Review

# Targeting the host or the virus: Current and novel concepts for antiviral approaches against influenza virus infection

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A B S T R A C T

Influenza epidemics and pandemics are constant threats to human health. The application of antiviral drugs provides an immediate and direct control of influenza virus infection. At present, the major strategy for managing patients with influenza is through targeting conserved viral proteins critical for viral replication. Two classes of conventional antiviral drugs, the M2 ion channel blockers and the neuraminidase inhibitors, are frequently used. In recent years, increasing levels of resistance to both drug classes has become a major public health concern, highlighting the urgent need for the development of alternative treatments. Novel classes of antiviral compounds or biomolecules targeting viral replication mechanism are under development, using approaches including high-throughput small-molecule screening platforms and structure-based designs. In response to influenza virus infection, host cellular mechanisms are triggered to defend against the invaders. At the same time, viruses as obligate intracellular pathogens have evolved to exploit cellular responses in support of their efficient replication, including antagonizing the host type I interferon response as well as activation of specific cellular pathways at different stages of the replication cycle. Numerous studies have highlighted the possibility of targeting virus–host interactions and host cellular mechanisms to develop new treatment regimens. This review aims to give an overview of current and novel concepts targeting the virus and the host for managing influenza.

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# 1. Introduction

Influenza epidemics and pandemics are constant threats to human health. A large gene pool of influenza A viruses resides in diverse animal reservoirs (Webster et al., 1992), thus influenza viruses with novel genetic composition are able to emerge through genetic reassortment over time, as recently seen with the A(H1N1)pdm09 virus (Smith et al., 2009). The error-prone RNA-dependent RNA polymerase (Lauring and Andino, 2010) of the influenza virus further increases the diversity of the influenza genome through random mutation, further increasing the challenge of controlling influenza infections.

Southeast Asia has long been termed the "epicentre" for influenza virus (Shortridge and Stuart-Harris, 1982), where a unique ecology and large human and domestic animal populations contribute to the human-animal interface, which may have facilitated the emergence of 1957 and 1968 pandemic influenza (Kawaoka et al., 1989). The highly pathogenic H5N1 virus that causes zoonotic human infections also emerged from this region in the late 1990s and has spread to a number of countries in Eurasia and Africa and evolved into different lineages. Long-term surveillance has been established in this region for decades, and the results have provided information on the evolution of animal influenza viruses and mechanisms leading to interspecies transmission events (Smith et al., 2009; Vijaykrishna et al., 2011). The challenge remains for scientists to forecast the emergence of a potential influenza pandemic.

Currently available control measures for influenza include vaccination and two classes of antiviral compounds, the M2 ion channel blockers and the neuraminidase (NA) inhibitors. The use of vaccines for the control of influenza requires timely vaccine production (which takes more than 6 months) and knowledge of the antigenic properties of the new circulating strains. This has been a challenging task for annual seasonal influenza vaccine production, as suboptimally matched vaccine strains or delays in production could lower the benefit of vaccination. Furthermore, vaccination may not achieve full protection in certain age groups or patients, as the effectiveness of a vaccine depends on the immune competence of its recipients (Fiore et al., 2009).

Traditional antiviral compounds such as M2 ion channel blockers or NA inhibitors that target functionally conserved domains of viral proteins generally provide inhibitory activity against different influenza viruses, including newly emerged pandemic influenza, and can be used for both treatment and prophylaxis of infection. The major challenges of using antiviral compounds for the control of influenza is the development of drug resistance and the difficulties in treating patients with severe influenza (Hayden, 2009). Resistance to both drug classes has become a major public health concern, and viruses with decreased sensitivity to both classes of antivirals have been reported. Thus, there is an urgent need to develop alternative influenza treatments. Novel antiviral compounds are under development. Strategies include improving currently available drugs in design, route of delivery, or potency; identifying new classes of compounds that target different viral proteins; and the application of combination therapy.

Other approaches, which remain under-explored, may have the potential to be developed into useful therapeutic interventions. One under-appreciated therapy is traditional Chinese medical herbs. With a 3000-year history, traditional Chinese medicine (TCM) has its own unique system in diagnosis and therapy. Unlike modern medicine which focuses on specific targets, TCM emphasizes body homeostasis (Yin-Yang Theory). For example, Kakkon-to, a TCM prescription for treating colds and influenza, has been shown to have immunomodulatory effects. Treatment of influenza virus-infected mice with Kakkon-to significantly reduced weight loss in the infected animals (Kurokawa et al., 2002). Instead of investigating the effect of TCM crude extracts in influenza treatment, future research should focus on identifying effective compounds isolated from TCM and developing a scientific and systemic platform to categorize TCMs.

In response to influenza virus infection, host antiviral mechanisms are triggered to defend the cells against the invaders. At the same time, the viruses have evolved to hijack cellular mechanisms for their own efficient replication (Nagata et al., 2008; Watanabe et al., 2010). Severe influenza, leading to tissue damage, results both from viral replication and host immune responses. Numerous studies have highlighted the possibility of targeting virus–host interactions and host cellular mechanisms to develop new regimens of influenza therapy (Ludwig, 2011). Specifically blocking certain signaling pathways has led to reductions in viral titers as well as modulation of host responses to infection, and some immune modulators can be applied in combination with antiviral compounds to both dampen tissue damage and effectively control infection. In this review, we will discuss current and novel concepts of antiviral approaches against influenza, from the point of view of targeting the virus and the host.

# 2. Antiviral approaches that target the influenza viruses

Antiviral compounds target functionally conserved domains of viral proteins to achieve cross-protection against different strains of influenza viruses. These conserved domains play critical roles during the viral replication cycle.

## 2.1. Replication of influenza A virus

Influenza viruses belong to the family *Orthomyxoviridae* and have single-stranded, segmented RNA genomes of negative polarity (Palese and Shaw, 2007). The eight-segmented genome of influenza A virus encodes the RNA polymerase complex (PB2, PB1, PA), the nucleoprotein (NP), the surface glycoproteins HA and NA, which function as a receptor binding protein and sialidase, respectively, the matrix protein (M1), the M2 ion channel and the nonstructural proteins NS1 and NS2/NEP (Palese and Shaw, 2007). In addition, most influenza A viruses encode a mitochondria-targeting

nonstructural PB1-F2 protein of varying length, with a pro-apoptotic function (Chen et al., 2001). Furthermore, N40 and PA-x are newly identified polypeptides encoded by the PB1 and PA genes, respectively (Jagger et al., 2012; Wise et al., 2009).

Infection is initiated after the attachment of HA to sialic acid-containing glycoprotein and glycolipid receptors on host cells (Gamblin and Skehel, 2010). The entry of influenza viruses has been shown to occur via clathrin-dependent endocytosis and macropinocytosis (de Vries et al., 2011). The HA is an important host range determinant, as influenza viruses from different hosts show different preferred recognition patterns for sialyl receptors (Rogers and Paulson, 1983). The acidic environment within the endosome following virus entry triggers a conformational change of HA, exposing the fusion protein to mediate the fusion of the viral envelope with the endosome membrane (Skehel and Wiley, 2000). M2 ion channels permit proton flow from the endosome into the interior of the virion, disrupting protein–protein interactions and freeing the ribonucleoproteins (RNPs) from the M1 proteins. The M2 ion channel also regulates the pH of the trans-Golgi network to prevent premature conformational changes of HA at later stages in the replication cycle (Lamb and Pinto, 2006).

The RNPs are transported into the nucleus for viral mRNA synthesis and viral genome replication (Resa-Infante et al., 2011). The PB1 protein functions as a RNA-dependent RNA polymerase, while the PB2 contains a cap-binding domain, and the PA possesses the endonuclease activity required for cap-snatching and viral mRNA synthesis. Newly synthesized viral proteins (PB1, PB2, PA, NP) and genome segments are packaged as RNPs and transported to the cell membrane for final assembly and budding. The NA functions as a sialidase that cleaves terminal sialic acid residues from the receptor, and is essential for the efficient release and spread of virions from the infected host cell (Air, 2011).

### 2.2. M2 ion channel blockers

The proton-conducting ion channel regulates virion pH during its entry in the endosome; it is a homo-tetramer, consisting of 4 M2 integral membrane proteins of 97 amino acids. Amantadine (1-adamantanamine hydrochloride) was first approved for prophylaxis against H2N2 influenza in 1966 and subsequently approved for prophylaxis and treatment of influenza A infections in 1976 (Gubareva and Hayden, 2006). The adamantane derivatives, amantadine and rimantadine, are effective in blocking the M2 ion channel activity of influenza A viruses, but exhibit no inhibition of the BM2 ion channel of influenza B viruses (Mould et al., 2003). The mechanism of action is exerted via an "early antiviral effect," by preventing the dissociation of RNPs from M1 proteins, as well as a "late antiviral effect," by causing early HA conformational change in the trans-Golgi (Lamb and Pinto, 2006) (Table 1).

Besides the lack of an inhibitory effect against influenza B virus, the major limitation for the use of amantadine and rimantadine for the control of influenza A virus infection is the rapid emergence of resistant variants in vitro and in vivo. Single-amino-acid substitutions at multiple positions (residues 26, 27, 30, 31, or 34) within the trans-membrane domain of the M2 protein confer cross-resistance to amantadine and rimantadine (Gubareva and Hayden, 2006). Treatment of influenza virus infection with M2 ion-channel blockers can cause the emergence of fully pathogenic and transmissible resistant variants in 30–80% of individuals, depending on the detection method used (Gubareva and Hayden, 2006). V27A, L26F, and S31N mutations have been reported from surveillance results as transmissible variants (Wang et al., 2011), with S31N as the most prevalent mutation. The current use of adamantine for the treatment of influenza A infection has been limited for H3N2 viruses, since the resistant variant became dominant in 2005 (Deyde et al., 2007). Furthermore, highly pathogenic H5N1 viruses that evolved into different clades have exhibited variable degrees of sensitivity to amantadine (Gutierrez et al., 2009). In addition, the newly emerged A(H1N1)pdm09 viruses were also resistant to adamatanes, due to the presence of the S31N mutation (Gubareva et al., 2010).

Derivatives of adamantanamine or adamantanaminoalcohols have been reported which have a greater inhibitory effect than amantadine on influenza virus replication (Zoidis et al., 2010). Derivatives of the spirene guanidine analog, 2-[3-azaspiro(5,5) undecanol]-2-imidazoline (BL-1743) (Kurtz et al., 1995) have also been identified which have a stronger M2 ion channel-inhibiting effect than amantadine (Wang et al., 2009). Furthermore, spirene derivatives [spiro(5,5)undecan-3-amine] inhibit the L26F and V27A variants, which otherwise confer resistance (Balannik et al., 2009). Recent studies on the transmembrane domain of the M2 tetramer complexed with amantadine or rimantadine have revealed two potential inhibitor binding sites, a high-affinity binding site at the N′-terminal channel lumen, in proximity to the reported

**Table 1**
Antiviral agents for influenza virus in current use or under development.

| Class of antiviral agent | Stage of viral replication | Inhibitor | Route of administration |
|---|---|---|---|
| M2 ion channel blockers | Inhibit viral uncoating at the early phase and promote premature HA conformational change in the trans-Golgi during the late phase of the replication cycle | Amantadine | Oral |
| | | Rimantadine | Oral |
| NA inhibitors | Inhibit NA enzyme activity important for initiation of infection and release of viral progeny | Zanamivir | Inhalation, IV (under named-patient program) |
| | | Oseltamivir | Oral |
| | | Peramivir | IV, IM |
| | | Lanamivir | Inhalation, long-acting |
| Nucleoside analogs | Depletion of cellular GTP pools, RNA elongation, mutagen | Ribavirin | Oral |
| | RNA elongation | Favipiravir (T-705) | Oral |
| Nucleozin and derivatives | NP oligomer formation and nuclear transportation | Nucleozin | IP (mouse model) |
| Endonuclease inhibitor | Inhibit the cap-snatching endonuclease activity of PA | 4-Substituted, 2,4-dioxo-4-phenylbutanoic acid | - |
| Fusion blockers | Inhibit HA fusogenic conformational change | Arbidol | Oral |
| | | HA2 mAb | IV |
| | | Protein inhibitor (F-HB80.4) | – |
| NS1 inhibitors | Inhibit NS1 activity in type I interferon-competent cells | NSC109834 | – |
| | | NSC128164 | |
| | | NSC95676 | |
| | | NSC125044 | |

mutations, that confers resistance to the adamantanes, and a low-affinity binding site at the C′-terminal protein surface (Cady et al., 2010). As amantadine was not structurally designed to fit the M2 ion channel, future optimization of M2 ion channel blockers, based on recent structural information, may provide new possibilities for pharmaceutical investigation (Cady et al., 2010).

### 2.3. NA inhibitors

The sialidase activity of the NA has been shown to play an important role during the initiation of infection and the release and spread of viral progeny (Air, 2011; Matrosovich et al., 2004). Although the HA and NA surface glycoproteins are subject to humoral immune selection pressure, resulting in antigenic drift, the presence of conserved residues within the NA active site across all influenza subtypes has provided a desirable target for drug design (von Itzstein et al., 1993). Zanamivir (Relenza; 4-guanidino-Neu5Ac2en, GG167) was the first small molecule against influenza developed through structure-based drug design (von Itzstein et al., 1993). It is administered via inhalation. Further antiviral development resulted in the orally administered compound, oseltamivir phosphate [Tamiflu; ethyl(3R,4R,5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate, GS4104], which is converted into its active form, oseltamivir carboxylate [(3R,4R,5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid, GS4071] by hepatic esterases (Kim et al., 1996) (Ward et al., 2005) and the intravenously administered compound, peramivir [(1S,2S,3S,4R)-3-(1S)-1-acetylamino-2- hydroxycyclopentane carboxylic acid, BCX-1812, RWJ-270201] (Babu et al., 2000). Zanamivir and oseltamivir have been approved for the prophylaxis and therapy of influenza A and B since 1999 in the USA and subsequently in other countries, while peramivir is currently under phase III clinical trial in the USA, and is only available in Korea and Japan. NA inhibitors are currently the predominant antiviral drugs used for the treatment and prophylaxis of influenza. Oseltamivir is more frequently used than zanamivir clinically, due to its oral route of administration. It has also been approved for use in a wider age group, including children 1–6 years old.

As the NA inhibitors are analogs of sialic acids, the mechanism of their antiviral activity is through competition with the natural ligand, blocking the enzyme active site. NA inhibitors are effective against influenza A and B viruses, and even different NA subtypes, since the conserved enzyme site is relatively conserved, and only subtle structural differences have been identified (Russell et al., 2006). Specifically, different NA subtypes of influenza A virus can be grouped based on phylogenetic analysis. Group 1 contains N1, N4, N5, and N8, and group 2 contains the N2, N3, N6, N7, and N9 subtypes. Compared to Group 2 NAs, Group 1 NAs possess an extended cavity (the 150-cavity) due to the differences in amino acid residues within the 150-loop (residues 147–152) (Russell et al., 2006). The extended cavity has provided new insights for NA inhibitor design (Rudrawar et al., 2010). Interestingly, the A(H1N1)pdm09 virus has been reported to lack the 150-cavity (Li et al., 2010).

Resistant viral variants have been isolated in in vitro studies through serial passage in the presence of NA inhibitors, and also arise in patients treated with NA inhibitors (Gubareva, 2004; McKimm-Breschkin, 2000). Due to the differential interaction between oseltamivir and zanamivir with conserved residues at the NA enzyme site, mutations that confer resistance to oseltamivir often do not confer resistance to zanamivir or laninamivir (Hayden, 2009). While most of oseltamivir-resistant variants have emerged during therapeutic usage, clinically derived resistance to zanamivir occurs at lower frequency (Hurt et al., 2009). Furthermore, clinically-derived NA mutations that confer resistance to oseltamivir are subtype-specific.

As the NA inhibitors are structurally similar to the natural ligand, sialic acid, it was once thought that mutations at conserved NA residues that confer drug resistance would also decrease NA activity or stability, compromising viral fitness. We and others have attempted to understand the effect of different NA mutations on fitness, including the pathogenicity and transmission potential of resistant variants (Abed et al., 2006; Kiso et al., 2011; Yen et al., 2007). Among the NA mutations that confer resistance to NA inhibitors, the H275Y NA mutation (N1 numbering) has been the most extensively studied to date, due to its significance in the seasonal H1N1, highly pathogenic H5N1, or more recently in the A(H1N1)pdm09 virus. This mutation was first reported during clinical trials among patients receiving oseltamivir (Gubareva et al., 2001). It was generally detected at a low frequency among community isolates prior to the 2007–8 influenza season (Monto et al., 2006; Sheu et al., 2008). However, an increasing number of H1N1 seasonal influenza viruses encoding the H275Y mutation (N1 numbering) emerged in northern Europe in late 2007 and subsequently spread globally, resulting in dominance of the H275Y variant over the wild-type virus in 2008 (Dharan et al., 2009; Lackenby et al., 2008). The detailed mechanism that enables the transmission of the H275Y variant is still under research (Bloom et al., 2010; Rameix-Welti et al., 2011).

The H275Y NA mutation confers resistance to oseltamivir and peramivir, but not to zanamivir or laninamivir. While A/Brisbane/59/07-like viruses with the H275Y NA mutation were resistant to oseltamivir, they remained largely sensitive to zanamivir or M2-ion channel blockers. However, dual resistance to both oseltamivir and M2-ion channel blockers were detected at an increasing rate among H1N1 seasonal influenza viruses isolate between 2008–10, with a 28% (7/25) detection rate reported by the US Centers for Disease Control and Prevention (CDC) during the 2009–10 season, before seasonal H1N1 was replaced by the newly emerged A(H1N1)pdm09 virus (Cheng et al., 2010; Sheu et al., 2011). Interestingly, most dual-resistant strains emerged through genetic reassortment, acquiring the M gene from a clade 2C virus with a S31N mutation that confers resistance to amantadine into a clade 2B viruses that already carried the H275Y NA mutation (Sheu et al., 2011).

As mentioned previously, the A(H1N1)pdm09 viruses that emerged in 2009 carry the S31N mutation in the M2 protein that confers resistance to amantadine and rimantadine. Dual-resistant A(H1N1)pdm09 viruses that acquired NA mutations conferring resistance to NA inhibitors have been identified at <2% of the isolates tested for susceptibility (Hurt et al., 2012). The H275Y NA mutation was the most frequently identified mutation among A(H1N1)pdm09 viruses that exhibited resistance to NA inhibitors. Other variants carrying NA mutations I223R/K/V, H275Y + I223R, S247N, S247N + H275Y, Q313R + I427T, or I117V have also been reported (Hurt et al., 2012). Recent studies that characterized the fitness and transmission potential of the A(H1N1)pdm09 viruses carrying the H275Y NA mutation showed variable findings, with most transmission studies in animals supporting a comparable or slightly delayed transmission potential for the H275Y variant (Duan et al., 2010; Kiso et al., 2010; Memoli et al., 2011; Seibert et al., 2010; Wong et al., 2012).

The long-acting NA inhibitor laninamivir (R-125489) has been approved in Japan since 2010 for the treatment of influenza A and B. The prodrug, laninamivir octanoate (CS-8958 or R-118958) is processed into laninamivir in the lungs after being administered by inhalation (Kubo et al., 2010) (Table 1). A single nasal administration leads to long retention of the drug in the lungs, providing long lasting anti-influenza activity in vivo (Yamashita et al., 2009). Laninamivir and zanamivir possess a 4-guanidino group which has been shown to result in greater potency against group-1 NA subtypes with the cavity formed by the 150-loop

(Vavricka et al., 2011). Clinical trials have reported comparable effectiveness of laninamivir versus other NA inhibitors against A(H1N1)pdm09 virus, seasonal H3N2 influenza virus, or influenza B virus (Ikematsu and Kawai, 2011; Shobugawa et al., in press).

Besides novel NA inhibitors, improvements in the route of administration such as intravenous zanamivir delivery have been tested under the Emergency Investigational New Drug (EIND) scheme in the USA or the named-patient program in other countries, depending on their regulations (Boltz et al., 2010) (Table 1). Peramivir was also administered to hospitalized adults or children with severe viral pneumonia under EIND or Emergency Use Authorization (EUA) in the USA during the 2009 pandemic (Hernandez et al., 2011; Sorbello et al., 2012; Yu et al., 2012).

In addition, double- or triple-combination chemotherapy with oseltamivir, adamantane and ribavirin have shown additive or synergistic effects in vitro and in animal models (Hoopes et al., 2011; Ilyushina et al., 2008; Ilyushina et al., 2007; Nguyen et al., 2010; Smee et al., 2009). Combination therapy may also play a role in limiting the emergence of drug-resistant viruses. A recent clinical trial that applied combination therapy with oseltamivir and zanamivir did not show greater protection than monotherapy against seasonal influenza (mainly H3N2) infection in humans (Duval et al., 2010). Similarly, a retrospective study that compared the effect of oseltamivir mono-therapy to triple-combination treatment with oseltamivir, amantadine and ribavirin of A(H1N1)pdm09-infected patients that required ventilator support reported comparable clinical outcomes for the two groups (Kim et al., 2011). Further studies are required to understand the effects of combinational therapy in humans and their role in limiting the emergence of resistant variants.

## 2.4. Ribavirin

Ribavirin (1-(β-D-Ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide) is a nucleoside analog that exhibits a broad range of antiviral activities against RNA and DNA viruses (De Clercq, 2009; Sidwell et al., 1972). It is processed by cellular adenosine kinase into ribavirin monophosphate (RMP), followed by subsequent phosphorylation into di- or triphosphate (RTP) forms (Graci and Cameron, 2006). Its major antiviral activity is conferred by the RMP form, which is structurally similar to cellular inosine monophosphate (IMP). RMP and IMP therefore can compete for the IMP dehydrogenase (IMPDH) active site, leading to depletion of the cellular GTP pool (Table 1). Such depletion has a general effect on viral and host RNA, DNA, and protein synthesis, and is considered to be the cause of ribavirin's broad antiviral activity against different RNA and DNA viruses (De Clercq, 2009; Graci and Cameron, 2006).

As a nucleoside analog, ribavirin may also directly interact with the viral polymerase. For influenza viruses, RTP has been shown to interact with the RNA polymerase in a cell-free system and exhibit competitive inhibition with GTP and ATP, but not with UTP or CTP (Eriksson et al., 1977). However, a higher concentration of RTP is required to fully inhibit influenza virus RNA elongation than that in competition with the GTP or ATP, suggesting that RTP can be incorporated into the influenza viral genome (Parker, 2005). This mechanism may increase error-prone replication, leading to error-catastrophe and a reduction in yield of infectious virus (Vignuzzi et al., 2005).

Despite reports of the broad in vitro antiviral activity of ribavirin, its clinical application for influenza has been limited, due to toxicity and limited evidence of clinical efficacy. Variable results have been reported from trials using oral or aerosolized ribavirin for influenza A or B patients (Chan-Tack et al., 2009) or from patients receiving intravenous ribavirin under an EIND scheme (Riner et al., 2009). Ribavirin is available as an oral form for influenza treatment in Mexico (Eichelberger et al., 2008) (Table 1). Combina-

tion therapy using ribavirin and pegylated interferon-alpha was established in early 2000 and it has become the standard treatment for chronic hepatitis C virus (HCV) infection in adults (De Clercq, 2009). Treatment for severe respiratory syncytial virus (RSV) infection using aerosolized ribavirin was approved in 1985, although its clinical benefit is still debatable (De Clercq, 2009). For influenza, ribavirin monotherapy has been tested in clinical trials or under EIND for severe infections. The results of these studies are inconclusive, due to small sample size or differences in treatment dose and duration; the potential clinical benefit of ribavirin for the treatment of influenza infection therefore remains to be further clarified (Chan-Tack et al., 2009). Importantly, resistance to ribavirin has not been reported for HCV, RSV or influenza viruses.

## 2.5. Antiviral compounds under development

### 2.5.1. Nucleoside analog: favipiravir (T-705)

Favipiravir (T-705; 6-Fluoro-3-hydroxypyrazine-2-carboxamide) is a pyrazine derivative first identified in 2002, which inhibits influenza A, B, and C viruses in vitro and influenza A virus in a mouse model (Furuta et al., 2002). Its action is mediated via its processed forms, T-705 ribofuranosyl phosphates, which include the monophosphate and triphosphate forms. The triphosphate form is mis-recognized by the influenza virus polymerase as a purine nucleotide during RNA elongation, inhibiting polymerase activity (Furuta et al., 2005). Unlike ribavirin, the host enzyme can differentiate favipiravir and its phosphorylated form from the natural nucleotides; favipiravir therefore shows little inhibitory effect on host cells and less cytotoxity than ribavirin (Furuta et al., 2009). The antiviral activity of T-705 can vary, depending on the host cells used; differences in uptake efficiency and/or the effectiveness of conversion of favipiravir into its ribofuranosyl phosphates by host-cell enzymes may determine its antiviral activity (Furuta et al., 2009). Synergistic effects between oseltamivir and favipiravir have been reported in mice against H1N1, H3N2 or H5N1 influenza virus infections (Smee et al., 2010).

Besides influenza virus, favipiravir inhibits other RNA viruses, including arenaviruses, bunyaviruses, West Nile virus, alphaviruses, poliovirus, rhinoviruses and RSV. Favipiravir exhibits no inhibitory effect against DNA viruses, including herpes simplex virus-1, human cytomegalovirus and adenoviruses, with EC50 values above 640 µM (Furuta et al., 2009). As ribavirin has shown inhibitory effects against DNA viruses, it is possible that the DNA viral polymerase can differentiate favipiravir better than ribavirin. Limited resistance to favipiravir has been reported; no resistant variant was detected after 30 serial passages of the A/PR/8/34 influenza virus (Furuta et al., 2009). Favipiravir began Phase III clinical trial in Japan in October, 2009, and Phase II clinical trials in the USA in February, 2010. We may therefore see increased use of favipiravir in the near future for the treatment of influenza.

### 2.5.2. Other RNA polymerase inhibitors

The influenza polymerase complex is a heterotrimer formed by the PB2, PB1, and PA proteins. The conserved PA$^{C'-term}$–PB1$^{N'-term}$ binding domain has also been explored as a target for influenza antivirals (Das et al., 2010; Ghanem et al., 2007). Peptides derived from the PB1$^{N'-term}$ have been shown to bind to PA, inhibiting viral replication and transcription (Ghanem et al., 2007; Wunderlich et al., 2009). Combined with knowledge derived from the crystal structure of PA-PB1 complex (He et al., 2008; Obayashi et al., 2008), these studies have provided the first step in identifying and designing small molecules that specifically disrupt protein–protein interactions.

The synthesis of influenza genomic vRNA and cRNA occurs de novo, while mRNA synthesis for virus protein production requires host-derived mRNA-derived 5'-caps as primers (Das et al., 2010).

The cap-binding domain of the influenza polymerase has been identified within PB2 (Guilligay et al., 2008), while endonuclease cap-snatching activity is conferred by the N′-terminus of PA (Dias et al., 2009). Furthermore, compound (L-735882), which inhibits endonuclease cap-snatching activity, has been reported (Tomassini et al., 1994) (Table 1). With knowledge of the catalytic site (Dias et al., 2009; Yuan et al., 2009), two recent studies have solved the co-crystal structures of the PA endonuclease domain with known or predicted inhibitors (Dubois et al., 2012; Kowalinski et al., 2012). Together with *in vitro* assays that measure their anti-endonuclease or antiviral activity, new insights have been proposed on the development of potential PA endonuclease inhibitors (Dubois et al., 2012; Kowalinski et al., 2012).

### 2.5.3. Nucleozin and its derivatives

By screening chemical libraries using a cell-based influenza infection assay, three laboratories independently identified the small-molecule compound, nucleozin, which induces NP oligomer formation (Gerritz et al., 2011; Kao et al., 2010; Su et al., 2010) (Table 1). The inhibitory effect was associated with the inhibition of NP transportation from the cytoplasm into the nucleus (Kao et al., 2010). Resolution of the crystal structure of a nucleozin analog bound to NP has revealed that NP dimer formation occurs via two ligand binding sites on each NP monomer; a hexameric structure was also observed, consisting of three NP dimers (Gerritz et al., 2011). Analogs of nucleozin have been identified which possess potent inhibitory effects *in vitro* and *in vivo*; however, resistance can develop after just 5 *in vitro* passages (Kao et al., 2010). Resistance to nucleozin has also been observed among naturally circulating influenza strains, including the A(H1N1)pdm09 virus, which possesses mutations at the ligand-binding pocket (Gerritz et al., 2011; Kao et al., 2010; Su et al., 2010). These studies support the concept that NP is a potential target for novel antivirals, but further optimization of nucleozin analogs will be required to increase their specificity and minimize drug resistance.

### 2.5.4. Blocking the fusogenic conformational change in HA

The influenza HA mediates receptor binding and membrane fusion activity, via its variable globular head region and its conserved stem region, respectively. Structure-based design has resulted in small molecules that inhibit the fusogenic conformational change of HA; however, these compounds cannot presently provide protection across a range of different HA subtypes (Bodian et al., 1993; Vanderlinden et al., 2010). However, an exception is arbidol [(ethyl-6-bromo-4- [(dimethylamino)methyl]-5-hydroxy-1-methyl-2-[(phenylthio)methyl]-indole-3-carboxylate hydrochloride monohydrate)], which has been licensed in Russia for the treatment of influenza A and B viruses (Leneva et al., 2009). Arbidol exerts immunemodulatory effects, as well as broad antiviral activity against influenza virus, RSV, rhinoviruses, coxsackie virus, HCV and chikungunya virus (Delogu et al., 2011; Shi et al., 2007). The mechanism of its antiviral activity against influenza was demonstrated through the identification of resistant variants with single amino acid changes in the HA2, which affected the HA fusion pH and conformational change (Leneva et al., 2009) (Table 1). An alternative approach made use of computational design of protein inhibitors targeting the HA stem region to block fusion. Protein inhibitors with improved cross-reactive binding affinity for group 1 HA have been achieved through screening of DNA-encoded library of protein variants, using yeast surface display followed by deep sequencing (Whitehead et al., 2012).

In addition to the compounds mentioned above that target the fusion mechanism, other inhibitors under development that target the HA include a cyanobacterium-derived protein (Cyanovirin-N) that binds to high-mannose structures on the HA to block viral entry (Smee et al., 2008); a peptide derived from a fibroblast growth-factor-4 signal sequence that possesses an inhibitory effect on viral attachment to cells (Jones et al., 2006); nitazoxanide, which targets a post-translational stage, blocking HA maturation and transportation (Rossignol et al., 2009); and the high-molecular-weight sulphated polysaccharide iota-carrageenan, derived from red seaweed, that inhibits viral spread as well as entry (Leibbrandt et al., 2010).

### 2.5.5. Cross-reactive antibodies that target the HA

Recent studies have identified naturally occurring human antibodies from vaccinated or exposed individuals that can bind different HA types. These cross-reactive antibodies possess neutralizing activity, by inhibiting the HA fusogenic conformational change (Corti et al., 2011; Ekiert et al., 2011; Sui et al., 2009; Throsby et al., 2008) or by targeting the HA globular head, preventing viral attachment to cells (Yoshida et al., 2009). Defining HA-Fab interaction by resolution of the crystal structure has revealed a series of conserved epitopes within the HA stalk region (Corti et al., 2011; Ekiert et al., 2011; Sui et al., 2009). These antibodies have been tested *in vitro* and in mice and ferrets, and exhibited significant protection upon challenge using different viral subtypes (Friesen et al., 2010; Koudstaal et al., 2009). The use of adoptive immunotherapy may be considered for pandemic preparedness, as cross-protection can be achieved regardless of the subtype of the emerging strains. Research is ongoing to identify the immune determinants that lead to the cross-reactive antibody response targeting the HA2 domain (Wang and Palese, 2011).

### 2.5.6. NS1 inhibitors

The multifunctional NS1 protein interacts with many viral and host proteins during the replication cycle (Hale et al., 2008). Among these multiple functions, the most essential is the ability of NS1 to antagonize the type I interferon response, which is achieved through several mechanisms including (1) interacting with TRIM25 to prevent RIG-I activation, (2) binding to CPSF and poly-(A) binding protein II to limit the exportation of cellular mRNA, (3) directly binding to and inhibiting the activation of PKR, and (4) binding to double-stranded RNA from detection of 2′-5′-oligoadenylate synthase (OAS) (Gack et al., 2009; Hale et al., 2008).

A yeast-based assay was developed to screen small molecules for anti-NS1 activity (Basu et al., 2009). Four compounds were found to possess anti-influenza activity, specifically showed reduction in viral protein synthesis or viral RNA production (Table 1). They demonstrated specificity against influenza virus, but not against RSV, and were only effective in interferon-competent cells. Derivatives of one of the 4 compounds (NSC125044) were synthesized (Jablonski et al., 2012; Walkiewicz et al., 2011), and one of them showed an anti-influenza effect that was dependent on host RNase L activity (Walkiewicz et al., 2011). These results suggest that NS1 is a potential target for novel influenza virus inhibitors.

## 3. Targeting the host to block virus entry

### 3.1. Sialidase

At the earliest step of the viral replication cycle, influenza virus binds to host cells through the interaction between the viral HA and terminally-linked sialyl receptors present on the cell surface. Removal of sialic acid from the cell surface is therefore an approach that can be utilized to disrupt virus-host interaction (Table 2). DAS181 is a sialidase fusion construct designed to remove sialic acid from cell surfaces. It is composed of a catalytic domain of sialidase from the bacteria *Actinomyces viscosus*, with a human respi-

**Table 2**
Agents under development that target the host to achieve antiviral and/or immunomodulatory effects.

| Potential therapeutic candidates | Antiviral effect | Immunomodulatory effect |
|---|---|---|
| Sialidase | Removes sialic acid receptors on the cell surface, blocking interaction with the viral HA | – |
| Protease inhibitors | Inhibit cleavage of the precursor HA0 into functional HA1/HA2 | – |
| MEK inhibitors | Block the MAPK/ERK protein kinase cascade, suppress the function of nuclear export protein, resulting in nuclear retention of viral RNPs | – |
| NF-κB and IKK2 inhibitors | Suppress the action of caspase and inhibit the release of viral RNP from the nucleus.Inhibit SOCS-3 induction, removing the inhibitory effect on ISG production mediated via the JAK/STAT pathway | Decrease proinflammatory cytokine and chemokine production upon H5N1 infection |
| COX-2 inhibitors | Suppress viral gene transcription, viral protein expression and progeny virus production in H5N1-infected cells | Attenuate H5N1-hyperinduced cytokines in the proinflammatory cascade |
| S1P agonists | – | Suppress cytokine release by T-cells and affect the antigen presentation ability of dendritic cells |
| IPP and PAM expanded gamma-delta T-cells | – | Expanded Vγ9Vδ2 T cell population to enhance the host immune response |
| PPAR agonists | – | Suppress inflammatory cytokine expression through trans-repression of NF-κB and AP-1 |
| Statins | – | Suppress inducible MHC-II expression and activity of LFA-1 |

ratory epithelium-anchoring domain fused to its C-terminus. DAS181 is well tolerated by the human immune system, as *A. viscosus* is a common oral bacterium. The human respiratory epithelium-anchoring domain contains a heparin-binding sequence that increases retention time and drug-potency (Malakhov et al., 2006). The sialidase activity of DAS181 can remove both the SA-α2–3 and SA-α2–6-linked residues which are recognized by avian and human influenza viruses, respectively.

DAS181 has demonstrated prophylactic and therapeutic effects against H5N1 virus infection in mice (Belser et al., 2007). Treatment with DAS181 leads to desialylation in human lung or bronchial tissues *ex vivo*, as well as in primary pneumocytes or airway epithelial cells, and thereby inhibits a broad spectrum of influenza viruses including the H5N1, the newly emerged A(H1N1)pdm09 virus and oseltamivir-resistant viruses (Chan et al., 2009; Triana-Baltzer et al., 2009a,b). DAS181 is also effective against human parainfluenza viruses *in vitro* and *in vivo* (Moscona et al., 2010).

Reducing influenza virus infection by using DAS181 to remove sialic acids from the cell surface has raised concerns for the potential exposure of cryptic receptors that might allow bacterial adherence and increase susceptibility to bacterial infection (Zhang, 2008). However, recent data suggested that DAS181 treatment does not enhance the colonization of *Streptococcus pneumoniae*, either *in vitro* or *in vivo* (Hedlund et al., 2010).

Unlike M2 and NA blockers, DAS181 does not target the virus, but the respiratory epithelial cells of the host. Recent studies have demonstrated that influenza virus variants selected through serial passage in the presence of DAS181 are attenuated, and possess an unstable resistance phenotype. DAS181-selected H3N2 and influenza B viruses regained their sensitivity when the drug was removed (Triana-Baltzer et al., 2011). Thus, the development of resistance in the future could be low. DAS181 has also proven to be safe in mice exposed to high concentrations (up to 3 mg/kg/day) in dry-powder formulations. DAS181 was well tolerated at all dose levels; body size, food consumption and other parameters were not affected (Larson et al., 2011). In humans, an international phase II clinical trial was conducted, involving 297 patients with H1N1, H3N2 or influenza B virus infections. A significant decrease in viral shedding was seen in patients who received low-dose treatment with DAS181 for three consecutive days (Moss et al., 2011). No adverse effects were reported.

### 3.2. Protease inhibitors

As noted above, the influenza HA glycoprotein functions as a binding receptor and as a fusion protein. It is composed of two subunits, HA1 and HA2, which are cleaved by host proteases from their HA0 precursor (Skehel and Waterfield, 1975). The cleavage of HA0 into functional HA1/HA2 activates virus infectivity, and is important for pathogenicity in human and avian hosts (Steinhauer, 1999). Functional HA is able to undergo conformational change in the low-pH endosomal environment, to induce membrane fusion followed by infection (Skehel and Wiley, 2000). Two proteases, human airway trypsin-like protease (HAT) and transmembrane protease serine S1 member 2 (TMPRSS2), cleave the monobasic cleavage site of some HAs (Bottcher et al., 2006). Expression of these two proteases in MDCK cells supports the propagation of seasonal influenza viruses, in the absence of trypsin (Bottcher et al., 2009). Recently, TMPRSS4 was identified, and it was found to have a similar role in the activation of HA (Chaipan et al., 2009). The HA of some highly pathogenic influenza viruses possess a multibasic protease cleavage site, which is cleaved by furin and other related cellular proteases located in the trans-Golgi apparatus (Stieneke-Grober et al., 1992). However, the acquisition of a multibasic cleavage site within the HA does not always transform a low-pathogenic virus into a highly pathogenic one (Stech et al., 2009).

Earlier reports have described protease inhibitors as a means to suppress the replication of influenza viruses (Zhirnov et al., 1984) (Table 2). Recently, peptide-mimetic protease inhibitors of HAT and TMPRSS2 were demonstrated to block viral replication *in vitro*, by inhibiting HA cleavage at the cell surface and within cells, respectively (Bottcher-Friebertshauser et al., 2010). Improvement in selectivity and potency was achieved by incorporating a synthetic amino acid residue, norvaline, into the protease inhibitor (Sielaff et al., 2011). The use of single-stranded DNA-like antisense agents to block the expression of TMPRSS2 also inhibits influenza virus infection in human airway epithelial cells (Bottcher-Friebertshauser et al., 2011). All of these studies have highlighted protease inhibitors as an alternative therapeutic approach. In addition to influenza, a recent report has also demonstrated that protease inhibitors are potential therapeutic agents against other respiratory viruses, including the SARS-coronavirus (Matsuyama et al., 2010), and thus may have wider applications future.

# 4. Targeting host signaling pathways

## 4.1. Antiviral approaches

Like other RNA viruses, influenza viruses take advantage of activated cellular signaling to support their replication. Novel antiviral drugs that disrupt these hijacked signaling events could therefore be considered for development.

### 4.1.1. MEK inhibitors

The Raf/MEK/ERK signaling pathway belongs to the mitogen-activated protein kinase (MAPK) cascade, which consist of a series of serine-threonine kinases. This cascade is an important pathway which converts the ligation of extracellular molecules into intracellular signals for the control of cell proliferation, differentiation and survival (Su and Karin, 1996). Its activation is triggered by G-protein-coupled receptors or receptor tyrosine kinase, leading to stepwise phosphorylation and activation of Raf kinases, the serine/ threonine kinases that participate in a sequential cascade to activate MEK1/2, which in turn activates ERK1/2. Numerous stimuli, including growth factors, hormones and cytokines, can trigger this pathway.

Activation of the MAPK cascade is now recognized as a hallmark signaling pathway activated by influenza A or B virus (Pleschka et al., 2001), including the highly pathogenic avian H5N1 and pdmH1N1viruses (Droebner et al., 2011). Specific inhibitors such as U0126, which blocks the cascade at the MAPK/ERK kinase (MEK) level, result in nuclear retention of viral RNP complexes and impair the function of the nuclear export protein (NEP/NS2), suppressing virus production (Pleschka et al., 2001) (Table 2). A further study suggested that activation of the MAPK cascade leads to efficient RNP export as well as virus production, achieved by the accumulation of the viral HA on the cell surface, resulted from enhanced viral polymerase activity (Marjuki et al., 2007). U0126 exhibited high antiviral activity and low cytotoxicity *in vitro* and *in vivo* (Droebner et al., 2011; Pleschka et al., 2001), making it a promising drug.

Clinical investigation is the next important phase to test the efficiency of MAPK cascade inhibitors to block influenza virus replication in humans. To date, the evaluation of MEK inhibitors in virus-infected mice has been limited to U0126 administered by the aerosol route (Droebner et al., 2011; Haasbach et al., 2011), which is not commonly employed in humans. As more MEK inhibitors that have been used in clinical trials with cancer patients become commercially available, further research is warranted.

### 4.1.2. NF-κB and IKK2 inhibitors

NF-κB signaling is another important pathway that is commonly activated after influenza virus infection. Upon recognition of pathogen infection or tissue damage, the NF-κB pathway is strongly activated by cellular pattern recognition receptors, including toll-like receptors and multiple cytosolic receptors, such as retinoic inducible gene I (RIG-I)-like helicases and NOD family proteins. NF-κB-responsive genes affect a diverse array of cellular processes, including apoptosis and cell survival, and often directly control the course of infection by up-regulating a variety of antiviral genes (Iversen and Paludan, 2010). Influenza virus infection activates the NF-κB pathway through activation of IKK2, as a result of the production of various cytokines (Julkunen et al., 2001). NF-κB is known as the key regulator of cytokine expression, including interferon (IFN)-β (Pahl, 1999). IFN-β initiates the type I IFN defense program to mediate antiviral activities (Wolff and Ludwig, 2009), by binding to the cell-surface type I IFN receptor and activating the JAK/STAT and other pathways, which control the expression of various antiviral genes (Platanias, 2005).

Although the NF-κB pathway mediates antiviral activity, influenza viruses have also evolved to evade and control the activity of NF-κB. For example, the viral NS1 protein antagonizes the NF-κB pathway, IFN-β induction and signaling (Wang et al., 2000). As noted above, novel small-molecule inhibitors of NS1 have restored the interferon-induced antiviral state in a number of recent studies (Babu et al., 2000; Jablonski et al., 2012; Walkiewicz et al., 2011), suggesting the high potential of inhibitors of viral IFN antagonists as antiviral drugs.

Influenza virus infection also induces the expression of TRAIL and FasL, which stimulate caspase 3 action (Wurzer et al., 2003, 2004). Caspase-3 plays a major role in nuclear disassembly, by cleaving cellular proteins which increase the diffusion limit through the nuclear membrane (Faleiro and Lazebnik, 2000). Nuclear retention of viral RNP complexes was observed when inhibitors of caspase or NF-κB were applied to infected cells, suggesting that caspase activity is crucial for viral RNP trafficking (Mazur et al., 2007; Wurzer et al., 2003). On the other hand, influenza viruses could also induce the expression of suppressor of cytokine signaling-3 (SOCS-3), via NF-κB signaling. Induction of SOCS-3 leads to inhibition of IFN-induced activation of the JAK/STAT pathway, which controls the expression of antiviral IFN-stimulated genes (ISGs) (Kubo et al., 2003) (Table 2).

NF-κB is a novel therapeutic target for influenza therapy. Various studies have shown that inhibition of the NF-κB pathway can reduce the viral titer. For example, acetylsalicylic acid (ASA), commonly known as aspirin, an inhibitor of IKK2, inhibited influenza virus replication *in vitro* and *in vivo*, and did not generate resistant strains in a multi-passage experiment (Mazur et al., 2007). However, it should be cautioned that aspirin has been linked with Reye's syndrome in children with influenza (Eyers et al., 2010; Starko, 2009) and with prominent systemic symptoms in adults with H1N1 virus infection, compared to those who received amantadine alone (Younkin et al., 1983). Recently, pyrrolidine dithiocarbamate (PDTC), an inhibitor of the NF-κB pathway, was shown to increase survival in H1N1-infected mice (Wiesener et al., 2011), while others have shown that treatment with inhibitors of the Raf/MEK/ERK cascade and NF-κB significantly reduced virus titers and cytokine expression, both *in vitro* and *in vivo* (Pinto et al., 2011). Phosphatidylinositol-3-kinase (PI3K) has also been suggested to be a cellular factor that is activated in response to influenza virus infection, and to play an important role in the regulation of virus replication (Ehrhardt et al., 2006).

## 4.2. Immunomodulatory approaches

Although the immune system provides a necessary response to infection, it may not always successfully eliminate the threat posed by influenza viruses. Some viruses may be directly cytopathic, while others, such as the highly pathogenic H5N1 virus, cause cytokine dysregulation, upregulating proinflammatory cytokines and leading to a fatal outcome. The use of immunomodulators to return the immune response to homeostasis should therefore be a promising strategy to deal with the deleterious host response triggered by such viruses.

### 4.2.1. COX-2 inhibitors

Cyclooxygenase-2 (COX-2) is a component of the arachidonic-acid cascade that is thought to be involved in inflammation and immune responses, and is therefore a target of anti-inflammatory drugs. As noted above, it was shown that ASA, which blocks NF-B activation and the activity of COX, inhibits influenza virus replication (Mazur et al., 2007). However, treatment with indomethacin, a pure COX inhibitor that inhibits both COX-1 and COX-2, failed to show any effect on virus replication. It was therefore concluded

that ASA blocks influenza virus replication via inhibition of NF-B signaling (Mazur et al., 2007).

Unlike the inducible COX-2, COX-1 is constitutively expressed in most normal body tissues, including bronchiolar and alveolar epithelial cells and pulmonary alveolar macrophages in rats and non-human primates (Ermert et al., 1998; Khan et al., 2000; Wilborn et al., 1995). It is involved in important physiological functions, such as vasodilatation, bronchodilation and surfactant synthesis (Brannon et al., 1998). Treatment with indomethacin therefore not only blocks not only COX-2 activity, but the activity of the physiologically important COX-1, thus perhaps leading to detrimental effects on virus-infected cells. In fact, an *in vivo* study demonstrated that defects in COX-1 or COX-2 led to opposite effects in H3N2 virus-infected mice: while COX-1 deficiency was detrimental, COX-2 deficiency improved survival (Carey et al., 2005).

COX-2 has been shown to play a regulatory role in the induction of pro-inflammatory responses by the H5N1 virus. Selective COX-2 inhibitors that are commonly used to treat rheumatoid arthritis, osteoarthritis and acute pain had immunomodulatory effects, suppressing hyper-cytokine induction after H5N1 infection (Lee et al., 2008). In a recent study, we further demonstrated that selective COX-2 inhibitors suppressed H5N1 virus replication *in vitro*, suggesting that viral replication was dependent on the activation of COX-2 signaling pathways (Lee et al., 2011). Another study showed that treatment of H5N1 virus-infected mice with the COX-2 inhibitor celecoxib, in combination with mesalazine and zanamivir, significantly improved survival (Zheng et al., 2008). These studies have highlighted the possibility of targeting COX-2 to regulate both virus replication and host immune response for the development of new treatment regimens (Table 2).

Some studies have highlighted the role of COX-2 in the resolution of late-stage inflammation (Chan and Moore, 2010; Fukunaga et al., 2005), and its inhibition may have detrimental effects in treating acute lung injury (Lukkarinen et al., 2006). Future work should aim to identify host factors in COX-2-induced downstream pathways that are crucial for H5N1 virus replication. This may lead to the separation of antiviral effects from the induction of mediators that are important for the resolution of inflammation and recovery from lung injury.

### 4.2.2. S1P agonists

Sphingosine-1-phosphate (S1P) is a ligand for a family of 5 G-protein-coupled receptors (GPCRs), $S1P_1$ -$S1P_5$, which are differentially expressed on various cell types. The binding of S1P to its receptors enables the control of a variety of cellular activities, including adhesion, migration, cytokine secretion and barrier integrity, and plays an important role in inflammation and immunity (Rivera et al., 2008).

AAL-R, a broad-spectrum agonist of S1P receptors, was shown to impair the activation of dendritic cells, down-modulate influenza virus-specific T-cell responses and dampen the release of pro-inflammatory cytokines, leading to decreased lung injury during influenza virus infection (Marsolais et al., 2008) (Table 2). Interestingly, although there was significant T cell suppression, the production of protective antibodies by infected mice was not affected by AAL-R treatment. This differed from the action of antiviral drugs such as oseltamivir, in which the reduction in tissue damage was the result of reduced viral burden. If AAL-R is used to treat influenza A infection in combination with antivirals, it will promote viral clearance as well as the clinical course (Walsh et al., 2011). These data indicate that AAL-R may be a useful drug for influenza treatment, either alone or in combination, dampening cytokine responses and reducing lung injury, while maintaining the benefits of the protective immune response.

Although S1P signaling appears to be a promising therapeutic target, it also mediates various kinds of cellular activities; the development of agonists that target specific S1P receptors is therefore needed. A recent study by Teijaro et al. demonstrated that treatment with the $S1P_1$ receptor specific agonist, CYM-5442, significantly reduced cytokine and chemokine responses associated with influenza virus-induced injury *in vivo*. However, unlike AAL-R, it did not impair dendritic cell activation, enabling the initiation of a T-cell response (Teijaro et al., 2011) (Table 2).

### 4.2.3. Isopentenyl pyrophosphate and aminobisphosphonate pamidronate expanded gamma-delta T cells

Enhancement of innate immune responses, the first line of defense against viral infection, could also be considered as an alternative therapeutic approach for influenza. Natural killer (NK) cells are key effectors of innate immunity, keeping infections under control in the early phase, by killing virus-infected cells. However, influenza virus could evade this innate immune defense by infecting NK cells and inhibiting their cytotoxic activity (Mao et al., 2009, 2010). Human gamma-delta ($\gamma\delta$) T cells share the characteristics of T cells, NK cells and antigen-presenting cells, mediating innate and adaptive responses to infection (Born et al., 2006; Brandes et al., 2005). Unlike NK cells, $\gamma\delta$ T cells are not susceptible to influenza A virus infection. Expansion of $\gamma\delta$ T cells may therefore be considered an alternative therapeutic approach (Table 2).

$\gamma\delta$ T cells constitute only 2–10% of T lymphocytes in the peripheral blood. In humans, cells with the $V\gamma9V\delta2$ receptor are the major $\gamma\delta$ T cells in the peripheral blood and lymphoid organs (Carding and Egan, 2002). Isopentenyl pyrophosphate (IPP)-expanded $V\gamma9V\delta2$ T cells have shown specific cytotoxicity towards influenza virus-infected human monocyte-derived macrophages, in a dose-dependent manner (Qin et al., 2009). Expanded $V\gamma9V\delta2$ T cells have also demonstrated antiviral activity against seasonal H1N1, avian H5N1 and H9N2 influenza A viruses (Qin et al., 2009). Aminobisphosphonate pamidronate (PAM) was also recently shown to have an effect similar to IPP, resulting in the killing of infected cells and inhibition of influenza virus replication by $V\gamma9V\delta2$ T cells. PAM treatment of influenza-infected humanized mice has also demonstrated a benefit, by expanding the population of $V\gamma9V\delta2$ T cells (Tu et al., 2011). Expansion of $V\gamma9V\delta2$ T cells by IPP and PAM may thus provide a novel, safe approach to influenza therapy, by enhancing the immune system's first line of defense.

### 4.2.4. Peroxisome proliferator-activated receptor agonists

Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors which are categorized into three subtypes, $\alpha$, $\delta(\beta)$, and $\gamma$. Each subtype is expressed in a diverse array of cell types, and is activated by different ligands (Kliewer et al., 1994). PPAR-$\gamma$ is expressed in adipose tissue, monocytes, and macrophages, and plays a role in glucose metabolism and adipocyte differentiation (Tontonoz et al., 1994). Numerous studies have found that PPAR-$\gamma$ may be involved in regulating cytokine production.

Activation of the PPAR-$\gamma$ receptor by its ligand inhibits the production of TNF-$\alpha$, IL-1$\beta$ and IL-6 and other pro-inflammatory cytokines in many cell types, such as monocytes and T lymphocytes, through trans-repression of NF-$\kappa$B and AP-1 (Clark et al., 2000; Jiang et al., 1998; Pascual et al., 2005) (Table 2). In a recent study, treatment of mice with the PPAR-$\gamma$ agonist (pioglitazone) before influenza virus challenge led to suppression of CCL-2 production (Herold et al., 2008), a reduction in infiltration of TNF/iNOS-producing monocytes in the lungs and improved survival (Aldridge et al., 2009). These results suggest that PPAR agonists may be used to modulate the immune response to influenza virus infection, reducing immunopathology and tissue damage.

### 4.2.5. Statins

Statins have been widely used to treat high blood cholesterol levels. They reversibly and competitively inhibit the activity of 3-

hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, reducing synthesis of cholesterol and mevalonate (Chong et al., 2001). In addition to their lipid-lowering activity, statins exert immunomodulatory effects (Table 2). They suppress the MHC-II expression induced by IFN-γ, without modulating constitutive MHC-II expression, by reducing the inducible expression of CIITA, a general MHC-II controller, by inhibiting the activity of CIITA promoter IV. Inhibition of the inducible expression of MHC-II suppresses activation of CD4 T lymphocytes, reducing the inflammatory response (Kwak et al., 2000). Statins also have another immunomodulatory mechanism, via interaction with lymphocyte function-associated antigen-1 (LFA-1), a glycoprotein in the $\beta_2$-integrin family (Weitz-Schmidt et al., 2001). LFA-1 is activated by engagement of the T-cell or chemokine receptor, and binds to the intracellular adhesion molecule-1 (ICAM-1) to drive various downstream activities, including lymphocyte recirculation and leukocyte extravasation (Carlos and Harlan, 1994).

The therapeutic potential of statins for influenza therapy has recently been demonstrated, in a study showing a significantly reduced risk of death in influenza patients who were receiving statins (Frost et al., 2007; Kwong et al., 2009). However, such retrospective studies have potential biases, and the patients were being treated with statins before they acquired influenza. The beneficial effect of statins has recently been challenged, as no beneficial effects on influenza patients were observed (Fleming et al., 2010; Viasus et al., 2011). Further investigation should be carried out, especially to identify a possible beneficial effect of initiating statins near the onset of influenza illness or at the time of hospitalization.

The molecular mechanisms of statin activity in influenza infection also remain poorly understood. One possibility is that the inhibition and depletion of cholesterol biosynthesis disrupt the structure of membrane-resident lipid rafts, resulting in loose interactions between these micro-lipid domains. Activation of the Raf/MEK/ERK signal cascade required efficient nuclear cytoplasmic RNP localization, which is triggered by the accumulation of the influenza HA in cell membranes, and thus has a close association with lipid rafts. Statin treatment might interfere with this chain of events by lowering cholesterol biosynthesis during influenza infection, impairing the production of infectious virus.

## 5. Concluding remarks

Influenza virus infection in humans can lead to mild to severe illness, as a result of both viral replication and the host immune response. Early treatment with NA inhibitors and M2 ion channel blockers is currently the mainstay for patient management. However, the response to antiviral therapy has been variable, and the development of drug resistance has raised public health concerns. The identification of resistant variants with transmissibility or pathogenicity comparable to their wild-type counterparts highlights the need to improve the design of NA inhibitors and to develop new classes of antiviral compounds.

In this review, we have discussed approaches under development for the control of influenza virus infection, including (1) improving the design, potency and route of delivery for existing inhibitors; (2) identifying novel classes of compounds or biomolecules that inhibit virus replication; (3) blocking virus-host interactions, to reduce viral replication efficiency; (4) modulating the host immune response to alleviate tissue damage; and (5) combination therapy. Current research aims to improve existing therapies, by targeting either influenza viruses or cellular factors that affect viral replication and host innate immune responses (summarized in Tables 1 and 2).

For the control of influenza, is it best to target the virus or the host? While targeting the virus provides a specific and direct inhibitory effect, targeting the host certainly provides other advantages, including a lower likelihood of drug resistance and immunomodulatory effects that may decrease tissue damage. Novel approaches that have both immunomodulatory and antiviral effects therefore deserve special attention. Furthermore, the application of antiviral drugs in combination with immunomodulatory agents provides a promising direction, as seen in recent *in vivo* studies (Zheng et al., 2008; Walsh et al., 2011). Further preclinical and clinical trials will be needed to study the underlying mechanisms of action and confirm their beneficial effects against influenza in humans.

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