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Review
Approaches and strategies for the treatment of influenza virus infections

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Influenza A and B viruses belong to the *Orthomyxoviridae* family of viruses. These viruses are responsible for severe morbidity and significant excess mortality each year. Infection with influenza viruses usually leads to respiratory involvement and can result in pneumonia and secondary bacterial infections. Vaccine approaches to the prophy-laxis of influenza virus infections have been problematic owing to the ability of these viruses to undergo antigenic shift by exchanging genomic segments or by undergoing antigenic drift, consisting of point mutations in the haemagglutinin (HA) and neuraminidase (NA) genes as a result of an error-prone viral polymerase. Historically, antiviral approaches for the therapy of both influenza A and B viruses have been largely unsuccessful until the elucidation of the X-ray crystallographic structure of the viral NA, which has permitted structure-based drug design of inhibitors of this enzyme. In addition, recent advances in the elucidation of the structure and complex function of influenza HA have resulted in the discovery of a number of diverse compounds that target this viral protein. This review article will focus largely on newer antiviral agents including those that inhibit the influenza virus NA and HA. Other novel approaches that have entered clinical trials or been considered for their clinical utility will be mentioned.

Keywords: Influenza virus; haemagglutinin; neuraminidase; amantadine; ribavirin; zanamivir; GS 4104

Introduction

Infection with influenza viruses can result in an infectious disease for which there is no adequate control. Killed influenza vaccines against this virus have been available for nearly 70 years but, on a community-wide basis, the use of these vaccines has not resulted in a significant decrease in the morbidity or mortality of this disease and has not alleviated the severe financial loss which occurs as a result of hospitalizations and lost productivity. Currently, the only compounds approved by the US FDA for the treatment of influenza infections are amantadine and rimantadine, both of which target the M2 ion channel protein of the virus (Pinto *et al.*, 1992). However, these compounds are not effective against influenza B virus, which does not have an M2 ion channel. Also, virus mutants resistant to amantadine or rimantadine can be isolated readily from individuals treated with these drugs (Belshe *et al.*, 1989; Hayden *et al.*, 1989). Recently, it has been suggested that resistance to amantadine can be overcome by mixing amantadine with other amantadine derivatives that interfere with the ion channel function of M2 from amantadine-resistant viruses (Scholtissek *et al.*, 1998). Ribavirin, a broad-spectrum antiviral agent (Sidwell *et al.*, 1972; Witkowski *et al.*, 1972; Huffman *et al.*, 1973; Oxford, 1975) has been considered for use against influenza A and B viruses, but the clinical trials of this compound have not been encouraging (Cohen *et al.*, 1976; Togo & McCracken, 1976; Magnussen *et al.*, 1977; Smith *et al.*, 1980; Stein *et al.*, 1987) and it has not been approved for the treatment of influenza virus infection.

Historical accounts suggest that influenza has been around for centuries although influenza viruses have most likely been around for many thousands, perhaps millions, of years. Influenza viruses can cause highly contagious, febrile, acute respiratory illness in humans. Epidemics of varying magnitudes of severity have occurred almost annually and pandemics of influenza, which are generally more widespread and have more dramatic impact than annual epidemics, have occurred unpredictably at irregular intervals. In 1918, a pandemic of 'Spanish' influenza was responsible for 20–40 million deaths worldwide, many of

which were among young and previously healthy adults. In 1957, a pandemic of 'Asian' influenza occurred, and most recently, in 1968, there was a pandemic of 'Hong Kong' influenza. During seasonally occurring infections of influenza, newborns and the elderly are most likely to suffer the severest disease, but in the pandemics of 1918, 1957 and 1968 immunologically novel influenza viruses caused severe and often fatal disease in all age groups around the world. Each pandemic was associated with high rates of morbidity, had a considerable impact on social, political and medical structures and caused substantial economic losses.

Influenza viruses are able to undergo rapid and unpredictable antigenic change either through nucleotide changes, especially in the haemagglutinin (HA) and neuraminidase (NA) glycoproteins, as a result of antibody selection pressure (antigenic drift) or through reassortment of viral genomic segments between two different strains of influenza virus (antigenic shift). These evolutionary mechanisms result in the formation of immunologically novel strains of influenza and thus account for the ecological and epidemiological success of these viruses. Consequently, efforts to control influenza through vaccination have been hampered resulting in the highly visible position influenza has attained among emerging and re-emerging diseases (Webster & Kawaoka, 1994; Cox & Fukuda, 1998).

Antigenic drift results in the antigenic change of the surface glycoproteins HA and NA and occurs as part of the ongoing evolution of influenza viruses after they emerge in pandemic form and become established in the human population. In a continuous homeostatic interplay between environmental change and adaptive response, as antibody levels to the pandemic strain rise within the human population, the circulating influenza viruses must change antigenically in order to survive. Thus the newly emerged virus variants, which can escape neutralization by antibody to previously circulating strains, are able to cause disease in individuals that are immune to those previous strains. Annual epidemics occur because there are always sufficient numbers of people in the world that are susceptible to new virus variants. Of interest is new evidence obtained from PCR analysis of influenza RNA in lung tissue samples from victims of the 1918 pandemic that indicates the 'Spanish' influenza virus (A/South Carolina/1/18) underwent a period of adaptation in humans prior to 1918 (Reid *et al.*, 1999). Although the HA from this virus is closely related to that of avian strains, the virus may have been introduced into the human population sometime between 1900 and 1915, undergoing mutational events resulting in a more 'mammalian-like' HA and a highly virulent virus (Reid *et al.*, 1999).

Through antigenic shift, novel influenza A viruses can emerge in the human population as a result of the reassortment of viral genomic segments between two distinct strains of virus. Furthermore, certain influenza viruses, which normally infect swine or chickens and are unrelated to strains of influenza circulating in the human population, can be transmitted to humans. Evidence suggests that emergence of novel pandemic strains may occur either after genetic reassortment between human and animal influenza viruses (antigenic shift) or, alternatively, via direct transmission of an animal strain to humans (Webster, *et al.*, 1992; Webster & Kawaoka, 1994). In 1997, in Hong Kong, an avian influenza virus that previously had not been detected in humans infected 18 people, killing six of them. This influenza A virus was characterized as an H5N1 (H, haemagglutinin; N, neuraminidase) virus (Subbarao *et al.*, 1998; Suarez *et al.*, 1998; Bender *et al.*, 1999). Apparently this virus was transmitted to people from infected chickens and, fortunately, there is no evidence that the virus can spread from person-to-person because no more human cases were identified subsequent to the slaughter of all chickens in Hong Kong. However, the theoretical possibility exists that this virus may undergo some change, which enables person-to-person spread, with devastating results. This is of grave concern because it is generally thought that vaccination would not be adequate to control future pandemics of influenza. Importantly, amantadine and rimantadine might also be inadequate owing to their toxic side effects and the ability of influenza viruses to develop resistance to these drugs quite readily. Consequently, the availability of an effective antiviral agent would be of obvious value.

Virus structure and replication

Virus structure

Influenza A and B viruses belong to the Orthomyxovirus family. They are morphologically similar, being approximately 80–120 nm in size. Although the virus is pleomorphic in structure, the filamentous form is the most dominant in fresh clinical isolates. Embedded within the viral lipid envelope are protein spikes, which can be visualized by negative staining. These spikes consist of the HA and NA, more of which will be discussed below. Through the action of HA, both influenza A and B viruses possess the ability to agglutinate red blood cells. Both viruses also have the ability to induce pathogenicity in the human respiratory tract and it is thought that the NA, which cleaves sialic acid residues off carbohydrates and proteins, facilitates release of the virus from the cell and enables viral transport through the viscous mucus layer found in the respiratory tract.

Influenza A and B viruses are both enveloped viruses, and contain a negative-stranded RNA genome that exists in eight distinct segments. Through differential splicing of

two of the RNA genomic segments, the viral genome encodes a total of 10 proteins. Each genomic segment is packaged into a nucleocapsid structure. The RNA genome of influenza virus has an innately high rate of genetic recombination owing to the reassortment of the viral genomic segments. Also, there is a high mutation rate as a result of the error-prone RNA polymerase, which can enable the selection of pre-existing virus variants under antibody selective pressure.

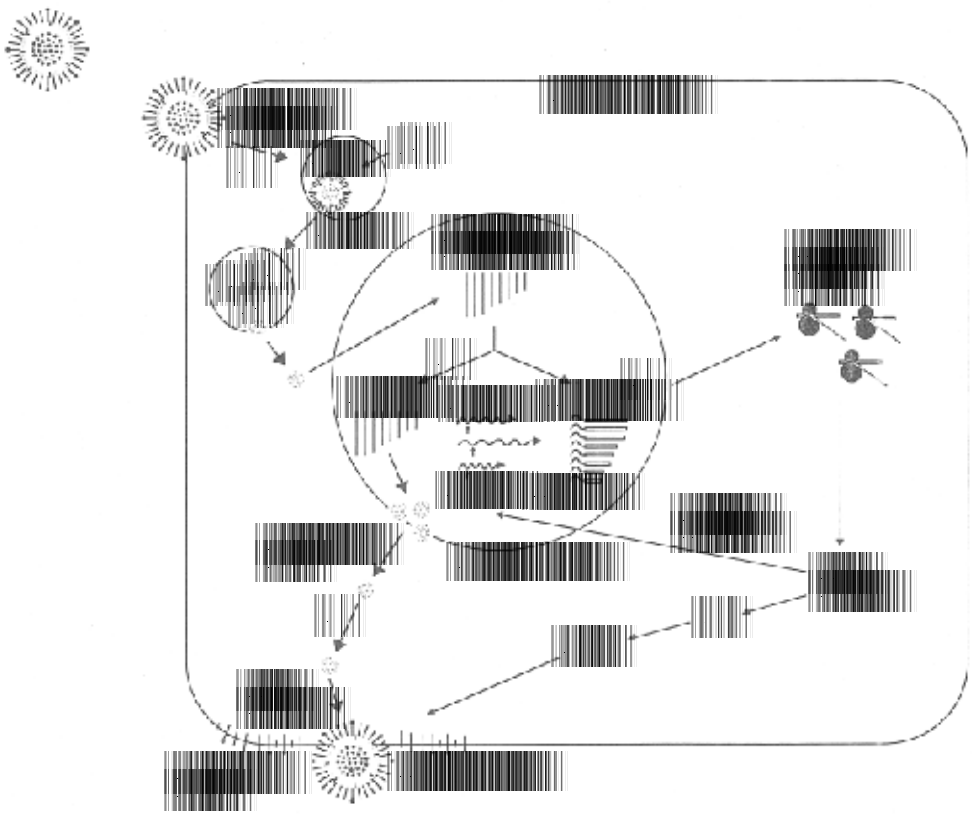
The 10 viral proteins can be categorized into four groups: (1) The nucleocapsid proteins include the nucleoprotein (NP) and the three largest proteins (P proteins). The P proteins make up the viral RNA polymerase, which is located as a complex at the 3′ end of each template genomic RNA, and are encoded by viral genomic segments one, two and three. All of the nucleocapsid proteins are functional in the nucleus of the host cell and thus contain nuclear localization signals that direct them to the nucleus subsequent to their synthesis in the cytoplasm. (2) The envelope proteins include HA and NA, which determine the predominant epidemic subtype, for example; H1N1, H2N2, H3N1, etc (as mentioned, the virulent avian influenza recently isolated in Hong Kong was subtype H5N1). HA and NA make up the characteristic 'spikes' seen during electron microscopic analysis of the virus. HA is encoded by RNA segment four and mediates attachment of the virus to the host cell receptor, which is a glycoconjugate that terminates with an *N*-acetyl neuraminic (sialic) acid. HA is also responsible for the ability of the virus to agglutinate red blood cells and it mediates the low pH induced fusion of the viral envelope to the endosomal membrane of the host cell. NA is encoded by RNA segment six and catalyses the enzymatic removal of sialic acid residues from any glycoconjugate including the oligosaccharide side chains that make up the HA and NA themselves. (3) The non-glycosylated matrix proteins of influenza A viruses include M1 and M2. Influenza B virus does not have a M2 protein. These proteins are encoded by RNA segment seven by differential transcription of a bicistronic gene. M1 is the major product of segment seven and is the major matrix protein, which is found on the internal side of the viral envelope. M1 is the most abundant structural protein of the virus and serves as a bidirectional 'transport chaperone', promoting export from the cytoplasm and inhibiting import to the nucleus of the newly synthesized viral ribonucleoprotein (Martin & Helenius, 1991). M2 is the smaller protein encoded by segment seven and is translated in a +1 reading frame more than 700 nucleotides from the 5′ end of the gene. It is a homo-tetrameric, non-glycosylated protein that is associated with the membrane and is present in virions in low molar amounts. M2 plays a pH regulatory function that influences the structural properties of HA (Ciampor *et al.*, 1992). Inhibition of the function of M2 by amantadine causes a conformational change in the HA of influenza A viruses (Ciampor *et al.*, 1992) and mutations in the M2 coding region confer resistance to this antiviral agent (Hay *et al.*, 1985). Using a *Xenopus* oocyte expression system, it was demonstrated that the transmembrane domain of M2 forms an ion channel, which is regulated by pH (Pinto *et al.*, 1992). (4) The non-structural proteins of influenza include NS1 and NS2, both of which are encoded by the bicistronic RNA segment eight through differential splicing. Neither NS1 nor NS2 is found in the virion and the functions of these proteins are not well understood. However, it has been demonstrated that viral replication can occur without the NS gene (Huang *et al.*, 1990) but NS proteins are necessary for the formation of infectious particles (Enami *et al.*, 1991). Furthermore, the transport of NS1 from the cell nucleus to the cytoplasm appears to play a major role in regulating the splicing of the mRNA for NS1 itself (Alonso-Caplen & Krug, 1991). In addition, NS1 has been shown to inhibit the interferon-induced double-stranded RNA-dependent protein kinase (PKR), which is a key mediator of the antiviral and antiproliferative effects of interferons (reviewed in Staehli, 1990). Recent work suggests that NS1 inhibits PKR by sequestering double-stranded RNA activators (Lu *et al.*, 1995) or by binding directly to PKR, preventing its activation (Tan & Katze, 1998). Furthermore, it was shown that influenza NS1 temperature-sensitive mutants of influenza A virus, which are deficient in RNA binding at the non-permissive temperature, failed to inhibit PKR in infected cells, and this was correlated with restricted viral protein synthesis (Hatada *et al.*, 1999). Moreover, it has been shown that an influenza virus lacking NS1 is highly pathogenic in STAT1 -/- mice, which are deficient in the interferon response pathway, but is not pathogenic in normal mice (Garcia-Sastre *et al.*, 1998). Taken together, these observations indicate that NS1 may play a role in virus pathogenicity.

Recently, NS2 was shown to contain a domain that has similarities to a nuclear export signal and that can interact with cellular nucleoporins. Furthermore, NS2 was demonstrated to act as an adapter molecule between viral ribonucleoprotein complexes and thus mediate the nuclear export of virion RNAs (O'Neill *et al.*, 1998).

Virus replication

Influenza virus replication can be divided into four discernable phases: (1) virus attachment and penetration into the host cell; (2) transcription of the viral genome and translation of viral proteins; (3) replication of the viral RNA; and (4) assembly of the virions and subsequent release from the host cell. The influenza virus replication cycle is shown in Figure 1.

Figure 1. The replication cycle of influenza viruses



The four discernible phases of influenza virus replication are: (1) virus attachment and penetration into the host cell; (2) transcription of the viral RNA and translation of viral proteins; (3) replication of viral RNA and (4) assembly of virion and subsequent release from the host cell. Adapted from Levine (1992).

Attachment and penetration. Attachment of the virus to sialoglycoproteins on the host cell membrane is mediated through the viral HA. HA is synthesized as a precursor (HA0) and is subsequently cleaved by a host cell trypsin-like protease to HA1 and HA2 subunits. Influenza viruses in which HA is not cleaved can attach to, but not penetrate the host cell and thus are not infectious (Klenk *et al.*, 1975; Lazarowitz & Chopin, 1975). The HA molecule exists as a trimer of identical HA subunits that fold in such a way as to accommodate the sialic acid residue of the host cell receptor. Also, at this time the cleavage of HA0 to HA1 and HA2 subunits is catalysed by a trypsin-like host cell protease. As a result of a low-pH-induced conformational change in HA, the highly conserved hydrophobic fusion domain at the N terminus of HA2 inserts into the lipid bilayer of the host cell and promotes fusion of the virus envelope to the endosomal membrane, thus allowing entry of the virus ribonucleoproteins (RNPs) into the cytoplasm.

Transcription and translation. Transcription of the negative strand influenza virus RNA is carried out by the viral RNA-dependent RNA polymerase (RdRp) complex within the nucleus of the cell (Herz *et al.*, 1981). The transcriptase complex is unable to initiate mRNA synthesis or to modify the 5′ termini of mRNA molecules by capping and methylation, so viral transcription is primed by capped and methylated 5′ termini, which are cleaved from host cell capped messages by the viral endonuclease in a process referred to as 'cap snatching' (Krug, 1989, and references therein). This process produces a host cell-derived oligonucleotide that is available for the priming of viral messages, each of which contains 10–13 nucleotides of host cell mRNA at the 5′ end. The viral mRNA is then transcribed from the viral genome template by the concerted action of the proteins PB1, PB2 and PA, which together form the P complex. It has been demonstrated that these proteins form a non-covalently linked complex (Detjen *et al.*, 1987) that moves along the template as the viral transcript is elongated (Braam *et al.*, 1983). The viral messages are then transported to the cytoplasm where they are translated into their respective viral proteins.

Replication. The replication of the viral RNA genomic segments is regulated in conjunction with viral transcription

by viral proteins. This process requires the nucleocapsid structural protein, NP. As the assembly of nucleocapsids ensues, the pools of NP proteins are depleted below the level required for translation. This signal switches on the translation of viral proteins, the end result of which is an increase in NP concentration. Viral RNA genome segments are copied into positive strand anti-genome segments, which then must be copied back into the negative strand viral genomic segments. Although the precise role of each of the P proteins in viral RNA replication has not been determined, data derived from experiments using temperature sensitive mutants of influenza virus indicate that PA and PB1 play important roles in this process (Krug *et al.*, 1975).

Assembly and release. The last phase of influenza virus replication includes the assembly of the virions and their release from the cell. The virus nucleocapsid is assembled in the nucleus and the viral envelope is acquired as the virus buds from the host cell. The matrix protein (M1) serves as a connector molecule between the nucleocapsid and the C-terminal cytoplasmic domains of the glycoproteins that are located in the nascent viral envelope patches. At the end of the virus replication cycle, the viral NA, which is located in the virus envelope, acts as a sialidase to cleave sialic acid moieties from the host cell receptor, thus facilitating the release of the virus from the cell. This desialation process also results in the removal of sialic acid residues from viral glycoproteins and thereby prevents the aggregation of viral progeny. NA may also facilitate movement of the virus through the respiratory tract by separating virus particles from inhibitory mucopolysaccharides and by releasing HA from host cell receptors.

Anti-influenza virus agents

With a working knowledge of the influenza virus replication cycle, a number of approaches, which target various points during the virus replication, can be considered. This review article will discuss a few of the most recent advances in the development of agents for chemotherapeutic intervention in influenza virus infection.

Sialic acid receptor antagonists

The viral HA protein plays two critical roles in the replication of influenza virus. The first is to mediate attachment of the virus to the host cell via the sialic acid receptor and the second is to promote fusion of the virus envelope to the endosomal membrane of the host. The three-dimensional structure of HA has been elucidated by X-ray crystallography (Wilson *et al.*, 1981) and the precise mapping of the binding site and fusion domains (Wiley *et al.*, 1981) will hopefully enable the design and development of selective agents that can interfere with the two functions of HA.

The interaction of HA with its receptor is weak but is facilitated by cooperative binding. Consequently, efforts to identify and develop single-ligand inhibitors of this binding interaction have not met with much success. The weak binding can be mechanistically explained by a deficiency of charge–charge interactions within the binding pocket of HA involved in the attachment to *N*-acetylneuraminic acid (NANA). The groups on NANA that participate in the binding of HA have been determined in experiments in which the HA crystals have been soaked in various NANA analogues (Kelm *et al.*, 1992). A systematic effort to identify sialoside HA inhibitors has resulted in the identification of a number of weak inhibitors of influenza HA binding, as evidenced by inhibition of influenza virus attachment to red blood cells (Gamian *et al.*, 1991; Spaltenstein & Whitesides, 1991; Sabesan *et al.*, 1991). One compound, 3,6-disialoside, displayed inhibition of HA binding with an IC_{50} value of 0.18 mM and appeared to be a specific inhibitor (Sabesan *et al.*, 1991). Clearly, however, much greater inhibitory activity would be required to validate the clinical feasibility of targeting HA-mediated binding.

Cleavage activation inhibitors

The fusion domain of HA2 is generated by the proteolytic cleavage of HA0 to HA1 and HA2, a process that is absolutely required for virus infectivity. The cleavage of HA0 has for a long time thought to have been catalysed by cellular endopeptidases (Klenk *et al.*, 1975; Lazarowitz & Chopin, 1975). Recently, evidence has been presented that furin, a cellular protease that is ubiquitous and has an acidic pH optimum, is the enzyme that activates avian influenza HA0 by cleaving it to reveal the fusion domain located in HA2 (Walker *et al.*, 1994). Furthermore, the virulence of a particular strain of influenza virus depends on the ability of its HA0 to be cleaved by cellular proteases (Klenk *et al.*, 1974; Garten *et al.*, 1981; Walker *et al.*, 1992).

Reasonably, it has been considered that inhibition of this cleavage would result in the inhibition of subsequent rounds of viral replication. Indeed, the protease inhibitors, camostat and nafamostat mesilate have been shown to inhibit both influenza A and B virus replication in tissue culture (Hosoya *et al.*, 1993). Along these lines, a number of low molecular weight protease inhibitors have been shown to inhibit influenza virus replication by 100-fold and reduce virulence in the mouse lung (Zhirnov *et al.*, 1984). Tryptase Clara is a trypsin-like serine protease that is secreted by the Clara cells of the bronchiolar epithelium and has been shown to catalyse the cleavage activation of influenza A virus HA0 (Kido *et al.*, 1992). Pulmonary surfactant, a specific inhibitor of Tryptase Clara, has been

Figure 2. The chemical structure of amantadine

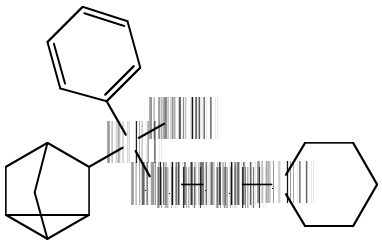
shown to prevent cleavage activation of HA0 (Kido *et al.*, 1993) and results from animal studies have led to consideration of the use of surfactant replacement therapy for treatment of respiratory failure caused by viral pneumonia (Van Daal *et al.*, 1992). However, because a cellular protease is known to catalyse the cleavage of HA0, any agent interfering with this process might have undesirable side effects.

Lysosomotropic agents

The low pH environment of the endosomal compartment, a result of the activity of vacuolar proton ATPase (v-[H^+]ATPase), is required to trigger the transition of HA from the non-fusogenic to the fusogenic conformation (reviewed in Hernandez *et al.*, 1996). Thus, this process might be considered as a target for chemotherapeutic intervention and agents that alter endosomal pH would be expected to prevent virus entry. In fact, early work showed that chloroquine, which causes a rapid rise in the pH inside lysosomes (from pH 5.6 to 6.5), can prevent the uncoating and hence the replication of influenza B virus in MDCK cells (Shibata *et al.*, 1983). It is well documented that amantadine (Figure 2), a basic tertiary amine, can inhibit the function of the M2 protein ion channel (reviewed in Hay, 1992). In addition to this activity, at high concentrations (100 μg/ml) amantadine can elevate endosomal pH (Hay *et al.*, 1985). Indeed, influenza viruses selected for resistance to high concentrations of amantadine contain sequence alterations in HA that enable it to undergo the conformational switch at a higher pH (Daniels *et al.*, 1985). Therefore, it is reasonable to consider that the *in vivo* activity of amantadine may be, in part, due to its ability to alter the pH of the endosome.

Other agents can alter the endosomal pH and thus inhibit virus entry. For example, the anti-Parkinsonism compound norakin (Figure 3; triperiden) inhibits influenza virus replication in cell culture by preventing virus entry (Presber *et al.*, 1984). In subsequent studies it was determined that influenza viruses selected for resistance to norakin contained sequence changes in HA, some of which were identical to those found in the HA of viruses selected for resistance to high concentrations of amantadine (Prosch *et al.*, 1988, 1990). Recently, norakin has been shown to elevate lysosomal pH, thus explaining its effects on virus entry (Ott & Wunderli-Allenspach, 1994). Of

Figure 3. The chemical structure of norakin (triperiden)



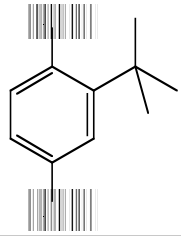
interest is bafilomycin A, a macrolide antibiotic, which is a specific inhibitor of v-[H^+]ATPase. In cell culture, bafilomycin A has been shown to prevent virus entry (Guinea & Carrasco, 1995) and to inhibit influenza A and B virus replication (Ochiai *et al.*, 1995).

Inhibitors of HA-mediated membrane fusion

Using genetic, biochemical, and crystallographic approaches, work from various groups has provided us with an in-depth understanding and appreciation of the structure and function of HA and its importance in the virus replication cycle (Wilson *et al.*, 1981; Daniels *et al.*, 1985; Carr & Kim, 1993; Bullough *et al.*, 1994; reviewed in White, 1990). As mentioned, HA is synthesized as a precursor protein (HA0) and is subsequently cleaved by a host cell protease resulting in the polypeptide subunits HA1 and HA2. Recently, the three-dimensional structure of the ectodomain of HA0 has been solved and compared to that of proteolytically cleaved HA (Chen *et al.*, 1998). The cleavage site of HA exists as a prominent surface loop, which is located adjacent to a novel cavity. Of interest is the observation that amino acid insertions at the cleavage site have been found in highly virulent avian influenza viruses and in the recently isolated Hong Kong (H5N1) virus. These insertions would extend the cleavage loop and thus facilitate intracellular cleavage and thereby enhance pathogenicity (Chen *et al.*, 1998).

The HA1 and HA2 subunits remain covalently linked by a single disulphide bond (reviewed in Wiley & Skehel, 1987). At neutral pH, HA exists in a metastable conformation that is present in the infectious virus particle. In this conformation, the HA mediates recognition of the host cell receptor to initiate the viral replication cycle. Subsequent to internalization of the virus, the low pH environment inside the endosome triggers a dramatic and irreversible structural rearrangement in HA, resulting in a more stable conformer of HA. The hydrophobic fusion domain, which is located in a buried, inaccessible location in the metastable form of HA, is displaced 100 Å toward the target endosomal membrane (Carr & Kim, 1993; Bullough *et al.*, 1994) so that the fusion peptide can be inserted into the target endosomal membrane. Apparently,

Figure 4. The chemical structure of tert-butylhydroquinone (TBHQ)

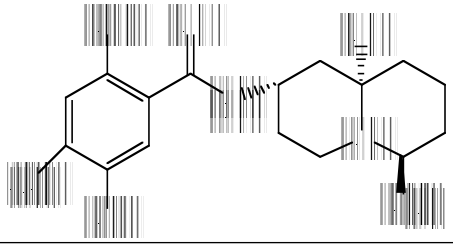


the HA trimers hold the cell and viral membranes in apposition, promoting the fusion of these two membranes and thus facilitating the release of viral RNP complexes into the host cell cytoplasm. Since this process appears to be absolutely essential for virus replication and is mediated by the influenza virus HA, a unique viral protein, the attractiveness of using inhibitors of this step in the virus life cycle has been noted (Daniels *et al.*, 1985; Carr & Kim, 1993).

Recently, a diverse array of small molecules that can affect the low pH-induced conformational change of HA have been identified by various groups. Using a structure-based approach and the DOCK program to identify potential protein domains for ligand binding, Bodian *et al.* (1993) identified tert-butylhydroquinone (Figure 4; TBHQ) as a compound with the potential to interact with the neutral pH conformer of HA. Several measures of the conformational transition of HA to the low pH structure, including haemolysis and fusion peptide exposure (Bodian *et al.*, 1993) and bis-ANS binding (Bethell *et al.*, 1995) were used to show that TBHQ in the low to high micromolar range, was able to inhibit this conformational change and thus influenza infectivity. Influenza virus selected for resistance to TBHQ was subsequently shown to contain sequence alterations in HA that enable the protein to undergo the conformational change at a higher pH (Hoffman *et al.*, 1997).

Another group identified 4-amino-5-chloro-2-hydroxy-N-9-αH-octahydro-6β-methyl-2H-quinolizin-2α-benzamide (Figure 5; BMY 27709) as an inhibitor of H1 and H2 subtypes of influenza virus (Luo *et al.*, 1996). Analysis of BMY 27709-resistant mutants revealed amino acid changes in HA near the 'fusion' peptide at the N terminus of HA2 (Luo *et al.*, 1997). It was suggested that BMY 27709 acts by preventing the movement of the fusion peptide or other domains in HA thereby preventing the low-pH-induced conformational change in HA (Luo *et al.*, 1997).

Figure 5. The chemical structure of 4-amino-5-chloro-2-hydroxy-N-9-αH-octahydro-6β-methyl-2H-quinolizin-2α-benzamide (BMY 27709)



Still another compound, diiodofluorescein (Figure 6; C22), that facilitates the low pH-induced transition of HA to the fusogenic conformation (Hoffman *et al.*, 1997) was identified. While C22 did not induce the conformation transition in HA at neutral pH, this transition occurred more readily at low pH in the presence of C22. Variants of influenza virus selected for resistance to C22 displayed a lower pH-of-haemolysis, suggesting that these variants contained a more stable HA (Hoffman *et al.*, 1997).

Recently, a compound related to podocarpic acid, methyl-O-methyl-7-ketopodocarpate (Figure 7; 180299) was identified in an antiviral screen as a specific inhibitor of influenza A viruses in tissue culture (Staschke *et al.*, 1998). In a standard plaque assay, 180299 was found to be active against influenza A/Kawasaki (H1N1) and influenza A/Ann Arbor/1/57 (H2N2). Generally, 180299 was not active against A/WSN/33 (H1N1), various strains of H3N2 influenza A viruses, or against influenza B/Lee/40.

Figure 6. The chemical structure of diiodofluorescein (C22)

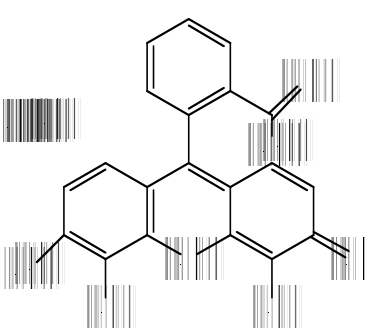


Figure 7. The chemical structure of methyl-O-methyl-7-ketopodocarpate (180299)

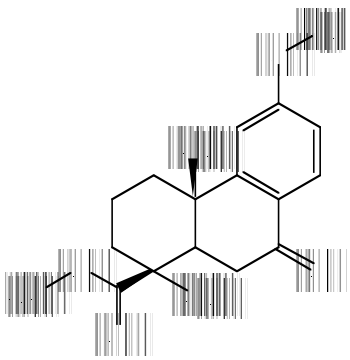


Figure 8. The chemical structure of the N-substituted piperidine CL 61917

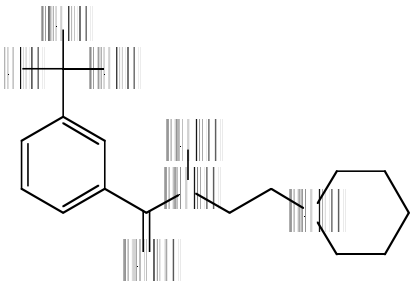
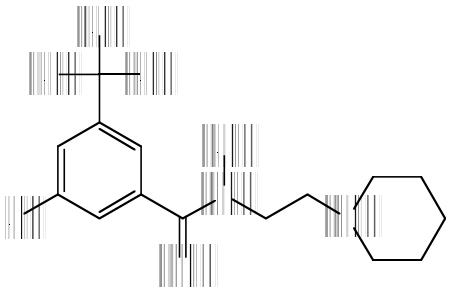


Figure 9. The chemical structure of N-substituted piperidine CL 38531



Reassortant experiments demonstrated that the HA from naturally resistant influenza viruses was necessary and sufficient to confer resistance to 180299. Influenza virus variants that were selected for resistance to 180299 were found to have mutations throughout the HA primary sequence, clustering in one of two regions: the interface between HA1 and HA2 and in a region near the fusion domain of HA2.

Treatment of influenza virions with low pH buffer in the absence of cells results in the loss of infectivity (Guinea & Carrasco, 1995) owing to the change in the conformation of HA to the fusogenic state outside the cell. When these viruses are added to cell culture, they are unable to attach to and penetrate the cells. The pH at which this inactivation occurs varies depending on the viral strain (Maeda & Ohnishi, 1980; Huang *et al.*, 1981; White *et al.*, 1981), thus each influenza virus strain has a characteristic pH-of-inactivation. In light of these observations, influenza virus variants that were resistant to 180299 displayed an elevated pH-of-inactivation. Furthermore, fusion of human erythrocytes by resistant viruses was not affected by 180299, in contrast to fusion mediated by susceptible influenza viruses, which is inhibited by this compound (Staschke *et al.*, 1998). This demonstrated a direct effect of 180299 on the influenza HA-mediated fusion process.

Several compounds have recently been described that inhibit the infectivity of H1, H2 and to a lesser extent, H3 subtypes of influenza A virus. The N-substituted piperidines, CL 61917 (Figure 8) and CL 38531 (Figure 9), appear to inhibit influenza virus replication by interfering with the fusogenic function of the viral HA, as demonstrated by the ability of these compounds to inhibit the low pH-induced haemolysis of red blood cells, infected cell–cell fusion and low pH-induced inactivation of virus infectivity (Plotch *et al.*, 1998). Independent variants of influenza that are resistant to CL 61917 and CL 38531 displayed single amino acid changes that occurred in the stem region of the HA trimer in and near the HA2 fusion peptide. Using computer-assisted modelling, the N-substituted piperidine CL 61917 could be docked in a pocket located in the region delineated by the amino acid changes, in the middle of the stem of the HA trimer in the vicinity of the buried fusion peptide (Plotch *et al.*, 1998). This putative binding site is close to, but not identical to, the putative binding site of the hydroquinone inhibitors discussed above (Bodian *et al.*, 1993; Hoffman *et al.*, 1997).

It is of interest that compounds belonging to such diverse classes have similar biological activities. Influenza virus variants resistant to 180299 were shown to have a higher pH-of-inactivation and the amino acid changes in these variants were similar to those observed in influenza virus fusion mutants (Daniels *et al.*, 1985), norakin^{r} influenza viruses (Prosch *et al.*, 1988, 1990), BMY 27709^{r} mutants (Luo *et al.*, 1997), TBHQ^{r} mutants (Hoffman *et al.*, 1997) and N-substituted piperidine^{r} influenza viruses (Plotch *et al.*, 1998). These mutations most likely lead to alterations in the interaction between subunits of the trimer, resulting in the elevation of the pH optimum for fusion or inactivation (the conformational transition of HA). As postulated previously (Daniels *et al.*, 1985), these amino acid changes may lower the activation energy necessary for the exposure of the N terminal peptide of HA2. It is clear from the work of these various groups that several amino acids are involved in the conformational switch. Although it is not clear if any of the above mentioned compounds will pass all of the hurdles on the way to clinical evaluation, the chemical diversity they represent may provide tools by which to study the conformational switch of HA in greater detail and may point to other compounds that may have clinical utility.

Inhibition of transcription, translation and replication

As previously mentioned, the negative strand influenza virus RNA must be transcribed into the positive strand mRNA for subsequent translation to viral proteins. Additionally, the replication of the viral genomic RNA segments proceeds through a positive strand anti-genomic phase. These processes are carried out by the virally encoded RdRp complex, an enzyme activity that is not present in uninfected mammalian cells. Consequently, it might be possible to find agents that interfere with the viral transcriptase complex and not with host cell RNA polymerase

Figure 10. The chemical structure of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin)

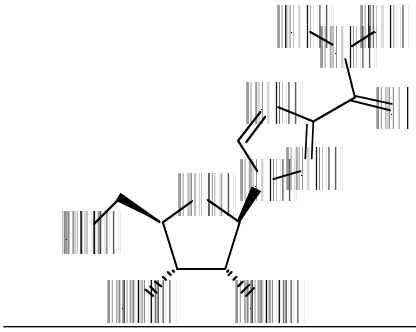
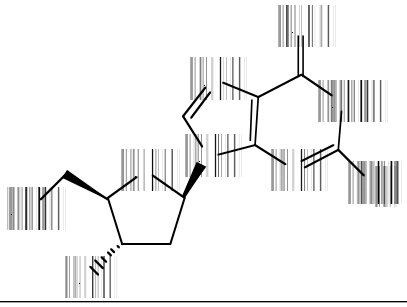


Figure 11. The chemical structure of 2′-deoxy-2′-fluoroguanosine (FDG)



II. Historically, the inhibition of the influenza replicase has been attempted using a number of approaches. In fact, notwithstanding the equivocal clinical trials of ribavirin (Figure 10), the 5′-triphosphate of ribavirin can selectively inhibit viral RNA polymerase (Erikkson *et al.*, 1977). The viral RNA polymerase is a zinc-containing metalloenzyme and chelating compounds, such as EDTA or phenanthrolines, could feasibly inhibit this enzyme by removing zinc. Along these lines, selenocysteine, another chelating agent, has been shown to inhibit influenza RNA polymerase (Ho & Walters, 1977). Other chelating agents, in particular 2-acetylpyridone-3-thiosemicarbazone (Oxford & Perrin, 1977), were found to inhibit the influenza RNA transcriptase *in vitro* but had no antiviral activity *in vivo*. Because metal ions such as calcium or zinc are important cofactors for cellular enzymes that carry out critical cellular metabolic reactions, it is doubtful that chelation offers a practical and selective approach to treating infections caused by influenza virus.

Nucleoside analogues have held a prominent place in the history of antiviral agents and analogues such as acyclovir and azidothymidine have been approved for the treatment of infections caused by herpesviruses and human immunodeficiency virus, respectively. In general nucleoside analogues, after phosphorylation to nucleotide triphosphates, act by interfering with the viral polymerase. Over the years, considerable work has shown that replacement of the hydroxyl group at the 2′ position of the ribose sugar yields nucleoside analogues that have unique biological activities. One such well-studied replacement group is fluorine, the electronegative nature of which lends increased chemical and enzymatic stability (Codington *et al.*, 1964; Montgomery *et al.*, 1986) to the glycosidic bond in the nucleoside analogue and alters the sugar conformation (Guschlbauer & Jankowski, 1980). Of interest to this discussion is the preclinical evaluation of 2′-deoxy-2′-fluororiboside analogues as inhibitors of the influenza virus RNA polymerase. A series of these nucleoside analogues was synthesized and it was determined that the most potent anti-influenza activity was associated with an amino group at the 2 position of the purine base. Within this series of compounds, 2′-deoxy-2′-fluoroguanosine (Figure 11; FDG) and congeners thereof which can be converted enzymatically to FDG, displayed the most potent anti-influenza virus activity in cell culture with antiviral IC_{50} values between 15–23 μM and minimal cytotoxicity (Tuttle *et al.*, 1993).

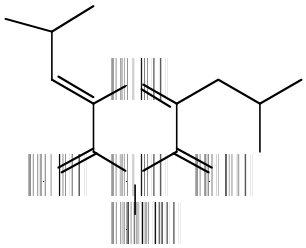
Further work with this series of nucleoside analogues demonstrated that they had potent anti-influenza virus activity in chick embryo fibroblasts (IC_{50} values of 0.01–2.9 μM) and, importantly, were able to reduce mouse lung virus titres to a greater extent (1–3 $\log_{10}$ units) than either amantadine or ribavirin when given orally (Tisdale *et al.*, 1993). It was also demonstrated that FDG is phosphorylated by deoxycytidine kinase and that anti-influenza virus activity correlated with intracellular levels of FDG-triphosphate (Tisdale *et al.*, 1993). FDG was greater than 25- and 120-fold more active against influenza A in human respiratory epithelial cells than in primary rhesus monkey kidney cells or in Madin–Darby canine kidney cells, respectively (Rollins *et al.*, 1993). Efforts to select FDG-resistant virus resulted in the isolation of a variant, which was fivefold more resistant than the parent, but attempts to produce greater resistance were not successful. In other *in vitro* studies, the anti-influenza virus activity of FDG was additive with the NA inhibitor GG167 (zanamivir) or with ribavirin or rimantadine (Madren *et al.*, 1995).

In biochemical studies, it was shown that the triphosphate of FDG is a competitive inhibitor of the influenza RdRp with a K_i of 1 μM. Selectivity at this level was demonstrated since cellular DNA polymerases were inhibited only weakly. In enzyme kinetic studies where GTP was replaced with FDG-TP, the elongation of virus RNA was blocked and the addition of a single nucleotide of FDG-TP to virus RNA caused chain termination, thus blocking subsequent virus transcription. Finally, the RdRp isolated from influenza virus selected for partial resistance to FDG was found to be 10-fold less sensitive to inhibition by FDG-TP (K_i=13.1 μM), thus demonstrating specificity of inhibition for influenza virus RdRp (Tisdale *et al.*, 1995).

In the ferret model of influenza virus infection, FDG was given as a single dose treatment (5–40 mg/kg of body

Figure 12. The chemical structure of 4-[N-benzyl-3-(4-chlorobenzyl)piperidin-3-yl]-2,4-dixobutanoic acid (L 735882)

Figure 13. The chemical structure of 2,6-diketopiperazine (flutimide)



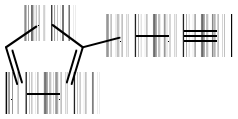
weight given intraperitoneally) 1 h after infection with influenza A virus. Virus replication was inhibited significantly in the upper respiratory tract and there was a demonstrable decrease in fever and nasal inflammation. To reduce viral replication by greater than 100-fold in the lower respiratory tract, three doses of FDG were required. While FDG was effective against influenza B virus in this model, it was not as potent as it was against influenza A virus (Jakeman *et al.*, 1994). Although preclinical data for FDG looks encouraging, to date no information regarding the clinical development of this interesting nucleoside analogue has been reported and the clinical utility of this compound remains to be determined.

As mentioned in the section on virus replication, the transcription of influenza virus mRNAs utilizes primers derived from newly transcribed host cell messages. These primers are 'snatched' from the host cell messages by a viral endonuclease activity that has been associated with the PB2 subunit of the RdRp complex (Nichol *et al.*, 1981; Ulmanen *et al.*, 1981; Braam *et al.*, 1983; Shi *et al.*, 1995). This process is unique and conserved among influenza A and B viruses, making it an attractive target for selective antiviral chemotherapy. Recently, a number of compounds have been identified that inhibit influenza virus endonuclease in a selective manner. One, known as L 735882 (Figure 12; 4-[N-benzyl-3-(4-chlorobenzyl)piperidin-3-yl]-2,4-dioxobutanoic acid) has a multicyclic structure and specifically inhibits the cap-dependent endonuclease function of the influenza virus RNA polymerase complex. L 735882 and related compounds inhibited the synthesis of influenza A and B virus with IC_{50} values of 6 μM and 2 μM, respectively. This compound had no effect on other viruses tested including vesicular stomatitis virus, Newcastle disease virus, murine encephalitis virus or La Cross virus and no cytotoxic effects at concentrations up to 100 μM (Tomassini *et al.*, 1994). Various 4-substituted 2,4-dioxobutanoic acid compounds inhibited cap-dependent influenza virus transcription with IC_{50} values of 0.2 to 29 μM and had no inhibitory effect on other viral or cellular polymerases even at concentrations 100- to 500-fold higher. It was shown that these compounds did not inhibit the initiation or elongation of influenza mRNA synthesis but only the cleavage of capped host cell mRNA molecules. The selectivity of L 735882 was demonstrated by the lack of any inhibitory activity against other nucleases including HIV RNase H, *Eco*RI restriction endonuclease and bovine pancreatic RNase A (Tomassini *et al.*, 1994). Currently, this compound is in preclinical development.

Another inhibitor of influenza endonuclease activity was identified in extracts of the fungal species, *Delitschi confertaspora*. This compound, known as flutimide (Figure 13; 2,6-diketopiperazine), the first natural product to selectively inhibit the cap-dependent transcriptase of both influenza A and B viruses, is a substituted 2,6-diketopiperazine. Like L 735882, flutimide had no effect on other polymerases tested (Tomassini *et al.*, 1996) and, similar to the dioxobutanoic acid derivatives, flutimide specifically inhibited the cap-dependent transcriptase activity (endonuclease) but did not inhibit the initiation or elongation of virus mRNA synthesis. Flutimide inhibited the transcriptase of various strains of influenza virus including A/PR/8/34, A/Japan/305/57, A/Port Chalmers/1/73, A/WSN/33 (H1N1) and B/Hong Kong/5/72 with IC_{50} values from 3.8 to 4.3 μM. Flutimide, like the dioxobutanoic acid compounds, is in preclinical development.

Another approach to the inhibition of influenza virus endonuclease is the use of oligonucleotides that can bind to the viral polymerase but do not prime transcription. It was shown that a 67 nucleotide RNA substrate with a ^{32}P-labelled type 1 cap structure ($m^7G^{32}pppGm$) was specifically cleaved by influenza virus endonuclease, resulting in the production of an 11 nucleotide RNA fragment that was capable of priming transcription (Chung *et al.*, 1994). In an elegant series of experiments, the RNA substrate was systematically truncated and it was demonstrated that only one nucleotide beyond the cleavage site was required for cleavage; only nine nucleotides were required for cleavage whereas only four nucleotides were required for binding to influenza RNA polymerase. These results demonstrated that short capped RNAs are potent, yet non-sequence specific oligonucleotide inhibitors of influenza virus RNA

Figure 14. The chemical structure of 1,3,4-thiadiazol-2-ylcyanamide (LY 217896)



transcription (Chung *et al.*, 1994). Although instructive biochemically and molecularly, it is not clear that this approach will prove technically feasible for the development of clinically useful anti-influenza virus agents.

LY217896 (Figure 14; 1,3,4-thiadiazol-2-ylcyanamide), was also found to inhibit influenza viral replication and is included in this section of the review because of its indirect effects on the viral RNA transcriptase complex. This compound was actually discovered by screening in an *in vivo* mouse model of influenza virus infection (Colacino *et al.*, 1990). Although LY217896 (or its sodium salt, LY253963) displayed no selective anti-influenza activity in cell culture (Colacino *et al.*, 1990; Hayden *et al.*, 1990), the compound displayed potent antiviral activity against respiratory syncytial virus (RSV) and parainfluenza virus type 3 (PIV-3) in cell culture with a selectivity index greater than 100 (Wyde *et al.*, 1990). Furthermore, it was shown that LY253963, when given intraperitoneally at dosages of 1–3 mg/kg/day, was protective against RSV and PIV-3 in cotton rats (Wyde *et al.*, 1990).

LY217896 was shown to be well tolerated and effective against both influenza A and B viruses in the mouse model of infection when given for up to 14 days (Colacino *et al.*, 1990). This compound is highly water soluble and is effective when given in drinking water, in food, by gavage, by intraperitoneal injection or by small-particle aerosolization (Colacino *et al.*, 1990). LY217896 was most effective when given within 3 days of challenge infection but significantly protected mice from influenza challenge even when given up to 96 h post-infection. Importantly, LY217896 proved to be more effective that amantadine in protecting mice against influenza A challenge and more effective than ribavirin in protecting mice against influenza B challenge (Colacino *et al.*, 1990). An antiviral effect was demonstrated by a 1–2 $\log_{10}$ reduction in lung virus titres in mice infected with influenza A/Ann Arbor and treated with LY217896 (50 mg/l in the drinking water, *ad libitum*) (Colacino *et al.*, 1990). In mice treated with LY217896, viral antigen expression in the bronchioles was slight and only patchy in alveolar macrophages and pneumocytes, whereas in untreated mice viral antigen expression was intense and diffuse (Engelhardt *et al.*, 1991). In the ferret model, LY21896 was able to reduce fever caused by influenza B virus infection when given only as a single dose as low as 18.7 mg/m^2. However, there was no demonstrable reduction in virus titre.

In efforts to determine the mechanism of action, two initial observations were very important. One was that influenza virus variants could not be selected *in vitro* for resistance to LY217896 or its sodium salt (Hayden *et al.*, 1990; JC Tang & JM Colacino, personal communication) suggesting the lack of a virus-specific target. The other was that the antiviral activity could be reversed by adding a molar excess of either guanosine or guanine (Colacino *et al.*, 1993). Previously, a related compound, 2-amino-1,3,4-thiadiazole, was shown to inhibit Sindbis virus replication *in vitro* (Malinoski & Stollar, 1981) and it was thought that this compound is converted to the mononucleotide form intracellularly, and as such it is a competitive inhibitor of inosine monophosphate dehydrogenase (IMPDH) with respect to the natural substrate, IMP (Nelson *et al.*, 1977). The end result of the inhibition of this enzyme is the selective reduction of intracellular pools of GTP and, indeed, the antiviral activity of aminothiadiazole was correlated to a drop in GTP pools (Malinoski & Stollar, 1981). Along these lines, it was shown that the antiviral activity and cytotoxicity of LY217896 were correlated with the formation of an intracellular metabolite (Colacino *et al.*, 1993). This metabolite was subsequently characterized as a mononucleotide, mesoionic species that was able to inhibit IMPDH (Ehlhardt *et al.*, 1993; Birch *et al.*, 1995). Thus the reversal of the biological activity of LY217896 by guanosine could be explained by the drop in GTP pools as a result of the inhibition of IMPDH by the metabolite of LY217896.

The efficacy and safety of LY217896 for the prevention of experimental influenza A/Kawasaki/86 (H1N1) infection in the clinical setting was evaluated (Hayden *et al.*, 1994). Although a 75 mg dose of LY217896 was well tolerated during short term administration (7 days), the rate of virus shedding, days of viral shedding, and virus titres in nasal washings did not differ significantly between the LY217896-treated group and the placebo group. Additionally, administration of LY217896 resulted in a dose-related increase in serum uric acid levels during the treatment period (Hayden *et al.*, 1994). The increase in uric acid level, the uricogenic effect, was consistent with the findings in patients receiving other thiadiazoles (Krakoff, 1965) and is consistent with the inhibition of IMPDH. Because of the apparent lack of efficacy at doses that elevated plasma uric acid levels, LY217896 was deemed not to have clinical potential for the treatment of influenza (Hayden *et al.*, 1994).

LY 217896 is one of several compounds that have been considered as agents that target the IMPDH of the cell, causing the depletion of GTP pools, which results in the deprivation of a necessary substrate for the viral polymerase. The mechanism of action of ribavirin is due, in part, to its phosphorylation to the monophosphate, which

can then inhibit IMPDH (Streeter *et al.*, 1973, 1977; Wray *et al.*, 1985). Consistent with this is the observation that the antiviral activity of ribavirin can be reversed by excess guanosine (Streeter *et al.*, 1973; Colacino *et al.*, 1993) and by inosine and xanthosine (Streeter *et al.*, 1973). Mycophenolic acid, which is also an inhibitor of inosine monophosphate dehydrogenase, displays potent anti-HIV activity *in vitro* by limiting the rate of *de novo* synthesis of guanosine nucleotides, thereby inhibiting HIV reverse transcriptase activity (Ichimura & Levy, 1995). This observation prompted the idea of the 'polymerase substrate depletion' strategy to antiviral therapy (Ichimura & Levy, 1995), similar to the use of LY 217896 as an inhibitor of influenza virus replication. Recently the structure of inosine monophosphate dehydrogenase (IMPDH) was solved, thus enabling the design of novel IMPDH inhibitors with increased potency and tolerability and pharmacokinetic properties advantageous for the treatment of chronic diseases (Sintchak *et al.*, 1996). A novel inhibitor of IMPDH, VX-497 was shown to be well tolerated in Phase I clinical trials. Phase II trials have been initiated evaluating this compound in patients infected with hepatitis C virus (HCV) who were unresponsive to interferon-α treatment (Corporate information from Vertex Pharmaceuticals, available on the internet; http://www.vpharm.com). Whether VX-497 will prove to be uricogenic remains to be seen, but considering the serious nature of HCV infection, transient increases in uric acid levels may be a tolerable side effect. Although this approach may have potential for the treatment of infections caused by HIV or HCV, the feasibility of targeting a cellular enzyme, for example IMPDH, in order to treat influenza virus infections has not been demonstrated.

Inhibition of assembly and release

Other points in the influenza virus replication cycle for antiviral targeting are the assembly and release of infectious virions. It might be expected that agents that interfere with the unique assembly process of the virus would be selective antiviral agents. Agents that interfere with the glycosylation of viral proteins might have the potential to block the formation of the viral envelope, the formation of which is dependent on the viral glycoproteins. Some known inhibitors of glycosylation, such as 2-deoxy-D-glucose or glucosamine, do interfere with the glycosylation of viral membrane glycoproteins and 2-deoxy-2-D-glucose has been shown to inhibit herpes simplex virus replication (Courtney *et al.*, 1973). D-glucosamine, 2-deoxy-D-glucose and 6-amino-6-deoxy-D-glucosone all are capable of inhibiting the replication of influenza virus *in vitro* or *in vivo* (Kilbourne, 1959; Kaluza *et al.*, 1972; Hruskova *et al.*, 1975; Ghandi *et al.*, 1972) in part by interfering with the glycosylation of the membrane glycoproteins, HA and NA. However, this inhibition is non-specific and these compounds, which interfere with the synthesis of all glycoproteins (Klenk *et al.*, 1972), have not been deemed clinically useful as anti-influenza virus agents.

Clearly a more productive approach for the development of agents that act at the end of the influenza virus replication cycle has been the inhibition of the viral NA activity. Nearly 30 years ago, it was demonstrated that a synthetic analogue of sialic or neuraminic acid could inhibit bacterial and mammalian NAs as well as influenza virus NA with K_i values of approximately 100 μM (Meindl & Tuppy, 1969). Subsequently, numerous derivatives of neuraminic acid analogues were made although none has had K_i values lower than in the micromolar range and, importantly, none has shown sufficient activity in animal models of influenza virus infection (Meindl *et al.*, 1974; Flashner *et al.*, 1983; Glanzer *et al.*, 1991). As will be discussed, the determination of the X-ray crystallographic structure of influenza virus NA in 1983 (Varghese *et al.*, 1983) has enabled a rational structure-based approach by which to identify NA inhibitors that can fit into the active site of the enzyme and thus display potent and selective inhibitory activity (reviewed by Wade, 1997).

Sialidase, or NA, activity was first observed over 50 years ago when it was noticed that red blood cells, after agglutination by influenza virus in the cold, dispersed when warmed and could not be reagglutinated even after the addition of fresh influenza virus (Hirst, 1942). It was then deduced that a receptor for the HA had been removed by some enzyme activity present on the virus (Hirst, 1942). Subsequently, it was determined that sialidase was an exoglycohydrolase with the ability to remove sialic acid residues that are attached to carbohydrates via an alpha-ketosidic linkage. Although much has been learned concerning the NA enzyme reaction and the structure of the enzyme, the precise role of this protein during infection is not fully understood. Using an NA-deficient virus it was demonstrated that NA does not have a role in the entry, replication, assembly, or budding of the virus (Liu *et al.*, 1995). However, when NA activity is completely inhibited by antibody (Compans *et al.*, 1969), inhibitors (Palese & Compans, 1976) or by temperature sensitive mutation at the non-permissive temperature (Palese *et al.*, 1974a; Shibata *et al.*, 1993), virus aggregates form in which virus particles are attached to each other and to the cell surface. These observations are consistent with the earlier hypothesis that NA facilitates the movement of influenza virus through the sialic acid-rich mucus layer in the respiratory tract (Burnet & Stone, 1947), enabling the virus to infect the epithelial cell layer (Klenk & Rott, 1988).

The early history of NA inhibitors, along with the elucidation of the structure of influenza virus NA and the ensuing structure-based drug design efforts for the discov-

Figure 15. The chemical structure of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA)

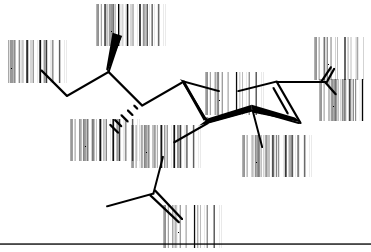
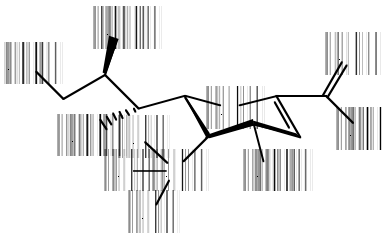


Figure 16. The chemical structure of N-trifluoro derivative of DANA (FANA)



ery of selective NA inhibitors, (Taylor, 1993) is one of the most exciting stories in antiviral drug development. The inhibition of this enzyme provided a rationale for the development of agents that could inhibit influenza virus replication and, accordingly, NA inhibition assays were developed for the purpose of finding such agents (Haskell *et al.*, 1970). Additionally, it had been shown that an antibody to NA could inhibit virus spread and decrease influenza virulence in animals and humans (Rott *et al.*, 1974). Thus it was considered that inhibitors of influenza virus NA would prevent the detachment of the virus from the host cell and from the mucopolysaccharide-containing proteins in the epithelium of the respiratory tract, thus inhibiting virus spread. However, early efforts to find compounds that could inhibit NA and display antiviral activity met with frustration. For instance, a class of NA inhibitors, the substituted β-aryl-α-mercaptoacrylic acids, were shown to be good inhibitors of the enzyme but had poor activity in the mouse model of infection (Haskell *et al.*, 1970). Also, it was demonstrated that compounds that could easily be selected for their ability to inhibit NA *in vitro* did not have *in vivo* antiviral activity (reviewed in Palese & Schulman, 1977).

Before the era of structure-based drug design, a diverse array of compounds, including non-specific inhibitors, high molecular weight natural products, small natural products, non-neuraminic acid compounds and compounds related to neuraminic acid, were shown to inhibit influenza virus NA (reviewed in Palese & Schulman, 1977). However, for the purposes of this review article, our discussion will be limited to compounds related to neuraminic acid which have proven to be the most potent inhibitors of influenza virus NA. These compounds include derivatives of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (Figure 15; DANA), which was first synthesized in 1969 (Meindl & Tuppy, 1969). DANA is active against bacterial, viral, and mammalian NAs. In structure–activity relationship studies it was found that if the N-acetyl group is replaced with N-fluoro, N-difluoro, N-trifluoro, or N-chloroacetyl moieties, inhibitory activity is increased (Meindl *et al.*, 1974). DANA and its N-trifluoro derivative, FANA (Figure 16), inhibit the replication of influenza and parainfluenza viruses in tissue culture (Meindl *et al.*, 1971; Palese *et al.*, 1974b; Schulman & Palese, 1975). Interestingly, it was demonstrated that influenza A/NWS, which has an N1 NA, was 50- to 100-fold more susceptible to inhibition by FANA than were influenza viruses containing an N2 NA. Importantly, the strain differences were due only to the differences in NA and not in HA. The specificity of FANA was demonstrated by its lack of activity against viruses that do not have NA, including measles virus and vesicular stomatitis virus (Palese *et al.*, 1974b); Kilbourne *et al.*, 1975). Furthermore, electron microscopy of cells infected with influenza and treated with FANA revealed aggregates of virus particles containing envelope-associated neuraminic acid, thus phenotypically resembling temperature-sensitive mutants of influenza that are deficient in NA activity at the non-permissive temperature (Palese & Compans, 1976).

Although FANA displayed potent antiviral activity in cell culture, this compound was not effective in the mouse model of influenza virus infection. In various experiments, FANA, when given intranasally or subcutaneously to mice infected with influenza A/NWS, was not active at doses that were predicted to be sufficient based on *in vitro* antiviral data (Palese & Schulman, 1977). The lack of *in vivo* activity for FANA led to the generally accepted belief that potent synthetic analogues such as DANA would not have *in vivo* activity. In addition, subsequent work demonstrated that sialic acid analogues were rapidly excreted (Nohle *et al.*, 1982), leading to the notion that DANA (Neu5Ac2en) analogues would not have potential clinical utility without modification of these molecules that would reduce the rapid excretion (reviewed in von Itzstein *et al.*, 1993a).

The elucidation of the X-ray crystallographic structure of influenza NA ushered in a new era of structure-based drug design for inhibitors of this enzyme. Early work that showed the HA and NA activities of influenza virus resided in two morphologically distinct surface 'spikes' (Laver & Valentine, 1969) led to the crystallization of NA 'heads' (Laver, 1978), which in turn, enabled the X-ray crystal structure of influenza virus HA and NA to be determined (Varghese *et al.*, 1983; Colman *et al.*, 1983; Varghese

Figure 17. The chemical structure of 4-amino-Neu5Ac2en

Figure 18. The chemical structure of 4-guanidino-Neu5Ac2en (zanamivir)

& Colman, 1991). The X-ray crystal structure of sialidase heads, obtained by pronase digestion of influenza virions, revealed that the enzyme consists of a tetramer of identical subunits, each with a molecular weight of 60 kDa. Each subunit is arranged into six four-stranded antiparallel β-sheets which resemble the blades of a propeller and there exists a nearly sixfold axis of symmetry that passes through the centre of each subunit. The active site lies close to the axis of symmetry and has been unequivocally identified by structural studies of enzyme–inhibitor complexes (Varghese *et al.*, 1983, 1992; Colman *et al.*, 1983; Varghese & Colman, 1991). The catalytic site comprises a deep cavity that is located on the surface of the enzyme. Several amino acids that line the active site of the enzyme and participate directly in the binding and catalysis of the substrate are invariant (reviewed by Colman, 1994). These structural findings have suggested that an active site inhibitor of influenza NA should, in principle, be active against all strains of influenza A and B viruses and have provided the impetus for a structure-based approach to the rational design of NA inhibitors. Two inhibitors identified by this approach, 4-amino- (Figure 17) and 4-guanidino-Neu5Ac2en (Figure 18), were found to have *in vitro* and *in vivo* activity against influenza virus replication with similar potency against influenza A and B viruses (von Itzstein *et al.*, 1993b). Both compounds bind entirely within the conserved catalytic site of NA, making no contacts with non-conserved amino acids outside the catalytic site. Furthermore, the bulkier guanidinyl group of the 4-guanidino compound is able to interact with the structural amino acids of the active site which, themselves, do not make contact with the natural sialic acid substrate (von Itzstein *et al.*, 1993b). Subsequently, the conformation of 4-guanidino-Neu5Ac2en in the active site was determined and agreed with one obtained using theoretical calculations. Importantly, the environment of the 4-guanidino group of the inhibitor was found to be strongly conserved and the secondary nitrogen of the 4-guanidinyl group was found to interact with the carboxylate of the Glu-119 and Asp-151 (Varghese *et al.*, 1995). These structural studies provided an explanation for the specificity of 4-guanidino-Neu5Ac2en for influenza virus NA and the broad spectrum of antiviral activity against a wide array of influenza virus strains.

Zanamivir. Recently much attention has been directed toward 4-guanidino-Neu5Ac2en (also known as zanamivir), which is a potent and selective inhibitor of the enzyme and is active against influenza A and B virus replication (von Itzstein *et al.*, 1993b; Woods *et al.*, 1993; Thomas *et al.*, 1994). Zanamivir is a potent inhibitor of influenza virus replication in cell culture and in animal models of infection. Although zanamivir inhibits the activity of NAs from a wide variety of influenza virus strains within a 1 $\log_{10}$ window (0.0002–0.003 μg/ml), in plaque assays there is much strain variation in antiviral potency, and over 500-fold variation (0.007–5.3 μg/ml) in the susceptibility of various clinical isolates has been demonstrated (Woods *et al.*, 1993). Consistent with the high degree of conservation of active sites in A and B influenza virus NAs, zanamivir was shown to inhibit human influenza A and B viruses in human respiratory epithelial cell explant cultures (Hayden *et al.*, 1994). Furthermore, zanamivir has shown activity in chick cell cultures against all of the nine NA subtypes from avian influenza viruses, although inhibitory concentrations varied over 200-fold.

In vivo, zanamivir has demonstrated good antiviral activity at low doses. Mice infected with influenza and treated intranasally with zanamivir showed a decreased mortality rate and lung consolidation. Also, zanamivir proved to be greater than 100-fold more potent than amantadine and 1000-fold more potent than ribavirin (von Itzstein *et al.*, 1993b). In subsequent studies using mice, influenza virus did not rebound after discontinuation of zanamivir treatment (Ryan *et al.*, 1994). In the ferret model of infection, intranasally administered zanamivir, at doses as low as 0.05 mg/kg beginning 1 day before challenge with influenza A/Singapore/1/57 and continuing for 5 days, resulted in significant decreases in viral titres and the prevention of influenza virus-induced pyrexia. Zanamivir was also effective in protecting ferrets from challenge with influenza A/Mississippi/1/85 (H3N2) and influenza B/Victoria/102/85 (Ryan *et al.*, 1995).

In pharmacokinetic studies, zanamivir was found to be readily bioavailable after nasal, intraperitoneal, and intra-

venous administration, but not after oral administration in mice (Ryan *et al.*, 1994). When given systemically, zanamivir does not partition into respiratory secretions from the blood and is rapidly cleared renally (Ryan *et al.*, 1994). Consistent with these findings is the inability of intra-tracheally administered zanamivir to protect chickens from a lethal and systemically disseminated infection by three different avian influenza viruses. However, zanamivir was able to inhibit these same viruses in culture (McCauley *et al.*, 1995). It should be noted that zanamivir (GG167) was able to inhibit influenza virus replication in the murine model when the compound was administered by gavage (Sidwell *et al.*, 1998).

In most evaluations of zanamivir, the compound has been administered by either inhalation of dry powder aerosols or intranasally as an aqueous solution. In studies that provided the first evidence that an agent which targets the influenza virus NA is clinically efficacious, intranasally administered zanamivir displayed a potent antiviral effect (Hayden *et al.*, 1996). Zanamivir was well tolerated in either prophylactic or therapeutic regimens. The drug was 82% effective in preventing laboratory infection and 95% effective in preventing influenza virus-induced febrile illness. It was shown that early treatment (1 day post-infection) with zanamivir reduced peak viral titres by 2 $\log_{10}$, shortened the median duration of viral shedding by 3 days and decreased the incidence of febrile illness by 85%. Also, twice daily dosing was found to be as effective as six times daily dosing (Hayden *et al.*, 1994). In human volunteers, zanamivir has also provided protection against challenge infection with influenza B virus (Hayden *et al.*, 1996) and when inhaled, zanamivir has been shown to provide a clinical benefit against uncomplicated acute influenza (Hayden *et al.*, 1996). Recently, 14 day chemoprophylaxis using zanamivir was shown to be comparably effective to the standard of care using rimantadine in preventing influenza-like illness and laboratory-confirmed influenza in nursing homes, but requires further testing (Schilling *et al.*, 1998).

In naturally occurring influenza virus infections, zanamivir, when administered topically, has shown efficacy (Hayden FG, Lobo M, Treanor JJ, Miller M & Mills RG; Efficacy and tolerability of oral GS 4104 for early treatment of experimental influenza in humans. *37th Interscience Conference on Antimicrobial Agents and Chemotherapy*, Toronto, Canada, 28 September–1 October 1997, Abstract LB-26). Intranasal (6.4 mg) plus inhaled (10 mg) zanamivir was compared to inhaled (10 mg) zanamivir alone or to placebo. In each group zanamivir was administered twice daily for 5 days. Of the participants in the trial, 63% had laboratory-confirmed influenza (either A or B). Treatment with zanamivir (inhaled plus intranasal) resulted in a statistically significant 1 day decrease in the median time to the alleviation of symptoms (4 days versus 5 days for the controls). Importantly, there was a 3 day decrease in the time to alleviation of all symptoms and return to normal activities in those participants who began the trial with febrile illness and were treated within 30 h of the onset of symptoms. Zanamivir treatment appeared to be well tolerated with no differences in the rate incidence of adverse effects between the treatment and placebo groups. In another study, intranasal zanamivir given as prophylaxis or early treatment of volunteers inoculated with influenza A/Texas/36/91 (H1N1) or influenza B/Yamagata/88 was efficacious in preventing middle ear pressure abnormalities, which are frequently observed during influenza virus infection (Walker *et al.*, 1997).

Taken together, these observations have demonstrated that topical delivery of zanamivir to the respiratory tract results in an overall improvement in influenza symptoms with a decrease in the time to alleviation of illness. As has been pointed out, it is not yet known whether treatment with zanamivir will result in a reduced risk of influenza-associated complications and further studies are planned to evaluate nebulized zanamivir for treating severe influenza in hospitalized patients who have lower respiratory tract disease (Calfee & Hayden, 1998).

More recently, a number of clinical evaluations have confirmed the efficacy and safety of zanamivir. Three consecutive daily intranasal doses of zanamivir beginning 4 h prior to influenza virus challenge resulted in protective efficacies of 87% for both viral shedding and infection (Calfee DP, Peng AW, Hussey EK, Lobo M & Hayden FG; Protective efficacy of reduced-frequency dosing of intranasal zanamivir in experimental human influenza. *38th Interscience Conference on Antimicrobial Agents and Chemotherapy*, San Diego, California, USA, 24–27 September 1998, Abstract H-68). Of interest was the observation that a single dose of zanamivir given at the time of influenza virus challenge is efficacious, indicating that influenza virus NA is essential for the spread of virus through the respiratory mucin (Calfee DP, Peng AW, Hussey EK, Lobo M & Hayden FG; Protective efficacy of reduced-frequency dosing of intranasal zanamivir in experimental human influenza. *38th Interscience Conference on Antimicrobial Agents and Chemotherapy*, San Diego, California, USA, 24–27 September 1998, Abstract H-68). In one study, zanamivir administered by inhalation (10 mg twice daily for 5 days) showed a clinically and statistically significant benefit on the time to reduction of influenza virus symptoms including fever, myalgia, weakness, cough and loss of appetite (Silagy CA, Campion KJ & Keene O; The efficacy and safety of zanamivir in the treatment of influenza in otherwise healthy and 'high risk' individuals. *38th Interscience Conference on Antimicrobial Agents and Chemotherapy*, San Diego, California, USA, 24–27

Figure 19. The chemical structure of (3R, 4R, 5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexane-1-carboxylic acid (GS4071)

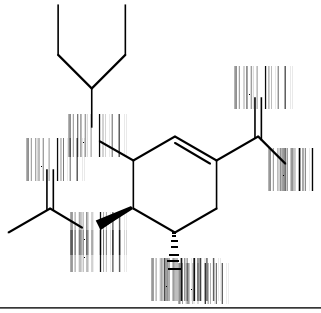
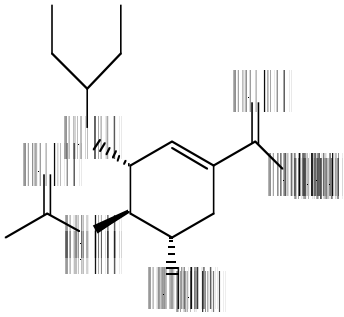


Figure 20. The chemical structure of the ethyl ester derivative GS4071 (GS4104)



September 1998, Abstract H-56). Another study demonstrated that intravenously administered zanamivir resulted in the abrogation of IL-6, TNF-α, MCP-1, MIP-1α, and MIP-1β responses, all of which are indicators of the severity of influenza virus infection (Fritz RS, Hayden FG, Calfee DP, Cass LMR, Peng AW, Alvord WG & Straus SE; Cytokine and chemokine responses during experimental influenza A infection: effect of intravenous zanamivir. *38th Interscience Conference on Antimicrobial Agents and Chemotherapy*, San Diego, California, USA, 24–27 September 1998, Abstract H-57). When given intravenously, zanamivir (600 mg infusions twice daily for 5 days) distributes to respiratory secretions and provides 100% efficacy against virus shedding and 86% protection against infection. Furthermore, significant reductions in incidences of fever and myalgia were noted (Calfee DP, Peng AW, Cass LMR, Lobo M & Hayden FG; Protective efficacy of intravenous zanamivir in experimental human influenza. *38th Interscience Conference on Antimicrobial Agents and Chemotherapy*, San Diego, California, USA, 24–27 September 1998, Abstract H-58).

Toxicity studies in several animal species have demonstrated that zanamivir, whether inhaled or given by the intravenous route, has no associated toxicities or adverse events (reviewed in Calfee & Hayden, 1998). Furthermore, in the clinical studies described above, zanamivir has proved to be well tolerated. The pharmacokinetic profile of zanamivir points out a limitation of the drug. In mice the compound is readily bioavailable when administered intranasally, intraperitoneally or intravenously, but not orally. Consistent with this, in humans zanamivir has low (less than 5%) oral bioavailability.

Relenza (zanamivir) has received a positive recommendation in Australia and an approval in Sweden but on February 24, 1999, the Antiviral Drugs Advisory Committee of the US Food and Drug Administration voted against recommending Relenza for FDA approval. According to a press release from GlaxoWellcome (http://www.glaxowellcome.co.uk/news-/business/1999/1-9990225.html), which is developing Relenza under license from the Australian company Biota Holdings (Melbourne), the committee members have expressed the view that Relenza had an acceptable safety profile and was a promising antiviral but voted against approval because of reservations about efficacy in one of three pivotal studies. GlaxoWellcome has stated that they will work with the FDA with the intent to make Relenza available in the United States as soon as possible.

GS 4071 and GS 4104. Other types of NA inhibitors have been designed with the intent of improving oral bioavailability. The approach taken was to synthesize a carbocyclic analogue of sialic acid and replace the polar glycerol moiety thereof with hydrophobic side chains. One compound of this class is GS 4071 [Figure 19; (3R, 4R, 5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexane-1-carboxylic acid]. GS 4071 is a transition state analogue inhibitor of influenza virus NA having potent (K_i <1 nM) and selective inhibitory activity (Kim *et al.*, 1997). X-ray crystallographic structures of several GS 4071 and other related analogues complexed with influenza virus NA demonstrated that the lipophilic side chains bound to the hydrophobic pocket of the active site consisting of Glu-276, Ala-242, Arg-224, and Ile-222. Structure–activity relationship studies demonstrated different inhibitory levels between influenza A and B NA, indicating a difference in the pattern of interactions between the lipophilic side chain of the inhibitor and the different NAs. Importantly, in these studies, GS 4071 was the most potent inhibitor of both influenza A and B NAs (Kim *et al.*, 1998). Furthermore, GS 4071 was shown to be a slow-binding, time-dependent inhibitor of both influenza A and B NAs and showed similar K_i values for both enzymes (Kati *et al.*, 1998).

In tissue culture, as an inhibitor of influenza virus replication, GS 4071 is equal in potency to zanamivir (Kim *et al.*, 1997; Sidwell *et al.*, 1998). However, like zanamivir, GS 4071 has low (<5%) oral bioavailability. GS 4104 (Figure 20) is an ethyl ester derivative of GS 4071 and is well

absorbed orally. Following absorption from the gut, GS 4104 is rapidly cleaved by esterases to yield the active compound, GS 4071. Oral administration of GS 4104 results in high levels of the active compound GS 4071 in serum (Li *et al.*, 1998) and the oral bioavailability of GS 4104 ranged from 30–73% in the rat, mouse, and dog. In these animals, GS 4104 was efficiently cleaved to active GS 4071 (Li *et al.*, 1998). Importantly, an oral dose of GS 4104 given to rats resulted in the presence of GS 4071 in the bronchoalveolar lining fluid (BALF), which reached a peak concentration at 2 h (Eisenberg *et al.*, 1997). Comparison of the ratios of the area under the curve values of GS 4071 in BALF to those in plasma indicated that there was significant penetration of active GS 4071 in the lower respiratory tract. The prodrug, GS 4104 was not detected in BALF (Eisenberg *et al.*, 1997). Considering that influenza virus replicates mainly in the surface epithelial cells of the respiratory tract, the penetration of GS 4071 into BALF after oral administration of GS 4104 is an important attribute for clinical efficacy.

The *in vivo* efficacy of GS 4104 is well-established. Mice infected with influenza A/NWS/33 (H1N1) and treated with oral GS 4104 at doses of 1 mg/kg/day, beginning 4 h prior to virus infection, had significantly lower death rates than did mice treated with placebo. At the lowest dosage of 0.1 mg/kg/day, mice displayed an increase in the number of days until death and also had increased oxygen saturation, an indicator of antiviral efficacy (Sidwell *et al.*, 1998). Oral GS 4104 (10 mg/kg/day) also demonstrated therapeutic efficacy since it provided protection against lethality and increased oxygen saturation even when administration was delayed 60 h post-infection (Sidwell *et al.*, 1998). In additional studies, oral GS 4104 (10 mg/kg/day) was efficacious in protecting mice against lethal infections of influenza A/NWS/33 (H1N1), A/Victoria/3/75 (H3N2) and B/Hong Kong/5/72. It was shown to be more efficacious than zanamivir when both drugs were given 4 h post-infection with influenza virus A/NWS/33 (Mendel *et al.*, 1998). GS 4104, at doses of 5 mg/kg and 25 mg/kg beginning 2 h after infection of ferrets with influenza A/England/939/69 (H3N2) was effective in reducing viral titres and virus-induced fever (Mendel *et al.*, 1998).

In fasting male volunteers, GS 4104 (also known as Ro640796) has demonstrated an advantageous pharmacokinetic and safety profile in double-blind, placebo controlled studies (Wood ND, Aitken M, Sharp S & Evison H; Tolerability and pharmacokinetics of the influenza NA inhibitor Ro-64–0802 (GS 4071) following oral administration of the prodrug Ro-64–0796 (GS 4104) to healthy male volunteers. *37th Interscience Conference on Antimicrobial Agents and Chemotherapy*, Toronto, Canada, 28 September –1 October 1997, Abstract A-123). Single doses of 20 to 1000 mg resulted in proportionately increasing values for the maximum plasma concentration and area under the plasma concentration versus time curve (AUC). Peak plasma concentrations for GS 4071, following administration of the prodrug GS 4104, occurred at 2.5 to 6 h with a mean terminal elimination half life ranging from 6.8 to 9.3 h. Peak concentrations of the prodrug were found to be 15 to 30% of those of the active metabolite. When 200 mg of GS 4104 were given after a meal, there was a 1 to 1.5 h delay in reaching the maximum concentration for both the prodrug and the active compound. However, no other effects of food on the pharmacokinetics of GS 4104 were observed. With twice daily dosing (50 to 500 mg) for 6 days, there was a small increase in the exposure to GS 4071, but not to GS 4104 and at day 3, mean pre-dose concentrations of 1280 mg/l were observed in the group receiving 500 mg twice daily. Importantly GS 4104 was well tolerated with no serious adverse events observed, although mild to moderate nausea was more frequently observed in the groups receiving the higher doses.

In a study to evaluate the therapeutic efficacy of GS 4104, susceptible adults were infected with influenza A/Texas/91 (H1N1) and assigned to one of five treatment groups: 20 mg orally twice daily; 100 mg orally twice daily; 200 mg orally twice daily; 200 mg orally once daily, or placebo (Hayden *et al.*, 1997). Treatment was begun 28 h after virus challenge. When compared with the placebo group, the treatment groups considered together displayed statistically significant decreases in viral shedding, the amount of virus shed, and in the duration of symptoms. No differences were observed among the treatment groups. In another study, doses of 100 mg once or twice daily were found to eliminate virus recovery from the upper respiratory tract and against influenza virus illness. Importantly, in these studies, GS 4104 was well tolerated and no serious adverse advents were observed (Hayden FG, Lobo M, Treanor JJ, Miller M & Mills RG; Efficacy and tolerability of oral GS 4104 for early treatment of experimental influenza in humans. *37th Interscience Conference on Antimicrobial Agents and Chemotherapy*, Toronto, Canada, 28 September–1 October 1997, Abstract LB-26).

More recently, in a multi-centre, placebo-controlled study of healthy, non-immunized adults treated within 36 h of respiratory diseases associated with fever, GS 4104 reduced the duration of illness by a median of 2.9 days (30% decrease). It also provided protection against the secondary complications of influenza including bronchitis and sinusitis (Treanor JJ, Vrooman PS, Hayden FG, Kinnersley N, Ward P & Mills RG, on behalf of the US Oral NA Inhibitor Study Group; Efficacy of oral GS 4104 in treating acute influenza. *38th Interscience Conference on Antimicrobial Agents and Chemotherapy*, San Diego, California, USA, 24–27 September 1998, Abstract LB-4).

Figure 21. The chemical structure of the phosphonate analogue of *N*-acetyl neuraminic acid (PANA)

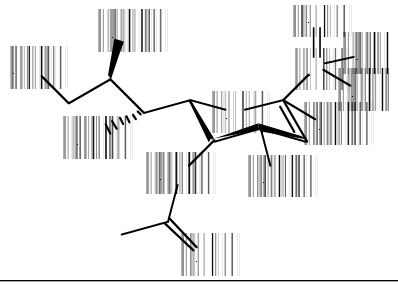
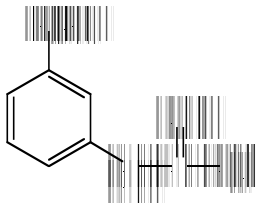


Figure 22. The chemical structure of 3-guanidinobenzoic acid



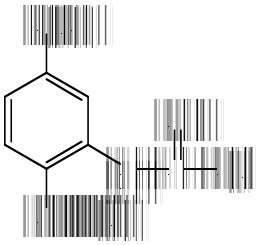
In another multi-centre placebo-controlled study, GS 4104 provided overall protective efficacy of 74% in a group of 1500 non-immunized adults (aged 18 to 65 years) who received the drug orally once or twice daily (Hayden FG, Atmar R, Schilling M, Johnson C, Poretz D, Parr D, Huson L, Ward P & Mills R; Safety and efficacy of oral GS 4104 in long term prophylaxis of natural influenza. *38th Interscience Conference on Antimicrobial Agents and Chemotherapy*, San Diego, California, USA, 24–27 September 1998, Abstract LB-6). In still another placebo-controlled treatment study conducted in Canada, Europe and China, healthy non-immunized adults with fever and presenting with at least one influenza virus symptom were treated with GS 4104 within 36 h of the onset of illness. Among 719 people so treated, GS 4104 provided protection by reducing the duration of illness by 30%. Earlier treatment (within 24 h of onset of symptoms) resulted in a 40% reduction in the duration of illness. Transient gastrointestinal effects were only slightly more common in those subjects receiving drug (Aoki F, Osterhaus A, Rimmelzwaan G, Kinnersley N & Ward P, on behalf of the NA Inhibitor Flu Treatment Investigator Group; Oral GS 4104 successfully reduces duration and severity of naturally acquired influenza. *38th Interscience Conference on Antimicrobial Agents and Chemotherapy*, San Diego, California, USA, 24–27 September 1998, Abstract LB-5).

Other NA inhibitors. Zanamivir and GS 4104 have demonstrated clinical potential and consequently have received much attention recently. However, with the aid of computer modelling and the availability of the X-ray crystallographic structure of NA, other inhibitors have also been identified. For instance, it was predicted that the replacement of a carboxylate moiety with that of a phosphonate would aid binding to the NA active site and, consequently, a phosphonate analogue of *N*-acetyl neuraminic acid (Figure 21; PANA) was designed and synthesized as the alpha (e) and beta (a) anomers (White *et al.*, 1995). The ePANA was a strong inhibitor of influenza A (N2) and B NA and a moderate inhibitor of influenza A N9 NA. In contrast, aPANA was not a strong inhibitor for any NAs tested (White *et al.*, 1995). X-ray structures of the PANA compounds complexed with influenza NA revealed that neither anomer distorted the enzyme active site upon binding and it was postulated that the increased inhibitor activity of ePANA was due to strong electrostatic interactions between the phosphonate group and the arginine pocket of the enzyme active site.

Another approach to the design of NA inhibitors has been to synthesize aromatic analogues of sialic acid (Williams *et al.*, 1995; Jedrzejas *et al.*, 1995; Luo *et al.*, 1995). One such inhibitor is based on the substitution of a benzene ring of 4-(*N*-acetylamino)benzoic acid for the dihydropyran ring of DANA and several derivatives were prepared as potential replacements for the glycerol side chain of Neu5Ac2en (Singh *et al.*, 1995). The most potent of such compounds was the 3-guanidinobenzoic acid derivative (Figure 22), the guanidino moiety of which is equivalent to the 4-guanidino grouping of 4-guanidino-Neu5Ac2en. This compound displayed a 50% enzyme inhibitory concentration of 10 μM and it was demonstrated that this compound occupied the glycerol-binding sub-site of the enzyme active site rather than the guanidino-binding sub-site, thus offering a novel interaction within the catalytic site of influenza virus NA (Singh *et al.*, 1995). No data concerning the clinical utility of this type of compound are currently available. Another series of benzoic acid-derived compounds was synthesized and tested for its ability to inhibit influenza virus NA and the most potent was 4-(acetylamino)-3-guanidinobenzoic acid (Figure 23), which displayed an IC_{50} value of 2.5 μM (Chand *et al.*, 1997). X-ray crystallographic analysis of this inhibitor complexed with influenza A N9 NA also revealed that it was oriented in the active site of the NA in a way that was not predicted from the observed active site binding of zanamivir. However, in a mouse model of influenza virus infection, intranasally administered 4-(acetylamino)-3-guanidinobenzoic acid did not protect mice from infection-related weight loss (Chand *et al.*, 1997). Thus it would appear that these types of compounds will not be as promising as zanamivir and GS 4104 have proved to be.

Still other agents have been considered for their potential as influenza virus NA inhibitors. The 9-amino- and 9-*N*-acyl-5-trifluoroacetyl methyl V-ketosides and

Figure 23. The chemical structure of 4-(acetylamino)-3-guanidinobenzoic acid



2,3-didehydro analogues thereof have been synthesized. In particular, one such 2,3-didehydro compound displayed an IC_{50} of >7.8 μM against influenza A N1 NA (Murakami *et al.*, 1996). Building off zanamivir as a prototypical structure, the triol group has been replaced with a lipophilic group attached via a carboxamide linkage at the C6 position, resulting in a novel series of NA inhibitors (Sollis *et al.*, 1996).

Of interest are the plant flavonoids, isoscutellarein and isoscutellarein-8-methyl ether (also known as F36), which are derived from the plant *Scutellaria baicalensis*. F36 was demonstrated to have *in vivo* activity in the mouse model of influenza virus infection (Nagai *et al.*, 1992) where it was shown to be well tolerated while it completely prevented the replication of mouse-adapted influenza virus A/PR/8/34 in the mouse lung when administered intranasally (0.5 mg/kg) or intraperitoneally (4 mg/kg). F36 was also found to be more potent than amantadine. Subsequently, F36 was shown to be a specific and non-competitive inhibitor of influenza virus NA (Nagai *et al.*, 1995).

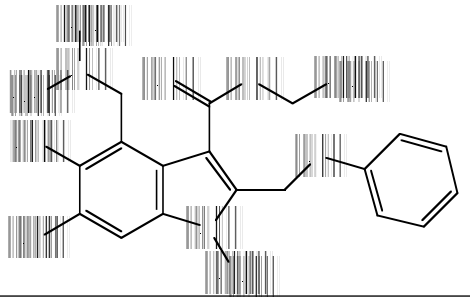
Resistance to NA inhibitors. In cell culture experiments, influenza virus variants have been selected for resistance to NA inhibitors that have been designed to interact with the active site of the enzyme. The mutations in these virus variants have been found in two gene segments, leading to amino acid changes in either the influenza HA or NA. In one study, influenza A/NWS/G70c was passaged in the presence of either 4-amino-Neu5Ac2en or zanamivir and variants were isolated that could replicate in the presence of inhibitory concentrations of drug (McKimm-Breschkin *et al.*, 1996). Although no differences in the NA activity of wild-type and resistant viruses were found, the variants were 100- to 1000-fold more resistant to zanamivir. Amino acid alterations were found, however, in the conserved regions of the HA involved in receptor binding. This observation has led to the idea that mutations in the HA that confer resistance to zanamivir lead to a decrease in the affinity of the HA for its receptor, thus allowing the virus to elute from infected cells more easily in the presence of an inhibited NA. Other studies have demonstrated mutations in the HA of variants resistant to NA inhibitors (Staschke *et al.*, 1995; Penn *et al.*, 1996; Gubareva *et al.*, 1997; McKimm-Breschkin *et al.*, 1998; Bantia *et al.*, 1998).

Studies have also demonstrated that resistance to zanamivir can indeed be conferred by mutations in the NA itself. Influenza A/NWS/-G70c (H1N9) and influenza B/Hong Kong/8/73 were passaged in stepwise increases of zanamivir and sequence analysis of resistant variants derived from each parent revealed the same alteration of a highly conserved amino acid which lies in a pocket beneath the enzyme active site, for example; Glu-119→Gly (influenza A) or Glu-117→Gly (influenza B) (Staschke *et al.*, 1995). The variant viruses displayed much lower enzyme activity (< 5%) than did the parent, yet were able to replicate to equal or greater titres in tissue culture and in embryonated eggs (Staschke *et al.*, 1995). In another study, a variant of influenza A/NWS/G70c was selected for resistance to zanamivir and the same Glu→Gly at position 119 was found (Blick *et al.*, 1995). This mutation, which was confirmed by X-ray crystallographic studies (Blick *et al.*, 1995, Colacino *et al.*, 1997) resulted in the loss of the slow binding to zanamivir but not to a related analogue lacking the guanidinium moiety at the C-4 position, 4-amino-Neu5Ac2en (Blick *et al.*, 1995). Still other studies, in which influenza A/Turkey/Minnesota/833/80 (H4N2) was passaged in zanamivir, have identified variants with alanine, aspartate, or glycine in place of the glutamate at position 119 (Gubareva *et al.*, 1996). Although mutants of this type (Glu-119→Gly, Ala, Asp) have a reduced infectivity in mice (Matsumoto *et al.*, 1996), a variant of A/NWS/G70c with the Glu-119→Gly was able to induce fever in ferrets (Colacino, 1996). The glutamate at position 119 (influenza A) or 117 (influenza B) is absolutely conserved in all influenza viruses studied so far and the change of this residue to a glycine eliminates the electrostatic interaction between the carboxylate of the glutamate at position 119 with the C-4 guanidinium moiety of the inhibitor, as has been described by structural studies (von Itzstein *et al.*, 1993b; Varghese *et al.*, 1995).

Another type of mutation in variants resistant to zanamivir is the change of the invariant arginine at position 292 to a lysine (Gubareva *et al.*, 1997). This arginine is one of a triad of arginines that forms a pocket in the enzyme active centre at the site of substrate binding. Variants with this mutation were found to be less resistant to zanamivir than variants with the Glu-119→Gly alteration but were found to have a 500-fold decreased infectivity in mice (Gubareva *et al.*, 1997).

Variants that are resistant to zanamivir have shown cross resistance to GS 4071. However, GS 4071 is active against Glu-119→Gly variants, most likely owing to the fact that GS 4071 does not contain a guanidinium moiety that can interact with the carboxylate of the glutamate at position

Figure 24. The chemical structure of 6-bromo-4(dimethylaminomethyl)-5-hydroxy-1-methyl-2-(phenylthiomethyl)-1H-indole-3-carboxylic acid ethyl ester hydrochloride monohydrate (arbidol)



119. In recent studies, influenza A/NWS/G70c was passaged in the presence of a NA inhibitor in which the triol group of zanamivir is replaced with a hydrophobic group linked via a carboxamide at the C-6 position. Resistant variants were of two types: (1) those with mutations in the HA resulting in mutants which bound to host cell receptors with lesser avidity; and (2) those with a mutation at the conserved Arg-292 in the NA (Arg-292→Lys). This NA mutation has also been observed in some variants that are resistant to zanamivir (Gubareva *et al.*, 1997). Interestingly, this NA was equally resistant to zanamivir or 4-amino-5Ac2en but showed much more resistance to the 6-carboxamide compound and was most resistant to GS 4071, which as discussed above, contains a hydrophobic group at the 6-position (McKimm-Breschkin *et al.*, 1998). The Arg-292→Lys mutation was associated with an 80% decrease in enzymatic activity relative to the parent virus and resulted in the decreased replication and spread of the variant in cell culture.

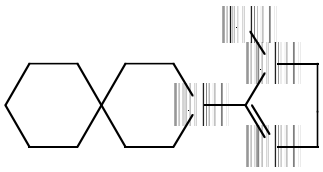
Most recently, variants of influenza A/Victoria/3/75 with increased resistance to GS 4071 were selected *in vitro* by passaging the virus in the presence of this compound (Tai *et al.*, 1998). Resistant variants that were selected after eight passages had mutations in the HA (Ala→Thr at position 28 in HA1 and Arg→Met at position 124 in HA2). However, after 12 passages in the presence of GS 4071, a resistant variant was isolated that contained these mutations and the Arg→Lys at the conserved position 292 in the NA. This was the same mutation seen in the NA of variants resistant to an inhibitor in which the triol group of zanamivir is replaced with a hydrophobic group linked via a carboxamide at the C-6 position. The mutant NA displayed a 30000-fold resistance to the inhibitor but only moderate (30-fold) resistance to zanamivir and 4-amino-Neu5Ac2en. Interestingly, the GS 4071-resistant NA also displayed a weaker affinity for the fluorogenic substrate 2′-(4-methylumbelliferyl)-β-D-*N*-acetylneuraminic acid and had a lower level of enzymatic activity when compared to the wild-type. In the mouse model of virus infection, the GS 4071-resistant variant was 10000-fold less infectious than was wild-type virus. In a recent study to clarify the structural and functional consequences of replacing the glycerol side chain of NA inhibitors with other moieties, such as carboxamide-linked hydrophobic substituents, it was found that the altered Arg (Arg-292→Lys) in the NA of the resistant virus is one of three arginine residues that surround the carboxylate group of the substrate (Varghese *et al.*, 1998). Thus it was concluded that this alteration in the NA affects the binding of the substrate by modifying the interaction with the substrate carboxylate and may be correlated to the reduced enzymatic activity of the variant. It was concluded from these results that a generally useful strategy for drug design, when the target is highly mutable, is to design the inhibitor to be as structurally similar as possible to the natural substrates or ligands of the target (Varghese *et al.*, 1998).

Because of the effect of the Arg→Lys alteration at position 292 on viral infectivity and virulence *in vivo*, it was suggested that this alteration will prove to be of limited clinical significance (Tai *et al.*, 1998). The clinical relevance of these *in vitro* studies is not yet clear because no zanamivir-resistant viruses have been isolated in an animal model of infection and treatment and attempts to isolate resistant influenza virus variants in clinical studies have been, in general, unsuccessful. However, recently during a prolonged treatment with zanamivir, a mutant virus was isolated from an immunocompromised child infected with influenza B virus (Gubareva *et al.*, 1998). A mutation was found in the HA (Thr-198→Ile), which apparently resulted in the reduced affinity of the HA for host cell receptors. Another mutation was found in the NA (Arg-152→Lys), which resulted in a 1000-fold increase in the resistance of the enzyme to zanamivir. Interestingly, the variant was less virulent than wild-type virus in ferrets, but did have a selective advantage in zanamivir-treated animals. Curiously, the variant virus was as sensitive to zanamivir as wild-type virus in Madin–Darby canine kidney cells.

Other anti-influenza agents

Arbidol. Arbidol [Figure 24; 6-bromo-4(dimethylaminomethyl)-5-hydroxy-1-methyl-2-(phenylth-iomethyl)-1H-indole-3-carboxylic acid ethyl ester hydrochloride monohydrate] has been launched by the Russian Federation as a treatment for influenza A and B virus infections. This compound is an antiviral and immunostimulatory agent that appears to inhibit the fusion of the influenza envelope with the host cell membrane (Leneva IA, Fadeeva NI & Fedykina IT; The study of effect of a new antiviral drug arbidol on different stages of viral reproduction. *7th International Conference on Antiviral Research*, 1994, Abstract 187). Additionally, the antiviral activity of arbidol may be related, in part, to the ability of this drug to

Figure 25. The chemical structure of the spirene containing compound, BL1743



induce interferon and activate 2,5-oligoadenylate synthetase (Guskova TA, Nikolaeva IS & Zakharova NG: Experimental and clinical study of arbidol, an antiviral drug. *9th Mediterranean Congress of Chemotherapy*, 1994, Abstract 82). Arbidol has been evaluated in clinical trials involving more than 9000 patients and no adverse events were reported (Glushkov, 1992). In clinical studies involving 2000 patients, arbidol was administered orally at a dose of 200 mg daily for 5–10 days and was shown to prevent influenza and other acute respiratory diseases in 85% of the contacts of infected patients. Also, arbidol prophylaxis was shown to be 80% effective during influenza outbreaks (Guskova TA, Nikolaeva IS & Zakharova NG: Experimental and clinical study of arbidol, an antiviral drug. *9th Mediterranean Congress of Chemotherapy*, 1994, Abstract 82).

SP303. SP303 is a polyphenolic compound that occurs naturally and is derived from the plant *Croton lechleri*. SP303 has demonstrated antiviral activity against a number of viruses including influenza. Topical SP303 (Virend) and oral SP303 (Provir) are in clinical trials for the treatment of herpes simplex virus (Orozco-Topete *et al.*, 1997; Safrin *et al.*, 1994) and respiratory syncytial virus infections, respectively. SP303 appears to bind to viruses extracellularly, thus blocking infection of host cells. The compound has shown selective activity against influenza A and B viruses and was evaluated in the mouse model of influenza virus infection (Sidwell *et al.*, 1994). When administered as a 1 h aerosol twice daily using a concentration of 4.3 mg/ml, SP303 caused a significant increase in the mean day to death and reduced lung consolidation in mice, although no decline of virus titre in the lung was observed. When SP303 (3, 10, or 30 mg/kg/day) was administered intraperitoneally once daily for 8 days beginning 48 h before or 4 h after exposure of mice to virus, the mean day of death was increased and lung consolidation was significantly reduced. However, the high dose was lethally toxic and the therapeutic index was less than that of ribavirin (Sidwell *et al.*, 1994).

BL1743. BL1743 (Figure 25) is a spirene-containing compound that was found in a novel assay designed to identify inhibitors of M2. The gene encoding M2 was expressed in *Saccharomyces cerevisiae* under the control of an inducible promoter. When expressed, M2 is toxic to the yeast cells as demonstrated by a reduced growth rate. Therefore, inhibitors of M2 would be expected to reverse this reduction in cell growth rate. Accordingly, BL1743 was able to restore the growth rate of the yeast cells and also inhibited the replication of influenza A/Udorn/72 with an ED_{50} of approximately 2 μM in plaque assays (Kurtz *et al.*, 1995). In experiments where M2 was expressed in frog oocytes, BL1743 was found to inhibit the function of this ion channel. Furthermore, mutant viruses selected for resistance to BL1743 are also resistant to amantadine and the amino acid changes that confer resistance to amantadine are the same as those found in BL1743-resistant variants. However, data indicate that BL1743 and amantadine may interact differently with the M2 transmembrane pore region since inhibition by BL1743 is complete and reversible whereas resistance by amantadine is irreversible (Tu *et al.*, 1996). To our knowledge, no further information regarding the clinical potential of this compound is available.

Antisense oligonucleotides. Antisense oligonucleotides have been demonstrated to inhibit virus replication *in vitro* and their use against influenza virus has been considered. Early studies showed that oligonucleotides covalently linked to an acridine derivative inhibited the replication of influenza A virus in cell culture (ED_{50}=50 μM), but did not inhibit the replication of influenza B/Hong Kong/8/73 owing to differences in the genomic sequence between the two viruses (Zerial *et al.*, 1987). More recently, a new type of antisense oligonucleotide, which contains two hairpin loop structures with RNA/DNA base pairs in the double helical stem (referred to as nicked and circular dumbbell DNA/RNA chimeric oligonucleotides), was designed to be more resistant to exonuclease attack. This type of oligonucleotide was shown to have activity against influenza A virus in cell culture (Abe *et al.*, 1997). Recent work has involved the liposomal encapsulation of phosphorothioate antisense oligonucleotides. These compounds have demonstrated specific inhibition of influenza virus RNA polymerase and nucleoprotein gene expression in MDCK cells (Abe *et al.*, 1998a). In other studies, phosphorothioate oligonucleotides, which target the PB1 and PB2 genes of influenza, were liposomally encapsulated and demonstrated to inhibit the influenza virus-induced cytotoxicity in MDCK cells as determined using the MTT assay (Abe *et al.*, 1998b). The liposomally encapsulated oligonucleotides displayed greater inhibitory activity than did the free oligonucleotides. To our knowledge, there have been no clinical trials evaluating antisense oligonucleotides for the treatment of influenza virus infections.

Zinc finger peptides. It has been shown that the influenza M1 (matrix) protein incorporates into lipid bilayers and

can inhibit the influenza virus polymerase, thus indicating a role of M1 in the regulation of viral replication (Ye *et al.*, 1987, 1988; Ye & Wagner, 1992). It was also shown that M1 could bind to RNA using a domain resembling a zinc binding motif common to RNA and DNA binding protein and prompted by this information, peptides based on the zinc binding motif in M1 were synthesized. One peptide, referred to as peptide 4, contains a zinc finger motif corresponding to amino acids 152 to 166 of M1 and was able to inhibit influenza virus transcription by 38% at a concentration of 0.05 μM (Judd *et al.*, 1992). Another peptide, referred to as peptide 6, was made by adding four more residues at the N terminus. This peptide contains a complete zinc finger including a seven amino acid loop or finger, a four amino acid tail at the C end, and eight amino acids that participate in the coordination of Zn. Peptide 6 was found to be more inhibitory to influenza virus transcription than was M1 itself (Judd *et al.*, 1992). In tissue culture, peptide 6 also inhibited the cytopathic effect of various type A and B influenza viruses (Nasser *et al.*, 1996).

More recently, peptide 6 was evaluated in the murine model of influenza virus infection. Mice infected with influenza A/PR/8/34 (H1N1) and treated with peptide 6 given intranasally at 30 or 60 mg/kg/day for 5 days beginning 4 h pre- or 8 h post-virus exposure were significantly protected from influenza induced death, arterial oxygen decline and lung consolidation (Judd *et al.*, 1997). In the delayed initiation of treatment protocol peptide 6, given intranasally at 30, 60, or 120 mg/kg/day, provided a similar protection pattern against influenza A/Victoria/3/75 (H3N2), as it did against the H1N1 virus. However, lung virus titres were not reduced in the day 5 assay (Judd *et al.*, 1997). Based on these experimental results, it was concluded that this zinc finger peptide might provide a novel class of antivirals useful for the treatment of influenza virus infections (Judd *et al.*, 1997). To our knowledge, no clinical evaluations of peptide 6 or similar peptides for the treatment of influenza virus infections have been initiated.

Oxygen free radical scavengers. The pathogenicity of influenza virus infections is characterized by infiltration of neutrophils and macrophages into the lung, oedema in the alveolar spaces and extensive haemorrhage. Neutrophils and macrophages produce superoxide free radicals (O_2^-) and hydrogen peroxide (H_2O_2) and although these radicals are useful in the phagocytic process, they can cause the lung tissue damage seen in adult respiratory distress syndrome and other inflammatory diseases (Tate & Repine, 1983; Blake *et al.*, 1983). Thus, the hypothesis that free radicals are involved in influenza pathogenesis was tested. In influenza virus-infected mice, O_2^- generation by alveolar phagocytic cells was studied and it was observed that on day 8 after virus infection, the O_2^--generating potency of alveolar phagocytic cells was approximately eightfold higher than was observed immediately following virus infection (Oda *et al.*, 1989). The specific superoxide radical scavenger, superoxide dismutase (SOD) or catalase can prevent the tissue damage induced by activated oxygen species, but delivery of SOD as a therapeutic agent is limited by the shortcomings of protein drugs. Therefore, conjugation of SOD and pyran copolymer (MW approximately 5600) was carried out and the conjugates were purified by gel filtration. Mice were infected with twice the LD_{50} of influenza A/Kumamoto/Y5/67 (H2N2) and treated daily with intravenous injections of the pyran-SOD conjugate at a dose of 200 U per mouse from days 5 to 8 after virus infection. This treatment dramatically protected mice from death, whereas native SOD at the same dose had little therapeutic effect (Oda *et al.*, 1989). These results suggested that O_2^- generated by neutrophils and macrophages in response to influenza virus challenge play an important role in the pathogenesis of influenza virus infection and, moreover, the oxygen free radical scavenger, pyran-SOD may represent a useful approach to therapy.

Another oxygen free radical scavenger, recombinant human manganese superoxide dismutase (MnSOD) was evaluated alone or in combination with ribavirin for its effects on influenza virus infection in the murine model of infection (Sidwell *et al.*, 1996). After challenge with influenza A/NWS/33 (H1N1), mice were treated parenterally with MnSOD at doses of 25, 50 and 100 mg/kg/day given every 8 h for 5 days beginning 48 h post-infection. The mice so treated displayed an increase in the mean day to death, a decrease in the decline of arterial oxygen saturation, a reduced lung consolidation and a reduction in lung virus titre (Sidwell *et al.*, 1996). The efficacy of MnSOD was found to be virus dose-dependent and, when administered by small-particle aerosolization, MnSOD displayed a dose-responsive efficacy. Also, intravenous administration of MnSOD was able to increase the mean day to death, lessen the decline in arterial oxygen saturation, and reduce lung virus titre in mice infected with influenza B/Hong Kong/5/72. The combination of MnSOD with ribavirin was somewhat more efficacious than was either agent alone (Sidwell *et al.*, 1996). These results suggest that the use of oxygen free radicals may provide an approach to the amelioration of the pathogenicity caused by influenza virus infections. To our knowledge, no clinical trials of oxygen free radical scavengers for the treatment of influenza virus infections have been reported.

Polyoxometalates. Polyoxometalate compounds consist of soluble inorganic cluster-like molecules made up of an oxide anion and early transition metal cations and can be grouped into the following families: (1) the Keggin class (For example α-$SiW_{12}O_{40}^{4-}$); (2) the Wells-Dawson class

(For example $P_2W_{18}O_{62}^{6-}$); (3) fragments from these structures (For example $PW_{11}O_{39}^{7-}$); (4) the Keggin-derived sandwich compounds [For example $K_{10}Fe_4(H_2O_2)(PW_9O_{34})_2$, code name HS-058]; (5) the hexametalates or the Lindquist class (For example $W_6O_{19}^{2-}$); (6) decatungstate ($W_{10}O_{32}^{4-}$); and (7) the Preyssler ion $(NaP_5W_{30}O_{114})^{14-}$ (Hill *et al.*, 1990a).

These compounds have displayed broad spectrum *in vitro* activity against human myxo-, herpes, retro-, toga-, rhabdo-, and arenaviruses (Yamamoto *et al.*, 1992; Ikeda *et al.*, 1993) and various polyoxometalates have demonstrated activity against HIV (Hill *et al.*, 1990a,b; Yamamoto *et al.*, 1992; Kim *et al.*, 1994), apparently by preventing the attachment of the virus to the host cell and thus preventing syncytium formation. Like HIV, myxoviruses, including influenza viruses, are known to bind to the host cell membrane by adsorption and penetration of the virus follows fusion of the virus envelope with the host cell membrane. This provided a rationale for examining the inhibitory activity of 25 polyoxometalates against influenza virus, among other viruses (Shigeta *et al.*, 1995). Of the 25 tested *in vitro*, 24 showed activity against influenza A virus, 11 showed activity against respiratory syncytial virus, six showed activity against measles virus, and 23 showed activity against HIV at concentrations which were not cytotoxic (Shigeta *et al.*, 1995). HS-058, which belongs to the Keggin sandwich class $[K_{10}Fe_4(H_2O_2)_2(PW_9O_{34})_2.nH_2O]$, inhibited influenza A and B viruses at a median effective concentration of 1.4 μM and displayed a median cytotoxic concentration for MDCK or Hep-2 cells of greater than 200 μM. Interestingly, this compound had no activity against PIV-3 or mumps virus (Shigeta *et al.*, 1995). If added before virus infection at a concentration of four times the effective median antiviral concentration, HS-058 completely inhibited the replication of influenza A virus in MDCK cells. HS-058 was also shown to in-hibit the low pH-induced and influenza virus-mediated haemolysis of chick red blood cells at a concentration of 58 μM but did not inhibit HA at a concentration of 100 μM. At a concentration of 200 μM, dextran sulphate did not inhibit HA or haemolysis (Shigeta *et al.*, 1995). These observations prompted the consideration that polyoxometalates do not inhibit the adsorption of influenza virus to the host cell but may inhibit the fusion of the viral envelope with the host cell membrane, a process that is mediated by the cleaved HA molecule (See above section, Inhibitors of HA-mediated membrane fusion). Similar results were reported for sulphated polysaccharides (Hosoya *et al.*, 1991).

Subsequently, a new series of germanium- or silicon-centred heteropolytungstates with the barrel, keggin, or double keggin structure were evaluated *in vitro* for their inhibitory activity against influenza A and B viruses (Huffman *et al.*, 1997). The EC_{50} values of these compounds against influenza A (H1N1) and B ranged from 0.1 to 7.8 μM and were usually 10-fold higher against influenza A (H3N2), as determined by the inhibition of virus induced cytopathic effects in MDCK cells. In virus yield reduction assays, EC_{90} values against these viruses ranged from 0.2 to 32 μM. The compounds showed 50% cytotoxicity (CC_{50}) at concentrations ranging from 38 to 189 μM (Huffman *et al.*, 1997). These polyoxometalates showed the greatest efficacy when given early in the viral replication cycle, as determined by time-of-addition studies. The most active polyoxometalates were evaluated in the mouse model of infection using intraperitoneal administration beginning 4 h prior to virus infection. One compound with a barrel structure and the other with a double keggin structure were found to be effective in preventing deaths, reducing the decline in arterial oxygen saturation, and decreasing lung consolidation. At most, lung virus titres were reduced by a maximum of 0.7 $\log_{10}$. When initiated 8 h post-infection, therapy with these compounds was not effective in the mouse model (Huffman *et al.*, 1997). As pointed out by the authors, although the barrel and double keggin structures were most efficacious *in vivo*, definitive structure–activity relationship studies with these compounds are difficult to carry out since all showed significant antiviral activity *in vitro* (Huffman *et al.*, 1997). Thus, selecting the most promising polyoxometalates as candidates for further development represents a challenge.

Studies in rats treated with HS-058-related molecules by single-dose intravenous administration indicated that the compounds were highly bound to serum in a dose-dependent manner, and had a long plasma half-life (measured in days), were stable and were non-toxic (Ni *et al.*, 1994). However, to our knowledge polyoxometalates have not been evaluated clinically for the treatment of influenza virus infections.

Conclusion

Many epidemics of influenza have been described and recorded during the past 500 years of history and still occur with unabated ferocity today. No control measures for influenza were available until vaccines were developed in the 1940's and these have proven to be less than perfect owing to the extensive antigenic variation that occurs among influenza viruses. Many hundreds of compounds have been evaluated for their ability to act as antiviral agents to control influenza virus infections but only two, amantadine and rimantadine, have been approved for clinical use by the FDA and these drugs have significant limitations as discussed in this review.

Therefore, up to the current time, when a victim of influenza virus infection sought advice from a physician, that advice usually consisted of 'go to bed, get some rest,

take aspirin, drink chicken soup, and hope for the best'. To make it worse, quite often antibiotics, which have no efficacy against influenza virus infections, were prescribed. With the help of sophisticated molecular biology and our ability to crystallize and elucidate the structure of various viral proteins, such as the influenza virus NA, structure-based drug design efforts have been implemented to identify and develop inhibitors of these proteins. Recently, inhibitors of influenza virus NA have shown much promise in clinical trials. It is hoped that with the approval of zanamivir and GS 4104 for general use, the physician will be able to prescribe a drug that will have therapeutic efficacy by preventing further replication of the virus. Of much interest and excitement regarding zanamivir and GS 4104 is that they appear to be well tolerated and may not have any serious adverse side effects. Importantly, the development of virus variants resistant to these inhibitors may not pose a threat to the clinical utility of these compounds.

The recent progress in the development of NA inhibitors and the demonstrated potential of these agents in the clinical setting has underscored the need for diagnostic tests to identify those individuals infected with influenza. There are two main reasons why accurate, rapid, sensitive and inexpensive diagnostic tests are needed if the NA inhibitors, or any anti-influenza virus agent, are going to be effective in the general population for treating influenza infections. First, infections of the respiratory tract caused by other viruses or bacteria result in symptoms that, to some extent, resemble those induced by influenza A or B viruses. For these infections, the NA inhibitors and other agents selective against influenza virus enzymes will not have any clinical value. Secondly, although disease symptoms are not felt until about 36 h after infection, during this time much virus replication has occurred, resulting in much damage to the body. Considering that influenza virus replication peaks about 48 h after infection, whereas disease complications are not felt until about 40 h after infection, any anti-influenza virus agent, including NA inhibitors, will be most effective when given soon after infection, when no disease symptoms are apparent but when virus shedding is detectable.

The ideal diagnostic for influenza virus infection therefore would be a simple 'dip-stick' immunological test that could be purchased over the counter and might involve sticking a piece of treated paper or plastic in the mouth routinely every day during seasonal outbreaks of influenza. A colour change on the 'dip stick' would indicate the presence and shedding of influenza A or B virus and would prompt the individual to seek treatment with any approved and available antiviral for influenza virus infection. The development of such a test is an active area and numerous large pharmaceutical companies and small biotech firms have made considerable progress in developing a rapid and easily performed diagnostic. In fact, recently the FDA has approved a rapid influenza test, FLU O1A, which can detect both influenza A and B within 20 min. The test uses optical immunoassay technology and can be performed by physicians in their offices. It is claimed that the test is faster and more convenient, yet as sensitive as 14 day viral culture tests (press release from Thermo BioAnalysis posted on PR Newswire, December 2, 1998: http://www.prnewswire.com/cnoc/exec/menu?877850).

In conclusion, it is hoped that the recently developed influenza NA inhibitors, zanamivir and GS 4104, perform as well in the general population as they have done so far in clinical trials. Additionally, it is expected that improvements in vaccine technology will continue to be made. Together, these approaches may bring influenza, which has plagued the world for so long, under control.

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