Influenza virus infection causes substantial morbidity and mortality worldwide, but current options for control are limited. Although major advances have been made in antiviral therapy of infections such as herpes and HIV, a "magic bullet" for influenza has proved elusive. The influenza neuraminidase enzyme is an attractive target for antiviral intervention, its active site is antigenically conserved in all clinically relevant strains and is critical to viral replication. The authors consider the subject of neuraminidase inhibition and discuss, in particular, the development of the oral agent Ro640796 (GS4104). The concept of neuraminidase inhibition is likely to lead to a major breakthrough in the control of influenza.

In recent years, major advances have been made in antiviral chemotherapy, most notably in the treatment of herpes virus, cytomegalovirus and human immunodeficiency virus infections. The core principle of antiviral drug intervention is the specific inhibition of the viral life cycle, which translates directly into an interruption of viral replication and a reduction of infectious particles.

Influenza virus infection presents a substantial medical, epidemiological and economic burden (see Boxes 1 and 2). The dramatic impact of morbidity and mortality associated with influenza has been recognized since at least the time of Elizabeth I of England. Excess mortality has been documented since 1889 and the infamous 1918 outbreak confirmed that influenza is truly "one of the last major plagues". With an annual attack rate of 5–20%, outbreaks of influenza virus affect a considerable proportion of the population each year. Such influenza outbreaks translate into millions of work-days lost, hundreds of thousands of hospitalizations, tens of thousands of deaths and billions of dollars in healthcare costs. In pandemics, which occur every 10 to 40 years, the impact of influenza can be even higher: the 1918 pandemic influenza killed an estimated 20 million people worldwide. The recent outbreak of a new highly pathogenic influenza strain (H5N1) in Hong Kong highlights the continuous nature of the threat.

Current options for the control of influenza are limited. An inactivated influenza vaccine is currently available and is the mainstay of public health preventative measures against influenza in the high-risk population. However, influenza vaccines provide only limited protection because of antigenic variation of the influenza virus, and new vaccine components and revaccination are needed annually (see Box 3). Antivirals have been available since the early 1980s, but they are not widely used due to a limited spectrum of activity, their side-effect profiles and the rapid development of influenza viral resistance. New therapeutic and prophylactic options for the control of influenza are therefore warranted. The discovery that the active site of influenza neuraminidase is highly conserved offers the potential for effective antiviral therapy and prophylaxis. This enzyme is essential for viral replication and its active site remains unchanged during genetic drifts and shifts, giving the potential for target drugs to be active against all influenza strains.
clinically relevant influenza strains. Influenza neuraminidase therefore became an attractive target for antiviral intervention and the focus for rational drug design. If used promptly after infection, drugs emerging from the concept of neuraminidase inhibition could limit the burden of disease by directly targeting the cause of influenza illness, the virus.

**Targeting influenza neuraminidase**

Influenza viruses belong to the orthomyxovirus family of viruses, and types A and B cause clinically relevant disease states. Figure 2 shows the structure of the influenza A virus. The major surface proteins, which project radially from the outer lipid bilayer, are haemagglutinin and neuraminidase. Haemagglutinin mediates the binding of the virion to sialic acid-containing receptors on the surface of target cells in the respiratory tract. It also mediates the fusion of the viral and cell membranes. This protein is the major target of neutralizing antibodies (and therefore influenza vaccines), but it is highly variable. The mushroom-shaped neuraminidase protein facilitates the release of newly formed virions from the host cell surface and prevents their aggregation by cleaving host terminal sialic acids. The enzyme’s active site is an excellent example of the importance of its three-dimensional structure for enzymatic function, and suggests that development of resistant neuraminidase strains could be hindered, as any change in this vital structure might reduce the viability of the virus. Once this important discovery had been made, neuraminidase inhibition became an attractive concept for antiviral intervention. Target drugs, unlike vaccines that protect only against certain influenza strains, would have the potential to show broad and persistent activity against all clinically relevant strains of influenza.

The development of sialic acid analogues to inhibit the neuraminidase active site is an excellent example of how rational drug design can be used to inhibit a target protein. The first analogue, 2-deoxy-2,3-dehydro-N-acetyl neuraminic acid (DANA or Neu5Ac2en), was developed in 1969 (Ref. 16). While this compound inhibited neuraminidase, it lacked specificity for viral neuraminidases. The elucidation of the crystal structure of this enzyme has revealed important features of the active site. The active site is deep and lined with charged residues that interact with the substrate (sialic acid) of influenza neuraminidase.

**Neuraminidase**

Neuraminidase is an essential enzyme for influenza virus replication, as it catalyses the release of newly formed virions from the host cell surface and prevents their aggregation by cleaving host terminal sialic acids. The enzyme’s active site is an excellent example of the importance of its three-dimensional structure for enzymatic function, and suggests that development of resistant neuraminidase strains could be hindered, as any change in this vital structure might reduce the viability of the virus. Once this important discovery had been made, neuraminidase inhibition became an attractive concept for antiviral intervention. Target drugs, unlike vaccines that protect only against certain influenza strains, would have the potential to show broad and persistent activity against all clinically relevant strains of influenza.
Influenza neuraminidase was a key turning point, allowing the rational design of more potent and specific inhibitors. Modifications to Neu5Acen were made using computerized analysis of the enzyme-substrate transition-state complex and were found to greatly increase affinity for the active site. This led to the development of 4-guanidino Neu5Acen (GG167 or zanamivir), which shows potent and selective inhibition of neuraminidase in vitro. More recently, innovative compounds that incorporate a carbocyclic structure into the molecule have been developed; the arrangement offers greater chemical stability and these groups of people are more likely to require hospitalization than the general population. As the influenza virus replicates in the upper and lower respiratory tract, complications caused by primary and secondary bacterial infections in these sites are common. Otitis media occurs in approximately 12% of children, and conjunctivitis is also a common complication in this patient population. Pneumonia can be caused by the influenza virus itself, but as the natural body defences have been weakened by the influenza infection, also by secondary infections of bacteria. Pneumonia is particularly common in children and the elderly and has a mortality rate of 7–42%, depending on the infecting organism. Less common complications include Reye’s syndrome, Guillain-Barre syndrome, encephalopathy, myopathy and myocarditis.
Proving the concept: in vitro activity and early clinical studies

Both Ro64-0802 and GG167 potently inhibit neuraminidase activity at low nanomolar concentrations in all human influenza A and B strains tested (Table 1). Ro64-0802 also inhibits neuraminidase activity in various avian influenza strains. Ro64-0802 is not cytotoxic in canine kidney cell culture assays, even at concentrations as high as 1 mM. As predicted during drug design, Ro64-0802 is highly selective for influenza neuraminidase, having little or no inhibitory activity against neuraminidases from human, bacterial or other viral sources.

The clinical efficacy of GG167 has been demonstrated in the prevention and treatment of experimental influenza infection. Hayden et al. reported the results of four randomized, double-blind, placebo-controlled trials that evaluated intranasal GG167 (3.6–16 mg two to six times daily) in the prevention and treatment of experimental influenza A (H1N1) infection in volunteers. Overall, GG167 prevented laboratory-proved infection and febrile illness in 82% and 95% of subjects, respectively (both \( p < 0.001 \) vs. placebo).

Early treatment of experimental infection with GG167 in these studies reduced peak viral titres, the duration of viral shedding, the frequency of illness and other measures of illness compared with placebo. GG167 treatment also reduces the impact of influenza virus infection on patients’ productivity and health status and the number of contacts made with healthcare professionals. GG167 is currently undergoing Phase III trials, and is at the pre-registration stage in Australia.

GG167 has low oral bioavailability and must be administered by the inhalation or intranasal routes.

**Box 3. Virus variability**

Influenza viruses are divided in types A, B and C, with only types A and B causing clinically relevant disease. Influenza A viruses can infect horses, pigs, seals and a large variety of birds as well as humans, while type B infects humans only. Type A viruses are further subdivided on the basis of their two surface antigens, haemagglutinin (H) and neuraminidase (N). The three strains of influenza A virus which commonly affect humans are H1N1, H2N2 and H3N2. Very recently an influenza virus, H5N1, was isolated from humans, which had originated in chickens in Hong Kong.

Influenza viruses undergo significant variation in their surface proteins. Infection with, or vaccination against, one subtype of virus confers little or no protection against a different one, and long-lasting immunity cannot therefore be achieved. Influenza B viruses have more antigenic stability than influenza A, but antigenic variation does occur. Antigenic variation results in major influenza epidemics, as new variants of influenza virus occur. The characteristics of the currently-circulating strains form the basis for selecting those strains to be included in each year’s influenza vaccine.

Two types of variation occur. Antigenic drift is the steady accumulation of point mutations that result in amino acid changes in the antigenic sites of the haemagglutinin and/or neuraminidase proteins. These changes reduce antibody binding, thereby reducing pre-existing host immunity within the population and facilitating the spread of epidemic influenza. Antigenic shift is a dramatic and more abrupt change, occurring in the haemagglutinin and/or neuraminidase surface proteins of influenza A viruses. Such changes occur as a result of the replacement of an entire viral gene segment with one from an animal (e.g. avian influenza virus). As the population has little or no immunity to these new strains, they cause pandemics associated with unusually high morbidity and mortality. In addition to antigenic drift and shift, reintroduction of an older strain of virus is a potential cause of outbreaks.

Unlike haemagglutinin, the structure and amino acid sequence of the active site of the neuraminidase surface protein is conserved across antigenically diverse strains, even though the rest of the protein may vary (Fig. 4). This makes neuraminidase an attractive target for influenza control.
Ro640796: an orally available neuraminidase inhibitor prodrug

A strategy for convenient administration and reliable drug delivery to the various sites of influenza infection is the development of orally administered neuraminidase inhibitors. Although Ro640802 is more lipophilic than GG167, its oral bioavailability is similarly low, and so an ethyl ester prodrug of Ro640802, known as Ro640796, was developed to improve oral bioavailability. This compound undergoes rapid enzymatic conversion to the active parent drug following gastrointestinal absorption (Fig. 5) causing high and sustained plasma concentrations of Ro640802 (the active drug) in all animals tested.

Whole body autoradiography in rats showed that radiolabelled Ro640802 is systemically distributed, with a half-life of approximately 5 h in most tissues. Systemic distribution of Ro640802 concentrations in the lung were approximately twice those in plasma at 6 h post-dose, and 30-fold higher at 24 h post-dose. Importantly, distribution of Ro640802 into brain tissue was minimal, indicating a low potential for CNS adverse effects.

Comparison of concentration–time profiles of Ro640802 in bronchoalveolar lavage fluid (BALF) and plasma showed that peak concentrations were similar, however, the elimination half-life in BALF was over fourfold longer than that in plasma. This suggests that the local antiviral effect of Ro640796 may be more prolonged than its plasma levels would predict. Pulmonary changes associated with infection may further increase Ro640802 penetration into lung tissue.

Antiviral activity of oral Ro640796 in animal models

Mouse pneumonia model

When experimentally infected with influenza virus, mice develop pneumonia and exhibit a high mortality. Oral administration of Ro640796 produced dose-dependent protective effects against various influenza viruses in this model. A dose of 1 mg kg\(^{-1}\) day\(^{-1}\) significantly reduced mortality from influenza A/NWS/33 (H1N1) affording 100% protection. A 10 mg kg\(^{-1}\) day\(^{-1}\) dose produced similar effects against...
influenza A/Victoria/3/75 (H3N2) and influenza B/Hong Kong/5/72. These effects were associated with substantial reductions in lung viral titres. Interestingly, Ro640796 (10 mg kg$^{-1}$ day$^{-1}$) increased survival following an 85% lethal dose of influenza A/NWS/33 (H1N1) when its administration was delayed for as long as 60 h after inoculation of virus$^{14}$.

Ferret model
In contrast to mice, ferrets infected with influenza show similar symptoms to those seen clinically in humans (i.e. fever, nasal signs and lethargy), the infection being primarily limited to the upper respiratory tract. Oral Ro640796 doses of 5 mg kg$^{-1}$ and 25 mg kg$^{-1}$ were administered to ferrets twice daily for three days beginning 2 h after inoculation with influenza A England/939/69 (H3N2)$^{22}$. Both the 5 mg kg$^{-1}$ and 25 mg kg$^{-1}$ doses reduced the febrile response to infection, decreasing the area under the curve of temperature increase over time by 58% and 93%, respectively. Ro640796 also prevented the appearance of nasal signs and lethargy, reduced peak viral titres and decreased the local inflammatory response to infection (as measured by the number of inflammatory cells in...
nasal washes). Oral Ro640796 has produced similar effects against influenza A Sydney/97 (H3N2), influenza A England/95 (H1N1) and influenza B Argentina/97 viruses. The distribution of Ro640796 to the various sites of infection was investigated by whole body autoradiography in ferrets. While greatest exposure was to the liver and kidney, concentrations in the lung were high, and exposure in this organ was greater than five times that of blood. There was also good penetration to the middle ear and nasal mucosa.

No drug-related toxicity with Ro640796 was observed in these studies in either mice or ferrets, nor in toxicological studies in rats, even after administration of 800 mg kg$^{-2}$ day$^{-2}$ for 14 days.

Oral administration of Ro640796 in man provides active drug levels and is well tolerated. The pharmacokinetic profile of oral Ro640796 has been investigated in a series of double-blind, placebo-controlled studies in healthy volunteers. Following administration of single Ro640796 doses (20–1000 mg), the maximum plasma concentration and area under the plasma concentration vs. time curve (AUC) for Ro640802 increased proportionately with dose. Peak plasma concentrations of Ro640802 occurred 2.5 to 6 h after administration of Ro640796. The decline in plasma Ro640802 concentrations was slower than that observed for Ro640796, the mean terminal elimination half-life ranging from 6.8 to 9.8 h.

No accumulation of Ro640802 was observed following multiple oral doses of Ro640796 (50–500 mg twice daily). Plasma concentrations of Ro640802 were significantly higher and longer lasting than those of the prodrug and greater than concentrations that have proved active against influenza A and B viruses in vitro and in vivo. The pharmacokinetics of Ro640796 were similar in healthy elderly volunteers and younger subjects, and little inter-subject variability was seen. The elimination half-life of Ro640802 in healthy elderly volunteers was also similar to that observed in younger subjects. These data suggest that no dosage reduction is required in healthy elderly patients.

Ro640796 was well tolerated at doses up to 1000 mg, given either as a single dose or as 500 mg twice daily for seven days. There were no clinically relevant changes in vital signs or laboratory values. Mild nausea and vomiting were seen in some patients at the highest dose used (1000 mg). No serious adverse events were observed.

Ro640796 is effective in experimental influenza infection in volunteers. Two double-blind, placebo-controlled, randomized studies have evaluated the antiviral activity, clinical efficacy and tolerability of Ro640796 in the prevention and early treatment of experimental influenza virus infection. Susceptible, healthy adults were inoculated with influenza A/Texas/36/91 (H1N1). In the prophylaxis study, oral Ro640796 100 mg once daily ($n = 11$) or twice daily ($n = 12$) was initiated 26 h before inoculation and continued for five days. Both Ro640796 regimens proved significantly superior to placebo, preventing viral recovery and influenza-associated illness in all participants.

In the treatment study, oral Ro640796 (20, 100 or 200 mg twice daily or 200 mg once daily) was initiated 28 h after inoculation with influenza virus. In patients with proven infection, oral Ro640796 reduced the median AUC of viral titre in nasal washes for all treatment groups compared with placebo, demonstrating a 100-fold reduction in viral load by 24 h and a 1000-fold reduction by 36 h after treatment. The median duration of influenza virus shedding was reduced from 10.7 h in the placebo group to 5.8 h in the oral Ro640796 group.
Influenza prevention (or reduction in severity) centres upon the administration of inactivated vaccine, which is given six to eight weeks before the start of the influenza season. Intact (whole) virus, split virus and subunit vaccines containing two strains of influenza A and one strain of influenza B are available, all types producing a similar serological response55. Each year the composition of the vaccine is based on those influenza strains expected to appear the following winter. Vaccination is recommended for use in populations at high risk of complications, such as the elderly and those with chronic pulmonary or cardiac disease54.

When the vaccine and epidemic strains are well-matched, high vaccination rates in nursing homes and other chronic care settings induce herd immunity and can reduce the risk of outbreaks. In addition, vaccination can reduce the rates of hospitalization and death due to influenza and its complications54. However, the vaccine has several limitations1,2,4,5,6,7. Antigenic drift in the haemagglutinin antigen and limited immunological response after vaccination necessitate the annual reformation of the vaccine and annual revaccination. In addition, vaccines have variable efficacy (70-90% in adults aged <60 years) depending on the accuracy of the match with circulating viral strains as well as the age and susceptibility of the vaccinee to infection. Efficacy is lower particularly in young children and the elderly, the two populations at an increased risk of complications, with protection rates of 30-70% in those aged ≥60 years.

The most common treatments taken for influenza infection are OTC medications, which give partial symptomatic relief of symptoms. Paracetamol and aspirin are commonly used, but aspirin should not be taken by children under 12 years as it has been linked with the development of Reye’s syndrome56. However, as OTC compounds are not target-specific, their use is further limited by their side-effect profile: adverse events associated with amantadine and rimantadine involve CNS and gastrointestinal disturbances. CNS effects are more common with amantadine (which is also licensed for the treatment of Parkinson’s disease) than rimantadine52,53.

The prodigal concept of Ro640796 allows convenient oral dosing and provides active drug levels of Ro640802 to the various sites of viral replication. Neuraminidase inhibition is likely to emerge as an important new concept in influenza treatment, and by providing additional agents to the anti-influenza armamentarium, could be the long-awaited ‘magic bullet’.

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therapeutic focus

Influenza virus infection causes substantial morbidity and mortality worldwide and creates an enormous economic burden55. Current options for the control of influenza virus infection are limited (see Box 4). The neuraminidase enzyme offers an attractive target for antiviral intervention, not least in view of its antigenic conservation, and it is likely that neuraminidase inhibitors will show broad and persistent activity against all clinically relevant strains of influenza. Two neuraminidase inhibitors have proved this concept in pre-clinical and clinical trials and are currently in Phase III development for the treatment of influenza infection. The prodigal concept of Ro640796 allows convenient oral dosing and provides active drug levels of Ro640802 to the various sites of viral replication. Neuraminidase inhibition is likely to emerge as an important new concept in influenza treatment, and by providing additional agents to the anti-influenza armamentarium, could be the long-awaited ‘magic bullet’.

Box 4. Current options for the control of influenza infection

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