	2300
	O	H	Ph	β-Et	0.8
	O	H	Ph	α–Et	12
	O	H	H	H	130
	CH_2	H	H	H	220
	S	H	H	H	212

Aromatic compounds

Sialidase inhibitors based on a benzene ring template have been independently reported by workers at Biocryst and Gilead (51-54). The prototype compound, 4-acetamido-3-guanidino-benzoic acid, has weak (micromolar) activity against influenza sialidases and adopts a different binding orientation to zanamivir when complexed with the enzyme. Thus, the guanidino group occupies the region in the active site occupied by the glycerol side chain of zanamivir. Introduction of a second guanidino group to the aromatic ring does not, however, improve activity since the flat aromatic ring cannot align all of the four pendant groups appropriately to achieve optimum binding for each of them. Recent reports have described fluoro aromatic analogs (53) and aromatic compounds bearing a lipophilic ether side chain analogous to GS4071 (52). A full paper on the SAR of these compounds has recently appeared (55). Although an extensive amount of work has been done in this area, none of the compounds described to date have shown inhibitory activity approaching that seen with the best of the nonaromatic inhibitors.

Other novel compounds

Synthesis of the phosphonic acid analogs of sialic acid and KDN have been reported. The compounds are more potent inhibitors than sialic acid itself and bind to the enzyme in the chair conformation rather than the twist boat conformation adopted by sialic acid (56).

Potent influenza sialidase inhibitors containing a novel 5-membered ring template were described recently by Biocryst (57). Although specific structures have not yet been disclosed, one compound, which is a mixture of four stereoisomers, has been claimed to be a potent inhibitor of both influenza A and B sialidases (IC_{50} <10 nM *vs.* influenza A, <100 nM *vs.* influenza B) and thus appears to be the prototype of an entirely new class of inhibitor. A recent patent describing this series has now been pub-

lished (58) and further developments are awaited with interest.

Drug resistance to influenza sialidase inhibitors

The emergence of drug-resistant strains depends on mutations giving rise to altered viral gene proteins that reduce the sensitivity of the virus to the inhibitory compound. Influenza viruses are known to have a high rate of mutation as the result of the absence of any proofreading activies associated with the viral RNA polymerase activity. Clinical studies with amantadine and rimantadine have shown that viruses resistant to these compounds rapidly emerge during treatment, and that they remain pathogenic for humans. The emergence of influenza viruses resistant to sialidase inhibitors has been the subject of extensive *in vitro* studies in recent years, with the majority of studies focusing on resistance to zanamivir. Resistant viruses with a number of differing phenotypes have been isolated, but all have in common a reduced sensitivity to zanamivir *in vitro*. Biological studies and sequence analysis of these viruses has shown that *in vitro* resistance to sialidase inhibitors can result from amino acid sequence changes either to the viral hemagglutinin, or to the viral sialidase, or to both proteins. The selection of drug-resistant variants *in vitro* may predict the possible genotype and phenotype of resistant viruses in the clinic but is very unlikely to predict the pathogenicity of these viruses.

Resistance to zanamivir

1) Mutations in hemagglutinin

Limiting dilution passage of influenza virus reassortant A/NWS/G70C in the presence of either zanamivir or its close analog, 4-amino-Neu5Ac2en, has led to the isolation of a series of viruses whose sensitivity to both compounds was reduced by at least 100- to 1000-fold in assays of antiviral activity (59). Some of these viruses were not only highly resistant to zanamivir but also had acquired a dependence on the presence of zanamivir or another sialidase inhibitor for optimal plaque formation. In contrast to this high level of apparent resistance, there was no evidence of a change in the affinity of zanamivir for sialidases of these viruses. Sequencing of the hemagglutinin gene of three such viruses led to the identification of a number of changes in hemagglutinin amino acid sequence in these viruses. All of the observed changes, V223I and R229I, R229S and T155A, are to amino acids close to the sialic acid binding site of hemagglutinin.

It is proposed that the effect of the mutations in hemagglutinin is to reduce the affinity of the hemagglutinin for the cellular receptor of the virus (59). A reduction in the affinity of the virus for cell surface sialic acids will reduce the dependence of progeny virions on sialidase activity for escape from the infected cell surface, and therefore reduce the sensitivity of the virus to sialidase inhibitors. This hypothesis is also able to account for the dependence on the presence of sialidase inhibitors for optimal plaque formation by some viruses, since the *in vitro* infectivity of viruses whose affinity for sialic acid has become very low will be increased in the presence of an inhibitor of the receptor-destroying activity of sialidase. The observation that the efficiencies of both viral adsorption and penetration are reduced in these mutant viruses relative to the wild-type control (60) supports this hypothesis.

Similar conclusions have been drawn from studies in which influenza virus A/Singapore/1/57 was passaged in the presence of zanamivir, resulting in a virus, AS4(+), whose sensitivity to both zanamivir and other sialidase inhibitors was more than 1000-fold lower than the wild-type virus in plaque reduction assays (61). A single amino acid substitution, G135D, in the hemagglutinin sequence of AS4(+), conferred the resistant phenotype. This residue constitutes part of the sialic acid binding site in hemagglutinin, forming a hydrogen bond to bound sialic acid through the backbone carbonyl group. There were no changes in the affinity of zanamivir for the sialidase of virus AS4(+), and the amino acid sequences of the sialidase from wild-type and resistant viruses were identical. The intranasal dose of zanamivir which effected a 90% reduction in the lung viral titer (ED_{AUC10}) of AS4(+) in an experimental mouse infection model was 7 μg/kg b.i.d. This dose is very similar to the ED_{AUC10} value of 27 μg/kg b.i.d. that has been determined for the wild-type virus (62). Virus recovered from the AS4(+)-infected mice had the same phenotype as the input virus, demonstrating that the observed sensitivity to zanamivir *in vivo* was not the result of reversion of AS4(+) to wild-type. Overall, the phenotype of the AS4(+) virus is very similar to that of influenza virus A/Stockholm/24/90, a wild-type virus (63). While the replication of both viruses appears resistant to sialidase inhibitors *in vitro*, both viruses have sialidases that have high affinity for zanamivir and both are fully sensitive to the compound *in vivo*.

Mutations within the hemagglutinin confer a broad resistance to all sialidase inhibitors *in vitro*, as would be expected from changes that have no direct effect on the binding of drugs to their target. To date no viruses which retain sialidases with high affinity for zanamivir, but which are resistant to zanamivir when replicating *in vitro*, have been shown to have reduced sensitivity to the drug *in vivo* (61, 63). The relevance of the mutations in hemagglutinin which have been selected *in vitro* to the replication of the virus *in vivo* may also be open to question, since it has been shown that there are differences in the relative prevalence of α-2,3- and α-2,6-linked sialic acids on the surfaces of MDCK cells, the cell line most often used for assays of drug sensitivity *in vitro*, and human respiratory epithelial cells (64, 65). Changes to hemagglutinin sequence can result in changes to the affinity of sialic acid binding which are dependent on whether the sialic acid is α-2,3- or α-2,6-linked (66), with the result that the same virus may have a different level of dependence on sialidase activity for replication *in vitro* and *in vivo*. Finally,

the pathogenicity to humans of viruses containing hemagglutinin mutations that confer resistance to zanamivir *in vitro* has not been studied.

2) Mutations in sialidase

Since zanamivir exerts its antiviral effect by inhibition of sialidase, mutations to the sialidase active site that reduce the affinity of the protein for zanamivir but maintain sufficient sialidase activity for replication may confer resistance to the drug. Passage of influenza virus reassortant A/NWS/G70C in the presence of zanamivir, under conditions which disfavor the selection of viruses with a reduced affinity for cellular receptors, has been shown to lead to the selection of a virus that is resistant to zanamivir *in vitro*. This virus, G70C4-G, contains a single amino acid substitution in sialidase, E119G (67), along with a mutation in the hemagglutinin, S186F (61). G70C4-G has a 100- to 1000-fold lower sensitivity to zanamivir in a plaque reduction assay (67, 68). However, zanamivir inhibits the replication of this virus in the mouse model of infection with an ED_{AUC10} value that is only 10-fold greater than that of the wild-type virus (61). A virus with the same mutation in the sialidase in influenza reassortant A/NWS/G70C has also been reported independently (69).

E119 is one of three acidic residues which interact with the 4-guanidino substituent of zanamivir, and the mutant sialidase, E119G, has been shown to have a 60-fold lower affinity for zanamivir than the wild-type sialidase (67). Proteolytic cleavage of the sialidase heads from viruses containing the E119G mutant sialidase resulted in enzyme preparations which differ markedly in specific activity (67, 69). These inconsistencies led to the discovery that sialidase protein containing the E119G substitution is less stable at all temperatures than the wild-type protein, both after proteolytic cleavage and purification and when the intact sialidase is present in complete virus particles (70). It is not known whether the instability of this zanamivir-resistant sialidase may reduce the pathogenicity of viruses containing this mutation.

Passage of three other influenza viruses in the presence of zanamivir has also led to the selection of mutations at E119. In the case of B/Hong Kong/8/73, the resistant virus contained the E119G (N2 numbering) mutation along with two mutations in the viral hemagglutinin, N145S and N150S, which results in the deletion of the potential glycosylation site at N145 and the introduction of a new glycosylation site at N148 (69). Replication of influenza virus B/Beijing/1/87 in the presence of zanamivir results in the selection of viruses which also have a glutamate to glycine change at position 119 (N2 numbering, codon 116 of the B/Beijing/1/87 sialidase) and also contain two mutations in the viral hemagglutinin protein sequence: V105A and L255Q (71). Passage of influenza virus A/Turkey/Minnesota/833/80 resulted in the selection of not only the E119G mutation but also of two other mutations at the same position: E119A and E119D (72). Again, these mutations were accompanied by a mutation to the viral hemagglutinin, G75E. The E119A mutant sialidase has a 50-fold reduction in affinity for zanamivir.

Passage of influenza virus A/Turkey/Minnesota/833/80 has also been shown to result in the selection of a different mutation to the sialidase, R292K. The R292K sialidase mutation, in an otherwise wild-type background, has been shown to confer resistance to zanamivir which is 1000-fold when resistance is assessed as plaque number but only 2-fold when resistance is assessed as plaque size (73). In the influenza A/NWS/G70C background, the same mutation, which was isolated after passage in the presence of a 6-carboxamide sialidase inhibitor, conferred only a 2-fold level of resistance to zanamivir when assessed by measurement of virus yield (74). R292 is one of three arginine residues which form the carboxylate binding region of the enzyme active site, and this mutation results in both a reduction in the catalytic activity of the enzyme and a reduction in the size of plaques formed in MDCK cell *in vitro* (74). It is reported that the pathogenicity of the A/Turkey/Minnesota/833/80 virus carrying the R292K mutation was lower than that of the wild-type virus size (73).

3) Interactions between hemagglutinin and sialidase mutations

The majority of resistant viruses with sialidase mutations have been shown to contain additional mutations within the amino acid sequence of the viral hemagglutinin. Experiments have been conducted, therefore, in order to determine the change in virus sensitivity to zanamivir that results from a sialidase mutation alone. Analysis of the *in vitro* sensitivity to zanamivir of reassortant viruses derived from resistant virus isolate G70C4-G has shown that both the mutations are required for high-level resistance to zanamivir *in vitro* (68). This confirms the result of another study which showed that the E119G sialidase mutation, in an otherwise wild-type background, confers approximately 20-fold resistance to zanamivir *in vitro* (69). Similarly, a virus containing both the R292K mutation in sialidase and the mutation N199S in hemagglutinin has been shown to be only 3-fold less sensitive to zanamivir than a virus which contains the same mutation in hemaglutinin but which has a wild-type sialidase sequence (74). The results demonstrate that the observed level of *in vitro* antiviral resistance reflects the combined effects of the sialidase mutation and the concomitant hemagglutinin mutations.

To date, no hemagglutinin mutations have been identified that confer resistance to zanamivir *in vivo*, while the reduction in sensitivity to zanamivir *in vivo* of virus that contains the E119G mutation in sialidase has been shown to be no greater than 10-fold. Any requirement for interdependent hemagglutinin and sialidase mutations for high-level resistance to zanamivir is likely to result in a rate of emergence of resistant virus which is substantial-

ly lower than is observed with amantadine or rimantadine, since for these agents a single amino acid mutation is able to confer very high levels of resistance.

4) Clinical resistance

Influenza virus isolates obtained from patients enrolled in clinical trials of zanamivir have been tested for sensitivity to the drug in both plaque reduction and sialidase inhibition assays. There is no evidence for a reduction in the sensitivity to zanamivir of any of these isolates from before and after administration (75). In contrast, data from a number of studies show that viruses resistant to amantadine and rimantadine are isolated from 11.5-50% of patients receiving treatment with these agents (76). The currently available data, therefore, indicate that the administration of zanamivir to humans infected with influenza virus is associated with a substantially lower rate of drug-resistant virus emergence than the administration of amantadine or rimantadine.

The only evidence for clinical resistance to zanamivir has come from a single immunocompromised patient infected with influenza B virus who received a 2-week course of treatment with zanamivir. Virus from an endotracheal aspirate sample obtained on day 12 of treatment was found to have sequence changes in both the hemagglutinin and sialidase protein sequences. However, the replication of the mutant virus in MDCK cells was found to be more sensitive to zanamivir than that of a virus sample obtained on the day when treatment with zanamivir began (77). There is currently no evidence that the emergence of this virus was responsible for any change in the clinical course of the infection.

Resistance to GS4071

Relative to zanamivir, far fewer studies of viral resistance to GS4071 have been conducted. It is expected, based on the arguments presented above, that those viruses whose *in vitro* resistance to zanamivir is conferred by mutations to hemagglutinin will be equally resistant to all other sialidase inhibitors, including GS4071. Similarly, any mutations in hemagglutinin that are identified during passage of viruses in the presence of GS4071 will confer equal levels of resistance to zanamivir.

No studies have yet been reported in which viruses resistant to GS4071 have been selected by passage of viruses in the presence of this drug *in vitro*. However, influenza A/NWS/G70C viruses containing the mutation R292K in the viral sialidase, as well as other mutations in the viral hemagglutinin, have been selected by passage in the presence of a 6-carboxamide analog of zanamivir (74) and reassortant viruses have been generated that contain either HA mutations or NA mutations. In view of the chemical similarity of the hydrophobic substituent on this compound and the 3-pentyl substituent of GS4071, the sensitivity to GS4071 of viruses containing the R292K mutation was assessed. Since the reassortant virus that contains the R292K mutation in the absence of HA mutations was found to plaque poorly *in vitro* (see above), the change in sensitivity was assessed in a virus yield assay. In the absence of HA mutations, the virus containing the R292K mutant was more than 3000-fold less sensitive to GS4071 than the wild-type virus, which is in marked contrast to the 2-fold resistance of the same mutant virus to zanamivir relative to wild-type. Viruses containing this NA mutation as well as the mutations to HA were found to have an even greater resistance to GS4071 *in vitro*.

Conclusions

The first clinical studies of zanamivir in naturally acquired influenza have confirmed the therapeutic potential of this new class of antiviral agent in the treatment of symptomatic influenza. The prophylactic efficacy of this drug has also been demonstrated in volunteers. The rate of emergence of viruses resistant to zanamivir appears to substantially lower than the corresponding rate for amantadine and rimantadine, with no reports of the isolation of resistant viruses from immunocompetent individuals and any of the clinical trials that have been conducted to date. Further application of medicinal chemistry and structure-based drug design is yielding sialidase inhibitors with different pharmacological properties from those of zanamivir, and early clinical studies are already in progress with an orally bioavailable prodrug of the sialidase inhibitor, GS4071.

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