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New Approaches to Influenza Chemotherapy

Neuraminidase Inhibitors

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Abstract

Epidemic influenza continues to be associated with significant morbidity in the general population, and mortality in the elderly and other high risk patients. Although the case fatality rate averages less than 0.01%, tens of thousands of deaths occur each year. Control through immunisation programmes has not been possible due to incomplete protective efficacy and antigenic variations that occur frequently.

Currently available anti-influenza medications (amantadine and rimantadine) have had limited success due to underutilisation, lack of activity against influenza B, the rapid development of viral resistance to the drugs, and adverse effects. A new class of antiviral agents designed to inhibit influenza neuraminidase, an important surface glycoprotein, is currently under active development for use in the prophylaxis and treatment of influenza A and B infections. Two of these compounds, zanamivir (GG167) and GS4104 (the ethyl ester prodrug of GS4071) have reached clinical trials.

Most studies of zanamivir have involved topical administration by inhalation of dry powder aerosols and/or intranasal doses of aqueous solutions. These routes rapidly provide high local concentrations at the sites of delivery. GS4104 is administered orally, which allows for greater ease of administration, and probably more uniform distribution of the parent compound GS4071 in the respiratory tract. Both have shown potent inhibitory activity against influenza in animal models and experimental human influenza with excellent tolerability profiles.

Zanamivir treatment has been shown to reduce the severity and duration of naturally occurring, uncomplicated influenza illness in adults. Clinical resistance to these drugs has not been recognised as a significant problem to date, although strains resistant to each agent have been produced in the laboratory. This class of agents shows considerable promise as a novel approach to prophylaxis and treatment of influenza infections. Ongoing studies should provide the data needed to allow the addition of 1 or more of the neuraminidase inhibitors to the clinician's anti-influenza armamentarium.

1. Influenza Virus Neuraminidase

Neuraminidase, or sialidase, is a surface glycoprotein that possesses enzymatic activity essential for viral replication found in both influenza A and B viruses. Approximately 100 molecules of this mushroom-shaped glycoprotein tetramer are present in each influenza virion. Related enzymes are found in many other viruses, bacteria and mammalian cells. The crystal structure of influenza neuraminidase was first determined in 1983 by Colman and colleagues.[1] The influenza sialidase is a tetramer of 4 identical subunits, each composed of six 4-stranded antiparallel β-pleated sheets. These sheets are arranged somewhat like the blades of a propeller with an axis of symmetry passing through the centre of the subunit.[2] The enzyme's active site is located within a pocket on the surface of each glycoprotein subunit that is lined by amino acids which are conserved among all influenza A and B neuraminidases characterised thus far.

This enzyme is responsible for catalysing the cleavage of the α(2-6)- or α(2-3)-ketosidic linkage that exists between a terminal sialic acid, *N*-acetyl neuraminic acid and an adjacent sugar residue. The breaking of this bond has several important effects that facilitate spread of the virus in the respiratory tract (table I). First, it allows for the release of virus from infected cells. Second, it prevents the formation of viral aggregates after release from host cells. Third, this enzyme, by cleaving the sialic acids found in respiratory tract mucus, may prevent viral inactivation and promote viral penetration into respiratory epithelial cells.[3]

Influenza neuraminidase may also contribute to viral pathogenicity. There may be potentiation of the virulence of some viral strains by alteration of carbohydrate moieties from the other surface glycoprotein, the haemagglutinin. In addition, influenza neuraminidase has been shown to directly activate latent transforming growth factor β (TGF-β).[4] This may occur due to cleavage of the sialic acid residues on the latency-associated peptide that is noncovalently bound to the mature, or active, molecule. Increased levels of the active cytokine induce cellular apoptosis which suggests yet another mechanism by which influenza virus neuraminidase may contribute to pathogenicity. Purified neuraminidase has also been shown to promote production of the proinflammatory cytokines like interleukin-1 (IL-1) and tumour necrosis factor (TNF) from macrophages.[5] Such cytokines could contribute to the pathogenesis of symptoms and of airway inflammation during influenza.

Immune-based inhibition of neuraminidase has been an active area of influenza research. Antibodies directed against neuraminidase have been shown to correlate with protection against influenza illness *in vitro* and in animal studies. This was demonstrated directly in humans in 1972[6] when volunteers were inoculated with wild-type influenza A/Hong Kong/68 (H3N2). This virus had a

Table I. Roles of influenza virus neuraminidase

Promotion of the release of virions from infected host cells
Prevention of the formation of viral aggregates after release from host cells
Prevention of viral inactivation by respiratory mucus and possible promotion of viral penetration into respiratory epithelial cells
Possible potentiation of pathogenicity by alteration of carbohydrate moieties from haemagglutinin
Induction of cellular apoptosis by activating latent transforming growth factor β
Induction of cytokine (interleukin-1, tumour necrosis factor) elaboration

haemagglutinin antigen that differed from recently circulating H2N2 influenza subtype viruses, but its neuraminidase antigen was similar. The chosen individuals lacked antibody to the viral haemagglutinin but had varying levels of antineuraminidase antibody. The severity of illness, duration of viral shedding and maximum amount of virus shed were inversely related to the level of serum antineuraminidase antibody.

The results of this study were supported by an independent study of 274 adults during the initial natural outbreak of influenza A/Hong Kong/68 (H3N2) in Tecumseh, Michigan, US,[7] which found that the proportion of individuals with respiratory illness attributable to that pandemic influenza strain decreased as the levels of pre-existing specific antineuraminidase antibodies rose.

Immunisation with neuraminidase-based vaccines has conferred partial protection against both experimental and naturally occurring influenza. Thirty-nine human volunteers were randomised to receive influenza B vaccine or vaccination with a recombinant hybrid virus that contained an irrelevant equine influenza haemagglutinin antigen (Heq1 or H7) and the neuraminidase antigen (N2) derived from influenza A/Hong Kong/68 (H3N2). The participants were later inoculated with wild type H3N2 influenza A virus. Although there was no difference between the groups in the frequency of isolation of virus from the respiratory tract, there were significant decreases in the number of participants with illness and fever in the hybrid vaccine group, and in viral titres in upper respiratory secretions. As in the earlier studies, an inverse relationship was seen between antineuraminidase antibodies and the severity of response to infection.[8] In another study, the protective efficacy of a neuraminidase-specific vaccine was found to be similar to that of a conventional biphasic vaccine.[9]

Passive protection against influenza has been investigated in animals by the administration of antineuraminidase monoclonal antibodies. In 1 study monoclonal antibodies to epitopes of 4 antigenic regions in the neuraminidase molecule were administered to chickens.[10] When later exposed to influenza virus, protection was shown in those animals receiving antibodies to 3 of the 4 antigenic regions.

2. Neuraminidase Inhibitors

Because of the importance of influenza neuraminidase in viral replication and pathogenesis, interest has focused on the development of selective inhibitors, in particular sialic acid analogues, of this enzyme. Unlike amantadine and rimantadine, inhibition of neuraminidase could potentially be effective for both influenza A and B virus infection. The first sialic acid analogue, 2-deoxy-2,3-dehydro-*N*-acetyl neuraminic acid (DANA) (fig. 1), was described in 1969 by Meindl and Tuppy.[11] DANA and its fluorinated derivative 2-deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid (FANA) were both able to inhibit neuraminidase enzyme activity and influenza virus growth *in vitro*.

In a study evaluating the effectiveness of DANA and 18 compounds derived from DANA, the parent compound and its halogenated derivatives were found to be the most active in neuraminidase inhibition assays against *Vibrio cholerae* and 4 viral neuraminidases (influenza A/Melbourne, influenza A/Vienna, influenza B/Lee and a parainfluenza virus, Newcastle disease virus). Twelve of the compounds were at least partly effective in inhibiting neuraminidase activity. For each compound the potency against influenza A and B strains were comparable. FANA was the most potent compound tested with 50% enzyme inhibition at a concentration of 5 μmol/L, whereas the parent compound (DANA) showed 50% enzyme inhibition at approximately 6-fold higher concen-

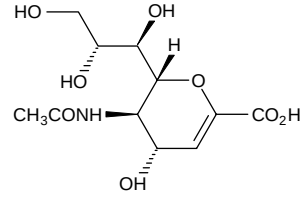

Fig. 1. Molecular structure of 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (DANA).

trations.[12] However, these compounds lacked specificity and were as effective, or more effective in inhibiting the bacterial sialidase as the viral neuraminidases.

Significant advances were made following the determination of the crystal structure of the enzyme in 1983[1] and of the co-crystal structure with the natural substrate sialic acid.[13] Knowledge of the structure has allowed molecular modelling to design more potent and selective inhibitors of the enzyme. Two of the compounds (zanamivir and GS4104) are now in clinical trials to assess their efficacy and tolerability in the prophylaxis and treatment of influenza infection.

2.1 Zanamivir (GG167)

2.1.1 Structure

Zanamivir (molecular weight 332) is a neuraminidase inhibitor derived from DANA that was designed using computer-based molecular modelling of the complex formed between influenza virus neuraminidase and sialic acid. This compound also referred to as 4-guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetyl neuraminic acid or GG167 has a guanidino group at the 4 carbon position of DANA (fig. 2). Potency and selectivity have been shown to be much greater for this newer compound than its predecessor. This is thought to result from hydrogen bonding and electrostatic and van der Waals interactions of the guanidino group with amino acids in a previously unoccupied area within the neuraminidase's active site of influenza neuraminidase.[13] These interactions result in classical competitive inhibition of the enzyme. Slow-binding

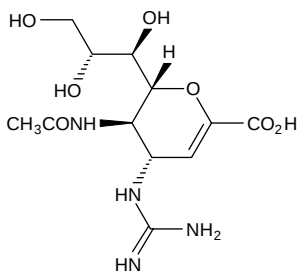

Fig. 2. Molecular structure of zanamivir.

competitive inhibition of influenza A/Tokyo/3/67 N2 enzyme and other influenza A neuraminidases has also been identified.[14] This is probably due to expulsion of a structural water molecule from the active site. Zanamivir is also a slow-binding inhibitor of influenza B virus.[15] Such inhibition has not been demonstrated in other, noninfluenza neuraminidases. Extensive preclinical and clinical testing of the anti-influenza activity of zanamivir has provided encouraging results and phase III trials are currently ongoing.

2.1.2 In Vitro *Activity*

Woods and colleagues[16] have provided the most extensive data regarding the *in vitro* activity of zanamivir. The 50% effective concentrations (EC_{50}) of zanamivir, 4-amino-Neu5Ac2en, DANA, amantadine, rimantadine, and ribavirin were determined by plaque formation inhibition assays in Madin Darby canine kidney (MDCK) cells with both laboratory-passaged strains and clinical isolates of influenza A and B viruses. Zanamivir consistently had lower EC_{50} values than the other compounds for both types of isolates, although the EC_{50} values had a much greater range for clinical isolates (0.002 to 16 μmol/L) than for laboratory-passaged isolates (0.004 to 0.014 μmol/L). However, when the EC_{50} was determined by inhibition of purified neuraminidase, zanamivir was shown to be a potent inhibitor of the neuraminidases from all isolates. The EC_{50} values (0.00064 to 0.0079 μmol/L) differed at most by a factor of 13, as compared with up to 8000 for the EC_{50}s determined by plaque reduction assay. In murine models of influenza (described below) the *in vivo* susceptibility of viruses correlates generally with enzyme inhibitory concentrations of zanamivir and not with activity in MDCK plaque assays. The discrepancies between *in vitro* sensitivities, and enzyme inhibition by zanamivir suggests that some viruses are less dependent from others on neuraminidase (NA) activity for growth in MDCK cells. Plaque assay studies also showed that zanamivir was active (at concentrations of 0.002 to 1.5 μmol/L) against clinical isolates resistant to amantadine and rimantadine.

Zanamivir has also been tested against the 9 neuraminidase subtypes of avian influenza A. Significant inhibitory activity has been demonstrated against these strains by plaque reduction assays and by decreasing viral yield from monolayer cell cultures.[17,18] Other studies have shown that zanamivir is capable of inhibiting replication of influenza A and B in explants of human respiratory epithelium.[19] In such explants, concentrations of <0.01 mg/L were capable of causing at least 1 $\log_{10}$ $TCID_{50}$/ml (50% tissue culture infectious dose) decrease in yield (90% reduction) compared with controls for clinical influenza A isolates of the H1N1 and H3N2 subtypes. Concentrations of 0.25 mg/L were necessary to achieve the same end-point for an influenza B/Hong Kong/5/72 virus.

Studies have also evaluated the cytotoxicity and selectivity of zanamivir. As compared with earlier sialidase inhibitors, zanamivir exerts much less inhibition of human and other noninfluenza sialidases. These are inhibited only at $>10^6$ higher concentrations compared with the influenza enzyme. Even at concentrations of up to 10 mmol/L, no cytotoxicity against human cells has been seen. Similarly, no significant inhibitory activity was observed against strains of herpes simplex virus types 1 and 2, varicella-zoster virus, cytomegalovirus, rhinovirus types 2 and 14, or parainfluenza virus types 2 and 3.[16]

2.1.3 Animal Studies

Pharmacokinetics and Tolerability

Pharmacokinetic studies of zanamivir have shown the drug to be readily bioavailable after nasal, intraperitoneal, and intravenous administration, but not after oral administration in mice.[20] 68% of the intraperitoneal dose was recovered from the urine. This percentage decreased to 43% for the intranasal route and further decreased to only 3% for orally administered drug.

In contrast, the bioavailability of intranasally administered zanamivir was found to be only 7.8% in a ferret model.[21] Peak serum concentrations were highest after intravenous doses followed in descending order by intraperitoneal, intranasal and oral doses. However, systemic doses are rapidly cleared by the renal route with no significant metabolism.

Zanamivir has been evaluated for toxicity in several animal species using both intranasal and inhalational administration as well as intravenous administration to maximise systemic exposure to the drug.[22] These studies revealed no toxicities or adverse events of clinical significance.

Efficacy

Zanamivir administered intranasally to mice infected with influenza A/Singapore/1/57(H2N2) was 100-fold more potent in inhibiting viral replication than amantadine and 1000 times more potent than ribavirin given by the same route.[2] The intranasal dose shown to decrease viral replication to 10% of controls (untreated, inoculated mice) was 0.027 mg/kg twice daily.

Efficacy of intranasal zanamivir given prophylactically to mice prior to inoculation with influenza A/Singapore/1/57 has also been evaluated using the end-points of viral titres in lung homogenates, lung consolidation and mortality.[20] With doses of 0.01 mg/kg, significant reductions were seen in mortality and lung consolidation. At the 0.4 mg/kg dose, significant effects were seen in all 3 end-points. Importantly, there was no rebound of virus replication after the treatment period (days 1 to 3) ended. In mice inoculated with influenza B/Victoria/102/85 doses as low as 0.4 mg/kg were able to significantly decrease lung viral titres. The dose required to decrease viral titres to 10% of controls (ED_{AUC10}) was 0.085 mg/kg/ dose. Zanamivir was shown to be 753 times more active than ribavirin given by the same route.

Similar studies of intranasal zanamivir in ferrets have used nasal wash viral titres, and the occurrence of pyrexia as primary end-points.[21] When the animals were treated with intranasal zanamivir at doses as low as 0.05 mg/kg/dose, beginning 1 day prior to inoculation with influenza A/Singapore/1/57 and continuing for 5 days after infection, significant decreases were seen in viral titres and development of pyrexia. Similar outcomes occurred in animals treated with intranasal ribavirin at 12.5 mg/kg/dose, but no significant de-

creases were found in the group receiving amantadine at 6.25 mg/kg/dose. For influenza A/ Mississippi/ 1/85(H3N2), doses of 0.06 mg/kg of zanamivir and 12.5 mg/kg of ribavirin produced significant reductions in viral titres and pyrexia. In influenza B/Victoria/102/85 infection, doses as low as 0.75 mg/kg of zanamivir and 50 mg/kg of ribavirin were able to produce similar results. Thus, zanamivir was 100 to 1000 times more potent than ribavirin and amantadine in ferrets experimentally infected with human strains of influenza A and B viruses. No decrease in serum antibody response to the infecting strains occurred.

A study of the prophylactic efficacy of intratracheal zanamivir in chickens inoculated intratracheally with 1 of 3 highly pathogenic avian influenza viruses [the reassortant SD1 (H7N7)] the reassortant SD17(H7N1) and A/Duck/Ireland/ 83(H5N8)] failed to show any protective activity.[23] The degree of pyrexia and death rate were unaffected by administration of zanamivir in doses of 1 to 1.5 mg/kg twice daily. There was a delay in the onset of pyrexia and death in the animals inoculated with the H7N1 virus. Virus recovered from the dead birds was no more resistant to zanamivir than the viruses used to inoculate the animals. It is possible that the failure was due to only partial inhibition of virus in the trachea and/or differences in distribution patterns of intratracheally administered drug and virus. Escape of virions from the respiratory tract to areas with insufficient concentrations of the inhibitor probably lead to dissemination of virus to other visceral sites.

A second avian study of the prophylactic efficacy of zanamivir using intranasally administered drug showed partial protection against infection by an H7N7 virus (influenza A/Chick/Vic/1/85) but no protection against the other highly virulent strains containing N1, N2 or N3 neuraminidase tested at doses of up to 1 mg/kg.[18]

The therapeutic efficacy of zanamivir has also been shown in animal models in which administration of the drug was delayed until after inoculation. In mice, intranasal doses of 12.5 mg/kg, but not doses of 0.4 mg/kg, begun 3 hours after inoculation produced significantly decreased viral titres [area under the concentration-time curve (AUC) <0.005% of controls].[19] In the abovementioned ferret model, effectiveness was seen even when the first dose ($5 \times ED_{AUC10}$) was delayed until 24 hours after inoculation.

Early studies showed no significant antiviral activity after oral administration as assessed by lung homogenate viral titres.[20] It has been hypothesised that intranasally administered zanamivir has been more effective due to the extracellular site of action of the neuraminidase enzyme. Newer data, however, have shown some beneficial effects of orally administered zanamivir. In mice given oral zanamivir beginning 4 hours before inoculation with influenza A/NWS/33 (H1N1), an increase in the mean number of days to death and improvements in oxygen saturation were seen in the groups receiving dosages of at least 1 mg/kg/day. The group receiving 10 mg/kg/day also experienced a significant decrease in mortality.[24] The reasons for the discrepancies in the reported activity of oral zanamivir are unclear and it remains to be determined whether oral zanamivir is active against other strains or in other animal models.

2.1.4 Human studies

Pharmacokinetics and Tolerability

Human pharmacokinetic and safety studies of zanamivir have obtained results similar to those in the animal models described above. In the human trials of zanamivir to date, the drug has been well tolerated with the incidence of adverse events comparable to that of placebo. The pharmacokinetics of zanamivir were studied following single and multiple doses of the drug given by intravenous, intranasal and inhaled routes of administration.[25] The kinetics were found to be linear for each route. In volunteers given 16mg doses by 1 of the 3 routes, maximum serum concentrations were highest in those receiving zanamivir intravenously (C_{max} = 1275 μg/L), followed by the inhalation group (C_{max} = 136 μg/L) and, finally, by the intranasal group (C_{max} = 4.2 μg/L). The terminal phase half-life was longer when the drug was administered by the topical routes (intravenous $t_{½}$ = 1.7 hours, inhaled $t_{½}$

= 2.9 hours, intranasal $t_{1/2}$ = 3.4 hours) which was felt to be related to slow or complex absorption rather than an alteration in the elimination phase. The absolute bioavailabilities, determined by urinary excretion data, of intranasal and inhaled zanamivir were approximately 10 and 20%, respectively. 90% of the intravenously administered drug was excreted unchanged in the urine. The drug was well tolerated in all groups with no reported serious adverse events.

The deposition pattern of zanamivir following inhalation of the drug by 2 inhalation devices was evaluated.[26] This was accomplished by administering zanamivir that had been radiolabelled with technetium followed by gamma scintigraphy of the lungs and oropharynx. It was found that both upper and lower respiratory tract mucosa were exposed to the drug, with 7 to 21% of the administered dose reaching the lungs and 70 to 90% depositing in the oropharynx. For that proportion of zanamivir reaching the pulmonary airways, deposition was fairly uniform throughout the lungs. Again, no serious adverse events were reported and only 2 adverse events (both headache) were considered to be related to the drug. No significant end-organ toxicity has been recognised with topical zanamivir. One tolerance study found no deterioration in clinical status or pulmonary function in asthmatic participants receiving inhalations of zanamivir.

Efficacy in Experimental Human Influenza

The first human efficacy trials assessed the activity and tolerability of intranasal zanamivir in the prevention and treatment of experimental influenza A/Texas/91(H1N1). In such studies the virus inoculation is given intranasally to susceptible healthy adults as determined by haemagglutination inhibition antibody testing. Nasal drops and sprays of various zanamivir concentrations were initiated either before or after virus exposure. When used for prophylaxis beginning 4 hours before viral inoculation, the drug was found to have protective efficacies of 96% against viral shedding and 82% against infection documented by serology and/or shedding and 95% against febrile illness.[27] Both nasal spray and drop forms of administration were able to significantly decrease overall symptom scores, paracetamol (acetaminophen) use, cough, upper respiratory tract symptoms and nasal mucus weight. The nasal drops were somewhat more effective than the sprays in reducing viral shedding and preventing infection although the reductions seen for both forms were significant. When given as early therapy, beginning at either 26 or 32 hours after inoculation, there was a reduction in viral shedding, viral titre AUC values and peak viral titres. The overall efficacy in the prevention of febrile illness was 84%. There were 40 to 65% reductions observed in total symptom scores, frequency of cough, nasal mucus weight and paracetamol use. When the initial dose was delayed until 50 hours after inoculation, there was a rapid reduction in viral titres, and the duration of viral shedding decreased by an average of 1 day. However, there was no significant change in symptom scores. In these studies, administration of twice daily dosages was equivalent in efficacy to that of 6 times daily dosages. These studies also found that early treatment with zanamivir significantly reduced the otological manifestations of experimental influenza with over 2-fold reductions in the frequencies of ear complaints and middle ear pressure abnormalities.[28]

The prophylactic efficacy of intranasal zanamivir has also been evaluated against experimental influenza B infection. This study involved 34 volunteers who were randomly assigned to 1 of 4 treatment groups: intranasal zanamivir 3.2mg twice daily, intranasal zanamivir 6.4mg twice daily, intranasal zanamivir 6.4mg once daily, and placebo. Administration was begun 4 hours prior to inoculation with influenza B/Yamagata/88 and continued for 5 days. The protective efficacy of the combined zanamivir groups was 60% against viral shedding and 32% against laboratory-documented infection. There tended to be a higher protective efficacy when zanamivir was administered twice daily than once daily. Symptom scores were also lower in the zanamivir groups, significantly so for the group receiving the higher twice daily dose of the drug.[29] However, due to the small number of

study participants, this could not be definitively determined and further studies of once daily administration for prevention are in progress.

Efficacy in Naturally Occurring Human Influenza

Several trials using topically applied zanamivir have been completed in naturally occurring influenza infection. Because naturally occurring disease frequently affects the lower respiratory tract, whereas experimental influenza infrequently causes lower tract disease, inhalation of zanamivir by dry powder aerosol was used in these studies.

In a randomised double-blind, placebo-controlled multicentre trial, intranasal (6.4mg) plus inhaled (10mg) zanamivir was compared with inhaled zanamivir (10mg) alone, and with placebo, in treating acute, uncomplicated influenza in previously healthy individuals.[30] Each of the 3 groups self-administered the treatments twice daily for 5 days.

Only 63% of the enrolled participants with an influenza-like illness had laboratory-confirmed influenza (56% influenza A, 44% influenza B). Among the 262 people with confirmed influenza, there was an overall decrease in median time to alleviation of major influenza symptoms of 1 day (4 days versus 5 days in controls) for the treatment groups although this was statistically significant only for the group receiving both inhaled and intranasal zanamivir.

In those who had fever at the time of enrollment or who were treated within 30 hours of symptom onset, there was a 3-day reduction in the time to alleviation of all major symptoms (4 days versus 7 days) in both zanamivir groups. Zanamivir treatment was also associated with more rapid return to normal activities.

In all of the enrolled patients, the treatments were well tolerated and no differences in the frequency of adverse events were observed between zanamivir and placebo groups. No differences were seen in the development of antibody responses to the infection among groups. Clinical benefit was observed in both influenza A and B virus infected individuals. Reductions in nasal wash viral titres were found in those receiving zanamivir by intranasal spray but not by inhalation alone. Whether such reductions in respiratory tract viral titres and symptoms would be associated with reductions in transmission of virus to contacts remains to be determined.

In another study of natural infection, 116 Japanese patients with an influenza-like illness of less than 36 hours duration were randomised to receive 1 of the 3 treatment regimens described above. Of the 116 patients, 73 had laboratory-documented influenza infections (34 influenza A, 39 influenza B). There was a significant decrease in the time until all major symptoms were alleviated in both of the zanamivir groups as compared with the control group. The study drug was well tolerated and no significant adverse events occurred.[31]

More recently, a multicentre trial compared the efficacy of combined intranasal and inhaled zanamivir given 2 or 4 times daily to placebo in acute influenza. In addition to providing symptom relief, this study found that zanamivir treatment decreased sleep disturbance, time confined to bed, additional healthcare contacts and interference with the ability to work and participate in leisure activities.[32]

Such results highlight the value of early antiviral therapy in acute influenza with respect to improving functional recovery and other health outcomes. Zanamivir has also been tested for preventing influenza-like illness and isolation of influenza virus from the patients in a nursing home prophylaxis study.[33] However, this was an open trial with low event rates and the prophylactic activity of zanamivir remains to be established in natural influenza.

These studies have clearly established that topical administration of zanamivir to the respiratory tract is associated with more rapid resolution of illness and functional recovery in acute influenza in ambulatory adults. Whether therapy can reduce the risk of influenza associated complication remains to be determined. Further studies in high risk patients are planned and a placebo-controlled study of nebulised zanamivir for treating severe influenza in patients hospitalised with lower respiratory tract disease is being undertaken by the

Collaborative Antiviral Study Group of the US National Institutes of Health.

2.2 GS4071/GS4104

One of the limitations seen thus far with zanamivir is its lack of oral bioavailability (estimated as <5% in humans). Attempts have been made to produce newer neuraminidase inhibitors that are more readily absorbed by the gastrointestinal tract. The greatest success has been seen with the development of a group of hydrophobic, carbocyclic, transition-state analogue inhibitors of neuraminidase.[34] The most promising compound in this group is (3R, 4R, 5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexane-1-carboxylic acid, also known as GS4071 (fig. 3). Its ethyl ester prodrug, currently designated GS4104 (fig. 4), provides excellent bioavailability of the parent compound following oral administration.

2.2.1 Structure

GS4071 (molecular weight 284) lacks the glycerol and guanidino groups found in zanamivir. Affinity for the enzyme is preserved because of the strong hydrophobic interactions that occur between the lipophilic 3-pentyloxy side chain of the compound and a hydrophobic pocket within the active site of the enzyme.[34] Thus, potential bioavailability is increased by the removal of 2 polar groups and affinity is maintained by formation of interactions within the active site of influenza neuraminidase.

2.2.2 In Vitro Activity

In general, GS4071 has an *in vitro* anti-influenza spectrum and potency comparable to that of zanamivir against the neuraminidase activity and virus replication of influenza A and B viruses. In an assay comparing GS4071 and zanamivir by their abilities to inhibit plaque formation by recent clinical isolates in MDCK cells, both agents had similar 50% effective inhibitory concentrations (EC_{50}). The median EC_{50} was <0.1 mg/L for the 18 influenza A (H3N2) strains, >10 mg/L for 8 influenza A (H1N1) strains and 0.1 mg/L for the 10 influenza B strains tested for both compounds.[35]

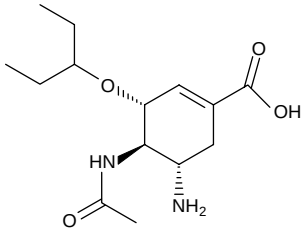

Fig. 3. Molecular structure of GS4071 (molecular weight 284.4).

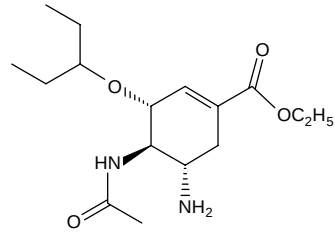

Fig. 4. Molecular structure of GS4104.

Other cell culture studies have found EC_{50} concentrations ranging from 0.7 to 150 nmol/L against various strains of influenza A and B viruses.[36]

Concentrations of 0.2 to 2.0 nmol/L are inhibitory for neuraminidase enzymatic activity.[36] The ability of GS4071 to inhibit the cytopathic effect of influenza A/WS/33 (H1N1), A/Victoria/3/75 (H3N2), A/Shangdong/09/93(H3N2), and influenza B/Hong Kong/5/72 has also been evaluated in cell culture. Inhibition was seen in all tested strains with 50% inhibitory concentrations ranging from 0.2 to 26 μmol/L.[24]

GS4071 appears to be several times more active against H3N2 subtype viruses in cell culture and in enzyme inhibition assays than zanamivir.[34] No cytotoxicity or inhibition of sialidases from other sources including human liver was seen with concentrations as high as 1 mmol/L, the highest concentration tested.[24]

2.2.3 Animal Studies

Pharmacokinetics and Tolerability

Animal studies have been used to establish the pharmacokinetics of GS4071 and GS4104. GS4071 administered intravenously to mice, rats,

dogs and monkeys had a plasma half-life of 1.6 to 1.8 hours.[37] Oral doses in rats and marmosets were found to have less than 5% bioavailabilty. Orally administered GS4104, however, provided bioavailabilities of GS4071 ranging between 30 and 73% among different species (mice, rats, dogs and monkeys) and the majority of the prodrug that was absorbed was hydrolysed to the active form (GS4071) in all species.[37]

Another study using carbon-14 labelled GS4104 administered orally to rats found 24% recovery of the drug in urine after 24 hours and 61% in the faeces.[38] In a study of rats given a single 10 mg/kg oral dose of GS4104, the peak plasma concentration of GS4071 was 0.52 mg/L and this was sustained for approximately 6 hours. This concentration is 10 to 1000 times higher than the 50% inhibitory concentrations of GS4071 in cell culture.[37]

Animal studies have also found that GS4104 given by the oral route is delivered to the respiratory tract, including the extracellular surface of the respiratory epithelium, a site important in influenza infection. In rats given oral GS4104 in doses of 30 mg/kg, drug concentrations in the lung exceeded those of plasma by 2 times at 6 hours. By 24 hours, this had increased to a 30-fold difference. When bronchoalveolar lavage was performed in rats following a 30 mg/kg oral dose of GS4104, maximum bronchoalveolar lavage (BAL) fluid concentrations of GS4071 (5.8 mg/L) were similar to those of plasma. However, the drug's half-life in the BAL fluid (3.2 hours) was longer than that seen in plasma.[39]

The toxicity of repetitive administration of GS4104 has been evaluated in rats and marmosets.[40] The drug demonstrated a wide safety margin and was well tolerated except for gastrointestinal irritation at doses markedly higher than those used clinically. It was neither teratogenic, nor affected fertility in either sex.

Efficacy

The efficacy of these drugs has been demonstrated with various doses of GS4071 and GS4104 by oral and intranasal administration.[24,36] Mice inoculated with influenza A/NWS/33 (H1N1) had significantly lower mortality than placebo when treated with oral doses of GS4104 as low as 1 mg/kg/day beginning 4 hours before exposure to virus. Other influenza strains, such as influenza A/Victoria/3/75 (H3N2), required doses of up to 10 mg/kg/day to achieve a similar protective effect. The mean number of days until death and oxygen saturation were significantly increased in mice receiving the lowest dosage tested (0.1 mg/kg/day). In comparison, animals receiving oral GS4071 required doses of 1 mg/kg/day to see beneficial effects and doses of 10 mg/kg/day to achieve a significant decrease in mortality. In an additional arm of the study, the length of time that initiation of therapy with GS4104 could be delayed and still produce a significant antiviral effect was assessed. In mice given an 85% lethal dose of influenza A/NWS/33 (H1N1), delay of GS4104 10 mg/kg/day as late as 60 hours after inoculation protected against mortality and decreases in oxygen saturation. When the virus inoculum was increased by 100 times, a significant decrease in mortality was still seen when treatment was initiated as late as 36 hours after viral challenge.

Other studies have found that dosages of 10 mg/kg/day provided protection against A/NWS/33(H1N1), A/Victoria3/75(H3N2) and B/Hong-Kong/5/72 lethal infections of mice.[36] Intranasal doses of GS4071 were compared directly with zanamivir given beginning 4 hours after inoculation with influenza A/NWS/33 (H1N1) and continuing for 5 days.[24] GS4071 was found to improve survival and the mean number of days until death with dosages as low as 0.01 mg/kg/day, while zanamivir required 10-fold higher doses to achieve comparable results. Both drugs were able to prevent decreases in oxygen saturation at doses of 0.01 mg/kg/day. Other experimental murine studies found greater antiviral activity with intranasal zanamivir than GS4071 in A/Singapore/1/57 (H2N2) infection.[41]

In experimentally infected ferrets, oral GS4104 in doses of 5 mg/kg and 25 mg/kg beginning 2 hours after inoculation with an influenza A/En-

gland/939/69 (H3N2) virus decreased the febrile response to infection. In the animals receiving the higher dose of GS4104, no fever was documented, and peak viral titres in nasal lavage were decreased by 8-fold compared with those of controls. The number of inflammatory cells in the upper respiratory tract was decreased 10-fold by this treatment regimen.[36] Another ferret study found that oral doses of 50 mg/kg but not 5 mg/kg twice daily reduced viral titres over 10-fold and protected against febrile responses following influenza A/Mississippi/1/85(H3N2) infection.[41]

2.2.4 Human studies

Pharmacokinetics and Tolerability

The pharmacokinetics and tolerability of GS4104 have been evaluated in human volunteers.[42] Single oral doses ranging from 20 to 1000mg were given to fasting male participants and plasma concentrations of the prodrug and the active drug were determined at specific intervals after administration. The plasma concentrations of GS4104 peaked early (0.5 to 3.5 hours) while a later peak was observed for GS4071 (2.5 to 6 hours). Mean peak concentrations of GS4071 ranged from 77.1 μg/L (±15.8) for the 20mg dose to 4490 μg/L (±2130) for the 1000mg dose. Peak concentrations of the prodrug were 15 to 30% of those of the active drug. Both maximum concentrations and plasma concentration-time curves of GS4071 increased in a dose-dependent fashion. GS4071 demonstrated a longer terminal half-life than GS4104 with approximately 35% of the maximum concentration remaining at 12 hours after the dose. When the 200mg dose was given following a standardised meal, a 1- to 1.5-hour delay in reaching maximum concentration occurred for each compound. No other effects on the pharmacokinetics were observed and the overall drug exposure was slightly higher in those ingesting drug with food. When doses ranging from 50 to 500mg were given twice daily for 6 days, a small increase in exposure to GS4071, but not to GS4104, occurred over time. Mean predose concentrations of up to 1280 μg/L were seen on day 3 in the group receiving 500mg twice daily.

In this study, GS4104 was well tolerated with no serious adverse events reported. The most frequently reported adverse event was headache. Mild to moderate nausea was most frequently reported by the group receiving higher doses of the drug and this was seen mainly in association with the first dose. Otherwise, there was no relationship between the number of adverse events and the dose of GS4104.

Efficacy in Experimental Human Influenza

In a placebo-controlled double-blind trial, 80 susceptible adults were inoculated with influenza A/Texas/91 (H1N1) and assigned to 1 of 5 treatment groups.[43] Treatment groups were: GS4104 20mg orally twice daily, 100mg orally twice daily, 200mg orally twice daily, 200mg orally once a day, or placebo. All treatments were started 28 hours after inoculation and were continued for 5 days. Significant decreases were seen in the combined GS4101 groups in the duration of viral shedding, the amount of virus shed (viral titre area under the curve) and in duration of symptoms as compared with the placebo group. No obvious differences in antiviral or clinical benefits were observed among the GS4104 groups. The study drug was generally well tolerated and no serious adverse effects were recognised. The only reported adverse effect was transient nausea of mild to moderate nature, especially at higher doses taken in the fasted state. A parallel prevention study found that doses of 100mg once or twice daily protected against viral recovery from the upper respiratory tract and against infection-associated illness. Phase III studies of the therapeutic and prophylactic activity of oral GS4104 in natural influenza are in progress.

3. Resistance to Neuraminidase Inhibitors

A concern for the neuraminidase inhibitors, as for other antimicrobial agents, is the potential for the development of *in vivo* resistance to their antiviral activity. To date, two different mechanisms of resistance have been recognised from the study of viral strains passaged in cell culture in the presence

of the compounds. These involve mutations in the viral haemagglutinin or neuraminidase.

Although this issue is far from settled, initial reports suggest that the frequency of resistance emergence is low during clinical use of zanamivir.[44] In uncomplicated influenza in adults, nasal wash samples obtained before and after treatment with zanamivir showed no evidence of development of resistance to the drug as determined phenotypically by plaque inhibition and neuraminidase inhibition assays.[44] Furthermore, the finding that the neuraminidase variants have altered enzymatic activity and reduced replication capacity suggests that they are biologically less fit.

3.1 Zanamivir

Influenza strains with resistance to zanamivir have been selected by *in vitro* passage (table II). Following passage of a recombined H1N9 (NWS/G70C) influenza virus in MDCK cells in the presence of either 4-amino-Neu5Ac2en or zanamivir, 3 variants were found that were able to grow despite the presence of the inhibitor.[45] These variants had sensitivities to the neuraminidase inhibitor that were 100 to 1000 times less than those of the wild-type virus as determined by plaque inhibition assay. Neuraminidase enzyme inhibition testing, though, found no difference between the variant viruses and the zanamivir-sensitive parent virus. In all 3 variants, mutations were found in conserved regions of the haemagglutinin gene and these coded for amino acid substitutions in areas associated with the haemagglutinin receptor-binding site. These findings suggest that 1 mechanism for resistance is due to a decrease in the affinity of the haemagglutinin for the cellular receptor, which allows virus to elute from infected cells without the need for significant neuraminidase activity.

Table II. Mutations in influenza virus haemagglutinin (HA) and neuraminidase (NA) conferring resistance to neuraminidase inhibitors

Reference	Virus	Mutation detected	Drug to which resistance was demonstrated	Method by which resistance was demonstrated
McKimm-Breschkin et al.[45]	A/NWS/G70c (H1N9)	HA: Thr^{155}Ala, Arg^{229}Ile, Arg^{229}Ser, Val^{223}Ile	Zanamivir, 4-amino-Neu5Ac2en	Plaque inhibition assay (not demonstrated in NA inhibition assays)
McKimm-Breschkin et al.[46]	A/NWS/G70c (H1N9)	HA: Thr^{155}, Val^{223}, Arg^{229}	Zanamivir, 4-amino-Neu5Ac2en	Plaque inhibition assay, liquid culture, HA-elution assay
Penn et al.[47]	A/Singapore/1/57 (H2N2)	HA: Gly^{135} Asp	Zanamivir	Plaque inhibition assay, tissue culture (not demonstrated in NA inhibition assays or *in vivo*)
Blick et al.[48]	A/NWS/G70c (H1N9)	NA: Glu^{119} Gly	Zanamivir, 4-amino-Neu5Ac2en	Plaque inhibition assay, NA inhibition assay (for zanamivir only)
Staschke et al.[49]	A/NWS/G70c (H1N9)	NA: Glu^{119} Gly	Zanamivir	Plaque inhibition assay, NA inhibition assay
Staschke et al.[49]	B/HK/8/73	NA: Glu^{117} Gly HA: Asp^{145} Ser, Asp^{150} Ser	Zanamivir	Plaque inhibition assay, NA inhibition assay
Gubareva et al.[50]	A/Turkey/Minnesota/83 3/80 (H4N2)	NA: Glu^{119} Ala, Glu^{119} Asp, Glu^{119} Gly, HA2: Gly^{75} Glu	Zanamivir	Plaque inhibition assay, NA inhibition assay
Gubareva et al.[51]; Gubareva et al.[52]	A/Turkey/Minnesota/83 3/80 (H4N2)	NA: Arg^{292} Lys HA1: Trp^{235} Leu, Thr^{280} Lys, Asp^{318} Asn HA2: Ala^{35} Thr, Lys^{68} Arg	Zanamivir	Plaque inhibition assay, NA inhibition assay
McKimm-Breschkin et al.[53]	A/NWS/G70c (H1N9)	NA: Arg^{292} Lys, HA: 3 residues in or around receptor binding site	Zanamivir, 4-amino-Neu5Ac2en, 6-carboxamide, GS4071	NA inhibition assay, cell culture

In a follow-up study of these same viral variants,[54] the variants were found to have a lower adsorption efficacy in MDCK cells than the parent viruses. When the variants were repassaged in the absence of zanamivir, reversion to the wild-type phenotype occurred and adsorption kinetics returned to those of the wild-type virus. This also suggests that the drug-resistance in these passaged strains was attributable to the mutated haemagglutinin that possessed reduced affinity for the cellular receptor. Other studies have confirmed these findings and provided more detailed information regarding the haemagglutinin mutations. One such study produced a resistant strain of A NWS/G70C with changes in conserved amino acids (Thr^{155}, Val^{223}, Arg^{229}) found in areas associated with haemagglutinin receptor binding.[46] In general, haemagglutinin mutations appear to confer cross-resistance to all current neuraminidase inhibitors in cell culture.

Another study utilised influenza A/Singapore/1/57 (H2N2) passaged through MDCK cells to produce virus with reduced sensitivity to zanamivir. In this study, the resulting virus was >1000 times less sensitive to inhibition by zanamivir as shown by plaque inhibition assay, but there was no decrease in the sensitivity to inhibition by zanamivir when determined by neuraminidase inhibition assay. When the variant influenza strain was administered as a challenge to mice, there was no decrease in the *in vivo* sensitivity to inhibition of viral replication by zanamivir as compared with challenge with the parent virus (ED_{90} of 0.007 mg/kg *vs* 0.027 mg/kg).[47] The observation that this haemagglutinin variant retained *in vivo* susceptibility to zanamivir suggests that they are not likely to be of clinical importance. However, *in vivo* susceptibility needs to be determined for a broader range of such variants.

Additional *in vitro* studies have determined that mutations in the neuraminidase gene can also produce strains resistant to zanamivir.[48,49] Resistant strains of influenza A/NWS-G70C(H1N9) and B/Hong Kong/8/73 were produced by passaging in the presence of zanamivir. Sequence analysis found glycine for glutamate substitutions in both viruses in a location beneath the active site of the neuraminidase enzyme (position 119 in influenza A, 117 in influenza B). These glutamate residues have been absolutely conserved in all known influenza neuraminidases and are thought to participate in the interaction between the neuraminidase and the guanidino group of zanamivir. No differences were found in the mutant influenza A virus haemagglutinin as compared to its parent virus. In the influenza B variant, 2 asparagine to serine mutations were identified at positions 145 and 150 of the haemagglutinin. These mutations could possibly result in a decrease in the affinity of the haemagglutinin for its cellular receptor by changing the site of attachment of a critical glycosyl group.

Other studies, in which influenza A/Turkey/Minnesota/833/80 (H4N2) was passaged in the presence of zanamivir, have produced resistant variants with substitutions of alanine, aspartate, or glycine for glutamate at position 119 of the neuraminidase.[50] Such mutants have reduced infectivity in mice[31] but can cause febrile responses in ferrets. An independent study using the same parent virus yielded a resistant strain with lysine substituted for the arginine invariantly located at position 292.[51] This is 1 of 3 arginines that form a pocket within the neuraminidase active site where the substrate is bound. This binding induces a conformational change in the substrate which is felt to be important for catalysis. These variants were less resistant to zanamivir than the glutamate 119 mutants by neuraminidase inhibition testing. Importantly, they were also found to have decreased infectivity in mice (approximately 500-fold compared with wild type), which indicates reduced viral fitness. The possible frequency and importance of neuraminidase variants during the clinical use of zanamivir is yet to be determined. It is possible that higher local drug concentrations may retard the development of resistance to neuraminadase inhibitors, especially if such variants are less replication competent *in vivo*.

The first clinical isolates demonstrating resistance emergence *in vivo* were recovered from an immunocompromised child who had persistent

viral shedding while receiving 2 weeks of nebulised zanamivir therapy for severe influenza B pneumonia.[55] After 8 days an isolate was recovered which possessed a haemagglutinin mutation (Thr 198 Lys) conferring altered antigenicity and reduced binding to Sia ∝(2-6)-containing receptors on human cells due to altered glycosylation near the receptor binding site. After 12 days a dual mutant also possessed a catalytic site change in neuraminidase (Arg 152 Lys) was recovered. This mutant showed a 1000-fold reduction in enzyme inhibition by zanamivir but also reduced enzymatic activity (3 to 5%) and poor replication *in vivo* in ferrets. It appears that the haemagglutinin mutation complemented the loss of neuraminidase inhibition by zanamivir to reduce viral dependence on neuraminidase function.

3.2 GS4071

Because of fewer studies, much less has been reported about the development of resistance to GS4071 by influenza viruses as compared with resistance to zanamivir. In general, variants which are resistant to zanamivir on the basis of haemagglutinin variants show cross-resistance to GS4071. In contrast, GS4071 retains antiviral activity against zanamivir-resistant variants with Glu^{119} Gly mutations, presumably because GS4071 does not possess a guanidino moiety that interacts with this amino acid. An initial study, however, suggests that resistance to this agent can also occur due to mutations of viral haemagglutinin and neuraminidase.[53] Resistant strains of A/NWS/G70C were produced after passage in the presence of 6-carboxamide, a newer neuraminidase inhibitor. Three haemagglutinin mutations were identified which were felt to be able to alter the affinity of the haemagglutinin for its cellular receptor. The neuraminidase mutations involved substitution of lysine for arginine in position 292 which disrupts the 3 arginine pocket important in substrate catalysis. This is one of the same mutations selected *in vitro* by zanamivir.[51] When tested against a panel of neuraminidase inhibitors (zanamivir, 4-amino-Neu5Ac2en, 6-carboxamide and GS4071) by enzymatic assays and in cell culture, each variant was resistant to all inhibitors with the greatest degree of resistance being to GS4071 (approximate 10 000-fold decrease in susceptibility). This is probably related to the lack of formation of a necessary hydrophobic pocket within the active site due to the altered amino acid sequence of the neuraminidase enzyme. This mutation was associated with an 80% decrease in relative enzymatic activity compared with the parent neuraminidase and this variant replicated and spread less efficiently in cell culture.

4. Other Neuraminidase Inhibitors

Due to the obvious importance of influenza virus neuraminidase in pathogenicity, and the early successes seen with neuraminidase inhibitors thus far, it is not surprising that further work is ongoing to find or design neuraminidase inhibitors with greater potency, more favourable pharmacological properties and other beneficial aspects in comparison with those described above. Using computer modelling and knowledge of the crystal structure of influenza neuraminidases, regions of the NA active site that are important in substrate binding have been identified.[56] This work has been able to accurately predict the binding regions for the carboxylate, acetamido and glycerol groups of DANA. It was also suggested that addition of an amino group at the C4 position of DANA would enhance inhibitor binding to the NA active site, as would replacement of the carboxylate with a phosphonate. This latter prediction was demonstrated by White et al.[57] who created a phosphonate analogue of DANA. The α anomer of this molecule (ePANA) showed 100-fold greater inhibition of the neuraminidase of influenza A (N2) than sialic acid, a weak inhibitor of influenza neuraminidase and 10-fold greater inhibition of the N9 neuraminidase. The crystal structure of this inhibitor-neuraminidase complex showed no distortion of the active site. It is postulated that the increased inhibitory activity of this new compound is due to strong electrostatic interactions between its phosphonate group and the arginine pocket of the NA active site and/or to a lower distortion energy requirement.

The substitution of the chemically simpler benzene ring of 4-*N*-acetyl aminobenzoic acids for the dihydropyran ring of DANA has also produced compounds with neuraminidase inhibitory activity.[58] The most potent inhibitor produced [as determined by *in vitro* inhibition of influenza A/Tokyo/67 (N2) and influenza B/Memphis/3/89] was 3-guanidinobenzoic acid (EC_{50} = 10 μmol/L). X-ray crystallography showed that this inhibitor occupied the glycerol-binding subsite as opposed to the guanidino-binding subsite of the neuraminidase active site. The symmetry of this compound prevents structural constraints and thus allows for binding at either the glycerol- or quanidium-binding site. The observation that binding preferentially occurs in the glycerol-binding site suggests that this is a more energetically favourable interaction. This feature may be useful in the development of clinically useful influenza virus neuraminidase inhibitors, but potency will need to be greatly enhanced.

Other agents under investigation as potential influenza virus neuraminidase inhibitors include the 9-amino- and 9-*N*-acyl-5-trifluoroacetyl methyl V-ketosides and their 2,3- didehydro analogues.[59] One of these compounds, of the 2,3-didehydro type, had an IC_{50} (concentration which inhibits 50% of strains) of >7.8 μmol/L when inhibition testing of the influenza A/PR/8/34 (H1N1) neuraminidase was performed. The substitution of the triol group of zanamivir with a hydrophobic group linked by a carboxamide at the C6 position has produced an additional group of neuraminidase inhibitors.[60] Other mechanism-based inhibitors with modest potency have been described.[61] The investigation of the anti-influenza and neuraminidase inhibitory activity of some naturally occurring compounds has also produced some interesting results. The plant flavonoids isoscutellarein and isoscutellarein-8-methyl ether (F36) derived from the leaves and roots of *Scutellaria baicalensis*, respectively, have shown inhibitory activity against influenza[62] and specifically against influenza virus neuraminidase[63] without significant toxicity in mice.

5. Conclusions

Neuraminidase plays a critical role in the pathogenesis of influenza virus infections. Interference with the activity of this enzyme by the sialic acid analogues zanamivir and GS4104 has been shown to provide significant antiviral effects. Decreases have been seen in both clinical and laboratory markers associated with influenza infection. These agents appear to offer the benefit of activity against both influenza A and B viruses with minimal adverse effects.

The data available at this time suggest that the neuraminidase inhibitors may soon fill a large void in the current anti-influenza armamentarium. However, several questions remain to be answered. Studies are ongoing to assess the efficacy of systemically administered zanamivir, because one of its current major limitations is its topical form of administration which may be poorly accepted by some patients, particularly young children and noncooperative elderly individuals. The results of studies of GS4104 in naturally occurring influenza infections are awaited to determine if the initial successes seen *in vitro*, in animal models and in experimental human influenza will be confirmed. Another hurdle is the development of formulations that can be used in paediatric populations. The potential for development of viral resistance to the neuraminidase inhibitors and its clinical and epidemiological significance will require continued investigation.

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