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Review

# Characteristics of human infection with avian influenza viruses and development of new antiviral agents

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Since 1997, several epizootic avian influenza viruses (AIVs) have been transmitted to humans, causing diseases and even deaths. The recent emergence of severe human infections with AIV (H7N9) in China has raised concerns about efficient interpersonal viral transmission, polygenic traits in viral pathogenicity and the management of newly emerging strains. The symptoms associated with viral infection are different in various AI strains: H5N1 and newly emerged H7N9 induce severe pneumonia and related complications in patients, while some H7 and H9 subtypes cause only conjunctivitis or mild respiratory symptoms. The virulence and tissue tropism of viruses as well as the host responses contribute to the pathogenesis of human AIV infection. Several preventive and therapeutic approaches have been proposed to combat AIV infection, including antiviral drugs such as M2 inhibitors, neuraminidase inhibitors, RNA polymerase inhibitors, attachment inhibitors and signal-transduction inhibitors etc. In this article, we summarize the recent progress in researches on the epidemiology, clinical features, pathogenicity determinants, and available or potential antivirals of AIV.

**Keywords:** avian influenza; human infection; pathogenicity; antiviral agent; M2 inhibitor; neuraminidase inhibitor; polymerase inhibitor; ribavirin; arbidol

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## Introduction

Avian influenza virus (AIV) is a potential source for the emergence of human influenza pandemics. Historically, several harmful influenza pandemics have originated from AIVs through genetic reassortment between human and avian influenza strains, as happened in the 1918 H1N1, 1957 H2N2, and 1968 H3N2 pandemics<sup>[1,2]</sup>. The unprecedented emergence of H5N1 human infections in 1997 provided the first evidence that AIV could directly transmit from poultry to humans<sup>[3]</sup>. Since February 2013, there have been cases of severe human infection with H7N9 AIV in China; this AIV strain consists of genes from three AIV strains and is viewed as a pandemic threat<sup>[4]</sup>.

Avian influenza virus (AIV) is an influenza A virus, which is a member of the genus *Orthomyxovirus*. The genome of AIV consists of eight minus-sense single-stranded RNA seg-

ments that encode a minimum of 10 unique viral proteins. The current classification into subtypes is based on the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). To date, 16 HA subtypes and nine NA subtypes have been described<sup>[5,6]</sup>. The unique genome of influenza virus H17N10 was recently discovered in bats in Guatemala<sup>[7]</sup>. One remarkable feature of influenza viruses is their inclination to undergo antigenic variation through antigenic drift and antigenic shift<sup>[8]</sup>. Antigenic drift consists of relatively minor mutational alterations in the antigenicity of HA or NA and occurs continuously as a result of selection pressure from host immunity<sup>[8]</sup>. Antigenic shift by genetic reassortment of the eight gene segments can result in the appearance of a novel HA/NA combination against which the human population has little or no immunity<sup>[9]</sup>. If the majority of people are immunologically naïve to novel strains and such strains can be transmitted efficiently from human to human, influenza pandemics may occur<sup>[9]</sup>.

Aquatic birds are the reservoirs of all influenza A virus subtypes. Most influenza viruses infect wild and/or domestic birds with limited or no signs of the disease and are thus

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classified as low pathogenic avian influenza (LPAI) viruses<sup>[10]</sup>. However, highly pathogenic avian influenza (HPAI) viruses can cause severe diseases in poultry, with fatality as high as 100%<sup>[10]</sup>. Currently, all HPAI viruses belong to subtype H5 or H7, but not all H5 or H7 viruses are HPAI.

## Human infections with AIV

### Epidemiology

Three different subtypes of AIV have been confirmed as capable of infecting humans: H5, H7, and H9 (Table 1). Humans can be infected by AIV primarily after their mucous membranes have come into direct contact with infectious secretions or excreta from infected birds or contaminated poultry products<sup>[11]</sup>. The main infectious route of AIV seems to be via the upper respiratory tract and conjunctivae<sup>[12, 13]</sup>. Direct introduction to the lower respiratory tract may occur only after massive exposure, such as the culling operations of infected poultry<sup>[14]</sup>. The role of infection through other routes (*eg*, gastrointestinal tract) remains to be explored.

The first warning that AIV could directly transmit from avian species to humans occurred in 1997 in Hong Kong and resulted in 18 infections and 6 deaths<sup>[3]</sup>. HPAI H5N1 influenza viruses were found to be endemic in poultry in most provinces of southern China, a fact that was supported by long-term surveillance<sup>[15–17]</sup>. Since 1997, HPAI H5N1 has spread from Asia to Europe, Africa, and the Far East through the poultry trade and migratory bird movements<sup>[18–22]</sup>. More than 600 cases of human infection have been confirmed in the past 10 years, and the fatality rate is approximately 60%<sup>[23]</sup>. Children and young adults appear to be more susceptible to the virus<sup>[24]</sup>.

The H7 subtypes of AIV have caused multiple cases of human infection since 2002 in Canada, China, Italy, Netherlands, the United States, and the United Kingdom<sup>[25]</sup>. In 2003, an HPAI H7N7 virus was found to transmit from ducks to humans; 89 cases of human infection were confirmed, and one patient died from severe pneumonia<sup>[26]</sup>. Several other AIV H7 strains, including HPAI H7N3, LPAI H7N3, and LPAI H7N2, have had sporadic outbreaks in recent years resulting in only mild illness in humans<sup>[12, 27–32]</sup>. The latest LPAI H7N9 outbreak in China, however, caused several deaths due to severe pneumonia and related complications<sup>[4, 33–36]</sup>. Although all H7 types of AIV are still zoonotic, they may be more likely to develop

into interpersonal pandemics because these viruses preferentially bind to  $\alpha$ -2,6-linked SA and/or a mammalian adaptation trait on the PB2 protein<sup>[4, 25]</sup>.

Since 1999, when the first human infection of LPAI H9N2 was detected in Hong Kong, this virus has been infrequently isolated from humans<sup>[30]</sup>. The symptoms associated with H9N2 infection are generally mild, and there is no evidence of human-to-human transmission<sup>[30–32]</sup>.

### Clinical features

The main clinical manifestations of AIV infection depend on the viral subtype. Some LPAI (*eg*, H7N2, H7N3, and H9N2) and HPAI strains (H7N7) only cause asymptomatic or mild symptoms, such as conjunctivitis or uncomplicated influenza-like illness, in humans<sup>[12, 37–42]</sup>. In current human infections with LPAI (H7N9), a typical influenza-like illness (*eg*, fever and cough) appeared in the early course of the disease. Blood biochemistry tests identified that aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and creatine kinase (CK) levels significantly increased, while white blood cell counts were normal or slightly decreased. Some patients progressed to severe pneumonia followed by acute respiratory distress syndrome (ARDS), and finally died from multi-organ failure<sup>[4]</sup>. Interstitial infiltration, lobar infiltration, and collapse/consolidation could be observed in chest radiographs 7 to 13 d after the onset of illness. These clinical features of H7N9 infections were comprehensively described by Gao *et al*<sup>[43]</sup>.

In HPAI H5N1 infection, the onset of disease occurred at a median of 3 to 4 d after exposure. The initial symptoms of disease were also influenza-like. Dyspnea could be observed in 42%–72% of patients, and extra-pulmonary symptoms such as conjunctivitis and gastrointestinal symptoms (*eg*, abdominal pain, diarrhea, and vomiting) occasionally occurred<sup>[44–46]</sup>. Other complications included Reye's syndrome and pulmonary hemorrhaging<sup>[44, 47, 48]</sup>. Many patients progressed to severe pneumonia, rapidly developed ARDS 4–13 d after disease onset, and finally died from multi-organ failure. Comparing with survivors of HPAI H5N1 infection, fatal cases were more likely to present with leukopenia, lymphopenia, thrombocytopenia, and elevated levels of AST, LDH, and CK<sup>[49–53]</sup>. Abnormal chest radiographic findings were the same as those for H7N9<sup>[46]</sup>.

**Table 1.** Human infections with avian influenza virus.

Subtype	Location <sup>a</sup> and year	Cases	Fatalities	References
H5N1	HK (1997); AZ, BD, CN, DJ, EG, HK, ID, IQ, KH, LA, MM, NG, PK, TH, TR, VN (2003–2013)	622	371	[3, 23]
H7N2	US (2002–2003); UK (2007)	3	0	[27, 28]
H7N3	IT (1999–2003); CA (2004); UK (2006); MX (2012)	12	0	[12, 28, 29]
H7N7	UK (1996); NL (2003)	90	1	[13, 26]
H7N9	CN (2013)	132	37	[35]
H9N2	CN, HK (1998–1999, 2003, 2007, 2009); BD (2011)	12	0	[30–32, 41]

<sup>a</sup> Abbreviation of Locations: Azerbaijan (AZ), Bangladesh (BD), Cambodia (KH), Canada (CA), China (CN), Djibouti (DJ), Egypt (EG), Hong Kong Special Administrative Region of China (HK), Indonesia (ID), Iraq (IQ), Italy (IT), Laos (LA), Mexico (MX), Myanmar (MM), Netherlands (NL), Nigeria (NG), Pakistan (PK), Thailand (TH), Turkey (TR), United Kingdom (UK), United States (US), Vietnam (VN).

The primary histopathological findings in fatal H5N1 human infections were extensive infiltration of lungs, disseminated intravascular coagulation, and multi-organ failure. In the lung, pneumocytes were the primary target of H5N1 infection, resulting in diffuse alveolar damage with interstitial fibrosis, hyaline membrane formation, patchy interstitial lymphoplasmacytic infiltrates, bronchiolitis with squamous metaplasia, and pulmonary congestion with varying degrees of hemorrhage<sup>[54–57]</sup>. The HPAI (H5N1) virus also infected multiple organs in addition to the lungs. Postmortem findings for patients who died from A/H5N1 infection showed edemas, degeneration of myocytes in the heart, extensive hepatic central lobular necrosis, extensive acute tubular necrosis in the kidney, disseminated intravascular coagulation, and cerebral involvement<sup>[50, 54, 58–60]</sup>. A fatal case of AIV (H5N1) infection in a pregnant woman revealed viral infection in the brain, placenta, and fetus<sup>[55, 61]</sup>. In addition, systemic cytokine activation resulted in hemophagocytic syndrome, lymphoid depletion, and skeletal muscle fiber necrosis<sup>[54, 56]</sup>.

The viral load of AIV varies in respiratory secretions, tissues, plasma, cerebrospinal fluid, and feces. Respiratory secretions and tissues commonly have the highest viral loads, and virus can be detected in all patients. Infectious virus and viral RNA have been detected in feces and intestines, suggesting that the virus sometimes replicates in the gastrointestinal tract<sup>[49, 59, 62]</sup>. Intestinal involvement in A/H5N1 virus infections may explain the common occurrence of diarrhea. Viral RNA in plasma is more often detected in patients with fatal disease than in those with nonfatal disease, indicating that levels of viral replication may influence the outcome<sup>[63]</sup>. A high viral load is also correlated with an increase in host response, as patients with HPAI (H5N1) infections rarely have detectable viral RNA in the respiratory tract for more than 3 weeks<sup>[63]</sup>.

### Host responses

The severe disease associated with AIV infection in humans could be caused by a variety of mechanisms, including viral dissemination, differences in tissue tropism and host response<sup>[64]</sup>. Generally, the host responses to AIV are complicated and include apoptosis and autophagy as well as immune responses (innate, humoral, and cell-mediated)<sup>[65–68]</sup>.

Autophagy, a tightly regulated homeostatic process for self-digestion of unwanted cellular subcomponents, has been suggested to be responsible for lung injury in AIV infection<sup>[65–67]</sup>. The autophagic death of alveolar epithelial cells may also be responsible for the high mortality rate of H5N1 infection because autophagy-blocking agents applied prophylactically or therapeutically in mice significantly increased the survival rate and alleviated the lung injury caused by H5N1 infection<sup>[67]</sup>.

Accumulating evidence suggests that virus-induced cytokine/chemokine dysregulation also plays a significant role in the pathogenesis of AIV infection<sup>[68]</sup>. Respiratory epithelial cells and macrophages are the primary innate immune cells involved in AIV infection<sup>[68]</sup>. Pronounced activation of the proinflammatory cytokine/chemokine cascade prolongs

the period of inflammatory response and contributes to further tissue damage and the persistence of the systemic inflammatory response syndrome<sup>[69]</sup>. Furthermore, cytokines can further sensitize neighboring cells by up-regulating RIG-I and amplifying the cytokine cascade in some HPAI infections<sup>[70]</sup>. It appears that cytokine responses may be driven by high-level viral replication, because plasma levels of macrophage- and neutrophil-attractant chemokines as well as pro- and anti-inflammatory cytokines (IL-6 and IL-10) were higher in patients with HPAI (H5N1) infection compared to the levels in patients with a conventional influenza infection<sup>[50]</sup>.

It should be noted that IL-17, Th-17 mediators, and IL-17-responsive cytokines were found in serum samples of 2009 swine-origin H1N1 influenza virus (S-OIV H1N1) infected patients<sup>[71]</sup>. IL-17 deficiency or treatment with monoclonal antibodies can significantly alleviate acute lung injury induced by the S-OIV H1N1 virus in mice<sup>[71]</sup>. In addition, IL-17 has been suggested to enhance the proinflammatory outcome of an antiviral response in human cells<sup>[72]</sup>. Thus, monoclonal antibodies against IL-17 may be helpful to reduce severe lung injury induced by AIV infections.

The suppression of interferon is also involved in human AIV infection. HPAI (H5N1) was found to attenuate the expression of IRF3 as well as the levels of type I IFNs, and subsequently substantially delay the phosphorylation of Stat2 and induction of IFN-stimulated genes (ISGs) *in vitro*<sup>[73, 74]</sup>. Viral protein M2 appeared to be the “main culprit” that correlated with the complete suppression of known viral inflammasome activation<sup>[75]</sup>.

In addition to cytokine response, apoptosis and autophagy, other humoral and cell immune responses are also involved in AIV infection<sup>[68, 76]</sup>. Surprisingly, although some of these responses exhibited antiviral activity, they did not affect virus replication, although some immune cells cannot be excluded from playing a role in the dissemination of the virus *in vivo*<sup>[60, 68]</sup>.

### Pathogenicity determinants

The virulence of AIV is determined by a constellation of genes<sup>[77–81]</sup>. These genes play important roles in viral replication and/or pathogenicity and are also potential targets of antiviral agents.

#### HA protein

HA influenza virus protein plays a crucial role in the early stages of the viral life cycle by binding to the viral receptor and mediating the fusion process<sup>[11]</sup>. Influenza virus infection begins when HA binds to sialic acid (SA)-linked glycoprotein receptors on the surface of the target cell. Usually, AIVs preferentially bind to  $\alpha$ -2,3 linked SA, which is located mainly on type II pneumocytes, alveolar macrophages and nonciliated cuboidal epithelial cells at terminal bronchioles, while human influenza viruses tend to use  $\alpha$ -2,6 linked SA as a receptor in the upper respiratory tract of humans<sup>[11, 82–85]</sup>. AIVs can infect human airway epithelium, but replication is limited due to the non-optimal cellular tropism<sup>[86]</sup>. Thus, the adaptation of AIV

HA for recognition of  $\alpha$ -2,6 linked SA located in the human upper respiratory tract would promote interpersonal transmission. Amino acids in the HA receptor-binding domain (RBD), formed by the 190-helix, 220-loop, and 130-loop, determine the receptor binding preference of influenza viruses<sup>[87–89]</sup>. However, several AIV (H5N1) strains with increased binding to human-type receptors isolated from humans still prefer binding to avian-type receptors and do not perform efficient human-to-human transmission<sup>[90–94]</sup>. An LPAI (H7N2) strain isolated from humans has also been reported to have dual binding affinity to both types of receptors, but no obvious inter-personal or inter-mammalian transmission was observed<sup>[95]</sup>. The H7N2 strain has an eight amino acid deletion in the 220-loop of the HA RBD<sup>[95]</sup>. Two specific mutations in H7-HA, Q226L and G228S, have also been suggested to contribute to the increased binding affinity for human receptors<sup>[96]</sup>. The Q226L mutation was identified in clinical isolates of H7N9 in 2013<sup>[4]</sup>. Five amino acid substitutions (or four with reassortment) in HA of the H5N1 virus have efficiently supported airborne viral transmission among mammals<sup>[97–99]</sup>.

The post-translational cleavage of HA0 into HA1 and HA2 subunits activates the membrane fusion potential of HA and is crucial for infectivity of the virus<sup>[100]</sup>. Most LPAI viruses possess a single arginine residue at the cleavage site of HA, which is cleaved by extracellular trypsin-like proteases restricted to the respiratory tract<sup>[101]</sup>. In contrast, HPAI viruses contain a motif of multiple basic amino acid residues at the equivalent cleavage site, permitting HA0 to be cleaved by ubiquitously distributed intracellular proteases, such as furin-like proteases, and enabling systemic infection<sup>[100, 102–104]</sup>. Notably, the circulating H7N9 virus possesses only a single arginine at the HA cleavage site, yet it has caused severe disease and death in humans<sup>[4, 34]</sup>. This fact indicates that the existence of a multi-basic cleavage site is not essential for the high pathogenicity of AIV in humans and other genes may also be involved. A flexible loop structure in the cleavage site between HA1 and HA2 is critical for the efficient cleavage of HA0. The relatively stable alpha-helix structure in the flexible cleavage loop (*eg*, key residue R328 hidden behind the helix) and the inaccessibility of the cleavage site may contribute to the low pathogenicity of a H16 subtype AIV<sup>[105]</sup>. The natural alpha-helix element might also provide a new opportunity for influenza virus inhibitor design<sup>[105]</sup>.

### NA protein

An evolutionary balance between the HA and NA proteins of AIVs is essential for the entry and release processes of the virus<sup>[106]</sup>. As a result, mutations within the active sites of either HA or NA can affect the enzymatic activity of both proteins. For example, some H5N1 viruses in sustained circulation in poultry possess additional glycosylation sites and a shortened NA stalk, both of which might enhance the virulence of AIV in mammals<sup>[107]</sup>. A deletion of five amino acids in the NA stalk region was also found in circulating H7N9 isolates<sup>[4]</sup>, which may help to explain the unusually high virulence of this strain.

### Ribonucleoprotein complex (RNP)

The polymerases PB2, PB1, and PA, and the nucleocapsid protein (NP) together form the RNP, which is critical for virus replication, virulence, and the determination of host restrictions<sup>[108]</sup>. Several amino acids in PB1 (*eg*, positions 99 and 368) and PB2 (*eg*, positions 89, 627, and 701) are basic molecular determinants associated with enhancing polymerase activity and pathogenicity of H5N1 virus in mammals<sup>[109]</sup>. Mutations of these positions in the H5N1 virus can not only promote higher viral yields in both the upper and lower respiratory tract of mice but also result in the conversion of a non-lethal H5N1 virus to a lethal one<sup>[110]</sup>. Similar mutations can also be observed in the currently circulating H7N9 virus<sup>[4, 34, 36, 111, 112]</sup>.

### PB1-F2 protein

Produced by an alternate reading frame in the PB1 gene, PB1-F2 is a non-structural accessory protein in influenza A viruses<sup>[113]</sup>. PB1-F2 protein is expressed by almost all avian influenza A strains, but strains from human and swine hosts have forms with premature truncations at either the C- or N-terminal end<sup>[114, 115]</sup>.

PB1-F2 has been shown to specifically target and sensitize alveolar macrophages to apoptotic stimuli<sup>[116, 117]</sup>. The presence of an intact PB1-F2 protein was also found to contribute to viral pathogenicity; an N66S mutation in H5N1 PB1-F2 led to the delayed activation of IFN-stimulated genes and increased cytokine/chemokine levels in mice due to the inhibition of early type I IFN responses at the level of the mitochondrial antiviral signaling protein (MAVS)<sup>[118, 119]</sup>.

The precise function of the PB1-F2 protein remains unclear. Research on this protein has indicated that the PB1-F2 protein has strain-specific functions that could vary in different hosts<sup>[120, 121]</sup>.

### NS1 protein

A multi-functional nonstructural RNA-binding protein, NS1 is critical for inhibiting both IFN production and the antiviral effects of IFN-induced proteins, such as dsRNA-dependent protein kinase R (PKR) and RNase L<sup>[122, 123]</sup>. A P42S mutation in NS1 dramatically increased the virulence of an H5N1 strain in mice and was also found in the circulating H7N9 virus<sup>[4, 124]</sup>. This substitution, in association with other mutations in HA, NA, PB1, and PB2, contribute to the pathogenicity of AIV H7N9 in humans.

### Antiviral agents against AIV

Several safe and effective drugs can be used to manage human and avian influenza infection. These agents include M2, neuraminidase, polymerase, attachment and signal-transduction inhibitors as well as ribavirin, arbidol, and herbs. Several M2 and neuraminidase inhibitors have been approved for prophylaxis and the therapeutic treatment of influenza (Table 2). Other agents are still being studied in preclinical or clinical investigations. Here, we review the basic pharmacokinetics and properties that support the use of these agents against AIV.



**Table 2.** Main Characteristics of current available antivirals<sup>[125–127, 131–134, 138–146]</sup>.

Characteristics	Amantadine	Rimantadine	Oseltamivir	Zanamivir	Laninamivir	Peramivir
Molecular weight	187.7	215.8	312.4 (free base)	332.3	472.53	328.4
Prophylaxis dosing (Adult)	100 mg bid	100 mg bid	75 mg qd	10 mg qd	– <sup>a</sup>	– <sup>a</sup>
Treatment dosing (Adult)	100 mg bid	100 mg bid	75 mg bid for 5 d	10 mg bid for 5 d	40 mg single dose	600 mg qd for 5–10 d
Route	Oral	Oral	Oral	Inhaled	Inhaled	Parenteral
Half-life (h)	12–18	24–36	6–10	4.14–5.05	>240	12–25
Use status and major adverse reactions	Pregnancy class C drugs, neuro-psychiatric reactions	Pregnancy class C drugs, neuropsychiatric reactions	Few major adverse effect, nausea, vomiting and transient neuropsychiatric reactions	Few major adverse effect, nausea, cough, and fatal bronchospasm (patients with underlying pulmonary disease)	Few major adverse effect, nausea, vomiting, and dizziness	Few major adverse effect, diarrhea, nausea, vomiting and decreased neutrophil count
Inhibitory activity on Avian influenza	EC <sub>50</sub> to AIV: H5N3: 0.1 µmol/L H7N2: 0.1 µmol/L H9N2: 0.5 µmol/L (plaque assay)		IC <sub>50</sub> S of NA activity (N1–N9): 1.4–3.6 nmol/L; EC <sub>50</sub> to AIV (N1–N9): 1.0–42.0 µmol/L (ELISA); 0.1–0.9 nmol/L (plaque assay) N1 NA: I223R <sup>*</sup> ,	IC <sub>50</sub> S of NA activity (N1–N9): 1.4–11.5 nmol/L; EC <sub>50</sub> to AIV (N1–N9): 4.0–58.3 µmol/L (ELISA); 0.6–3.6 nmol/L (plaque assay)	IC <sub>50</sub> S of NA activity (N1–N9): 1.8–27.9 nmol/L; EC <sub>50</sub> to AIV (N1–N9): 0.3–2.5 nmol/L (plaque assay)	IC <sub>50</sub> S of NA activity (N1–N9): 0.9–4.3 nmol/L; EC <sub>50</sub> to AIV (N1–N9): 0.5–11.8 µmol/L (ELISA)
Reported mutations confer to drug resistance	M2: L26F, V27A <sup>*</sup> , V27D <sup>*</sup> , V27T, V27S, I27T <sup>*</sup> , I27S <sup>*</sup> , I27A <sup>*</sup> , A30E <sup>*</sup> , A30T, A30P <sup>*</sup> , A30V, A30G, S31N <sup>*</sup> , G34E <sup>*</sup> , W41A; HA: G23C		H275Y <sup>*</sup> , N295S <sup>*#</sup> , N2 NA: E119V <sup>*</sup> , D151E <sup>#</sup> , R224K, E276D <sup>#</sup> , R292K <sup>*</sup> , N294S <sup>*#</sup> , R371K; N9 NA: H274Y, R292K	N1 NA: I223R <sup>*</sup> ; N2 NA: E119A, E119D, R224K, R292K <sup>*#</sup> , R371K, E276D, N9 NA: E119G	N1 NA: N295S	N1 NA: H275Y <sup>*</sup> , N295S <sup>*</sup> ; N2 NA: E119V, R292K <sup>*</sup>

<sup>a</sup>Not identified; <sup>b</sup>Data from independent research different from other neuraminidase inhibitor; <sup>\*</sup>Mutations have been found to arise naturally drug-resistant in avian influenza; <sup>#</sup>Low resistance.

## M2 inhibitors

The migration of H<sup>+</sup> ions into the interior of viral particles is mediated by the M2 channel and allows for the uncoating of virus particles in the endosome<sup>[125, 126]</sup>. There are two commercially available M2 inhibitors: amantadine and rimantadine (Table 2). They have been widely used in the treatment of both human and avian influenza infections for many years and have been shown to shorten the disease duration and facilitate symptom resolution<sup>[127]</sup>.

Unfortunately, most of the currently circulating animal viruses, including H5N1, H9N2, the 2009 swine-originated H1N1, and current H7N9, are all M2 inhibitor resistant due to overuse of the inhibitors<sup>[111, 128–130]</sup>. The widespread resistance of these strains precludes the use of these M2 inhibitors in most cases of human infection<sup>[131, 132]</sup>. Resistance mutations occur mainly in the trans-membrane portion of the M2 protein (position 26, 27, 30, 31, or 34) and result in an enlarged diameter of the M2 channel pore, thus reducing the binding of M2 inhibitors<sup>[126]</sup>. Different subtypes or clades of AIV exhibit different frequencies of M2 inhibitor resistance<sup>[133]</sup>. For example, all 24 cases of clade 1 H5N1 isolated between 2008–2011 had an S31N substitution, while the frequency of this mutation in clade 2 ranged from 0% to 67%. In addition, all 3 cases of H7N9 that have been isolated are S31N variations<sup>[133]</sup>. It

seems that the increase in adamantane-resistant influenza viruses is not mediated by continued selective drug pressure; therefore, the WHO recommends amantadine or rimantadine only be used to prevent or treat AIV that is known to be sensitive. Interestingly, one study suggested that a G23C mutation in the H7 HA protein might also play a role in amantadine resistance<sup>[134]</sup>. In addition, several novel M2 inhibitors, including new adamantane derivatives and biological agents (eg, annexin A6), have shown marked activity against human influenza viruses<sup>[135–137]</sup>. Whether any of these agents could replace amantadine and rimantadine as an influenza virus treatment needs to be further explored.

## Neuraminidase inhibitors

Viral neuraminidase (NA) enables progeny virus to be cleaved from its receptor and spread to other cells. The neuraminidase inhibitors, which are cyclopentane or pyrrolidine derivatives, can prevent the further spread of influenza by blocking the release of newly formed particles<sup>[138]</sup>. Four commercial neuraminidase inhibitors (oseltamivir, zanamivir, peramivir, and laninamivir) have been approved for use in humans<sup>[139]</sup>. The first two are widely used in most countries and are effective against influenza infection. Particularly for oseltamivir, early administration can not only shorten the duration of ill-

ness but also facilitate symptom resolution<sup>[139]</sup>. Neuraminidase inhibitors are also effective against different AIV subtypes *in vitro* (Table 2)<sup>[140–142]</sup>. Studies in animal models have demonstrated that oseltamivir given as treatment or prophylaxis was active against the H5, H7, and H9 avian influenza strains<sup>[140, 143]</sup>. The protective efficacy was influenced by the virulence of the strains, the dosage and treatment initiation time<sup>[140, 143]</sup>. Clinical trials also suggested that oseltamivir and zanamivir were useful in reducing the mortality of lethal H5N1 infection, while resistance to the treatments rarely emerged<sup>[132]</sup>. Therefore, the WHO recommends these two agents as the primary intervention for treatment and prevention of human AIV infections. However, continued monitoring of AIV for drug susceptibility is needed, because oseltamivir-resistant seasonal influenza strains have been spreading around the world since 2009<sup>[144, 145]</sup>. Notably, one of the current H7N9 isolates has an R292K substitution<sup>[4]</sup>, which has been associated with *in vitro* resistance to neuraminidase inhibitors in another N9 NA subtype AIV. This mutation has also been confirmed to lead to oseltamivir- and zanamivir-resistance in clinical N2 AIV subtype (Table 3)<sup>[146]</sup>. The presence of the NA R292K substitution in two H7N9 patients who also received corticosteroid treatment resulted in treatment failure<sup>[147]</sup>. Thus, it is crucial to assess the prevalence of drug-resistant H7N9 in future influenza surveillance. Among the newly developed neuraminidase inhibitors, laninamivir has an extremely long persistence time in the lungs<sup>[141]</sup>, increasing the prospect of a long-lasting antiviral that can effectively prevent influenza infection with a single dose.

#### RNA polymerase inhibitors

The influenza polymerase consists of several polypeptides, including PB1, PB2, and PA, and also contributes to the high virulence of AIV in humans<sup>[1, 4, 148]</sup>. Therefore, novel antivirals that target polymerase are anticipated to reduce the replication of AIV as well as inhibit the severe pathogenicity induced by the virus. Current influenza RNA polymerase inhibitors can be divided into nucleosides and non-nucleosides. These polymerase inhibitors, such as fluorodeoxycytidine analogs and favipiravir (T705), have been shown to be active in the treatment of influenza both *in vitro* and *in vivo*<sup>[149–151]</sup>. The most potent inhibitor among fluorodeoxycytidine analogs was 2'-deoxy-2'-fluorocytidine (2'-FdC) which inhibited various strains of HPAI and LPAI with 50% inhibitory concentrations (IC<sub>50</sub>s) ranging from 0.21 to 3.2  $\mu\text{mol/L}$  in MDCK cells<sup>[149]</sup>. 2'-FdC 60  $\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$  (ip, bid $\times$ 8 d) could protect 60%–80% of mice from lethal HPAI H5N1 infection when administered 24 h before virus exposure<sup>[149]</sup>. T705 is a pro-drug that needs to be converted to its active form, T705-RTP<sup>[150]</sup>. T705-RTP then competitively inhibits the RNA synthesis activity of influenza polymerase. T705 was effective against several H5N1 strains with an IC<sub>50</sub> range of 1.3–7.7  $\mu\text{mol/L}$  *in vitro*, while the range of oseltamivir was 0.007–0.92  $\mu\text{mol/L}$ <sup>[151]</sup>. *In vivo*, T705 300  $\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$  with different delivery methods was able to protect 100% of mice from a lethal H5N1 infection<sup>[151]</sup>.

Several other polymerase inhibitors are in development

against AIV infection. A GTPase induced by type I and type III IFNs, Mx1, inhibits influenza virus infection by interacting with the ribonucleoprotein complex and interfering with viral assembly by disrupting the PB2-NP interaction<sup>[152]</sup>. A short peptide derived from PB1 (731–757) was also reported to inhibit virus polymerase<sup>[153]</sup>. Several small interfering RNAs (siRNAs) targeting the overlapping gene of PB1 and PB1-F2 were found to reduce virus-associated cell apoptosis and virus titers in chicken embryos<sup>[154]</sup>. A novel compound, THC19, inhibited influenza viral genome replication and/or transcription in a PA-dependent manner<sup>[155]</sup>. Another novel compound, ASN2, induced IFN production and inhibited growth of influenza A viruses through ASN2-mediated inhibition of viral polymerase function and the subsequent loss of expression of the viral IFN antagonist, NS1<sup>[156]</sup>.

Although the efficacy of these polymerase inhibitors needs to be further examined in clinical investigations, these leading compounds have resulted in the development of a series of novel anti-influenza agents that target the viral RNA polymerase.

#### Ribavirin and arbidol

Ribavirin and arbidol have long been recognized as broad-spectrum antiviral agents against viruses from different families. Viruses resistant to these treatments have rarely been observed<sup>[157–162]</sup>. The target of ribavirin is a cellular enzyme, inosine 5'-monophosphate (IMP) dehydrogenase, which is involved in viral RNA synthesis and cellular biosynthesis of GTP. The IC<sub>50</sub> of ribavirin on H5N1 ranged from 6 to 22  $\mu\text{mol/L}$  *in vitro*<sup>[157]</sup>. Ribavirin 75  $\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$  (po, bid $\times$ 8 d) protected 70% of mice from lethal H5N1 infection<sup>[163]</sup>. The clinical efficacy of ribavirin on influenza was less effective than that of adamantanes or NA inhibitors and more dependent on the delivery manner<sup>[162, 164, 165]</sup>. Aerosolized ribavirin effectively eliminated the influenza virus and shortened the duration of illness in clinical observations, while orally administered ribavirin did not<sup>[162, 164, 165]</sup>. However, the clinical utility of ribavirin may be limited due to the risk of hemolytic anemia and teratogenicity<sup>[166]</sup>. A prodrug of ribavirin, viramidine, may improve the utility of ribavirin<sup>[157]</sup>. This prodrug had a similar activity against seasonal and H5N1 influenza viruses but was less toxic. Furthermore, viramidine effectively protected mice from lethal influenza, even when the drug was administered in drinking water<sup>[157]</sup>. This drug is now in Phase 3 development for HCV treatment<sup>[167]</sup>.

Arbidol has been widely used in Russia for almost 20 years<sup>[158]</sup>. The mechanisms of arbidol are complicated; both membrane fusion inhibition and immunomodulatory activity may contribute to its broad antiviral effects<sup>[158]</sup>. Arbidol was shown to stabilize HA by causing a 0.2 pH unit reduction in the pH required for transition to the low pH form of the protein. A recombinant arbidol-resistant strain possessed single amino acid substitutions in the HA2 subunit that abrogated this activity of arbidol<sup>[168]</sup>. Arbidol was active against H5N1, H9N2, H2N2, and H6N1 AIV with a range of IC<sub>50</sub> values from 19.4 to 58.3  $\mu\text{mol/L}$ <sup>[160, 169]</sup>. Our current research

also confirmed that post-treatment with arbidol decreased the mortality in influenza-infected mice by mitigating lung lesion formation and reducing viral titers<sup>[170]</sup>. Furthermore, it efficiently protected the host from virus-induced inflammation by modulating the expression of pro-inflammatory cytokines<sup>[170]</sup>. These data suggest that arbidol might also be effective in the treatment of severe AIV infections in humans.

#### Attachment inhibitors

As mentioned above, influenza virus needs to attach the SA receptor to enter host cells. A number of attachment inhibitors have been developed against AIV, including sialidase mimics, sialyl glycopolymers and hemagglutinin inhibitors. The sialidase recombinant construct DAS181 is an inhaled bacterial sialidase that prevents the attachment and subsequent infection of influenza virus by removing influenza-receptor interactions<sup>[171]</sup>. DAS181 was shown to have activity against H5N1 infection in both continuous and shorter treatments in human airway epithelium models<sup>[172]</sup>. Further studies were performed in lethally H5N1-infected mice models; 1 mg·kg<sup>-1</sup>·d<sup>-1</sup> DAS181 (inhale, qd×8 d) protected 100% of mice from viral infection in both prophylactic and therapeutic approaches<sup>[173, 174]</sup>. A phase 2 study of DAS181 on seasonal or pandemic influenza has been completed, and when patients were given 10 mg·kg<sup>-1</sup>·d<sup>-1</sup> DAS181 (inhale, qd×3 d), they showed a significant reduction in viral load over 5 d, but no symptom improvement<sup>[175]</sup>. Sialylglycopolymers and other hemagglutinin inhibitors (eg, Neo6, EB peptide, and NDFRSKT peptide) showed enhanced binding affinity for HA compared to normal SA, allowing them to block attachment of H5N1 and H9N2 AIV<sup>[176-180]</sup>. A fusion inhibitor, tert-butyl hydroquinone (TBHQ), bound in a hydrophobic pocket formed at the interface between HA monomers and consequently inhibited the conformational rearrangements required for membrane fusion<sup>[181]</sup>. Although most of these agents are still in pre-clinical studies, they are anticipated to be used against any newly emerging influenza strains, especially AIV<sup>[178, 179]</sup>.

#### Signal-transduction inhibitors

Aside from agents that directly target viral proteins, current antiviral strategies also focus on the intracellular cascade needed for viral replication<sup>[182-184]</sup>. Two signaling pathways that are required to ensure efficient virus replication have been considered suitable targets for antiviral approaches: the IKK/NF-κB module and the Raf/MEK/ERK cascade<sup>[184]</sup>. Both of these pathways are also critical for cytokine and interferon synthesis during influenza infection<sup>[182-184]</sup>. Thus, inhibitors targeting these cascades may not only inhibit the replication of virus but also moderate the severe systemic inflammation in AIV infection<sup>[184]</sup>. This hypothesis has been confirmed by several laboratory experiments *in vitro* and *in vivo*<sup>[182-185]</sup>, although the efficacy in humans needs to be further investigated. However, some inhibitors targeting NF-κB, such as aspirin, have been routinely used in influenza-like illness for many years<sup>[185]</sup>. Current research identified that a high dosage of aspirin efficiently blocked influenza virus replication both *in vitro* and

*in vivo*, and there are plans to use this drug in clinical studies with administration via inhalation<sup>[185]</sup>. Several other anti-inflammatory agents (eg, statins and sphingosine analogs) have also received attention and are being investigated<sup>[186-190]</sup>. However, results from these studies were mixed and sometimes even conflict<sup>[186-190]</sup>. Nonetheless, these studies have promoted optimization of these agents and suggested new strategies for therapies against AIV infection.

#### Herbs

Herbs may also be a potential choice for AIV treatment. Some Chinese herbs were recommended and authorized by the Chinese government during the 2009 H1N1 and 2013 H7N9 pandemics<sup>[191, 192]</sup>. Herbal medicines that contain *Isatis tinctoria* L, *Lonicera japonica* Thunb, *Saposhnikovia divaricata* (Turcz) Schischk, *Bupleurum chinense* DC, *Forsythia suspensa* (Thunb) Vahl, *Citrus reticulata* Blanco, and *Perilla frutescens* (L) Britton are commonly taken in a formula used for the prophylactic and therapeutic treatment of influenza infection. A number of clinical trials for these herbs for the treatment of influenza have been conducted, but systematic reviews on the utility of these herbs for H1N1 influenza treatment have revealed that few herbal medicines showed a positive effect on viral shedding, and most of the medicines had a positive effect only on fever resolution or relief of symptoms<sup>[193, 194]</sup>. Although more rigorous placebo-controlled and randomized trials are needed to reach further conclusions<sup>[193, 194]</sup>, many Chinese herbs exhibit beneficial immunomodulatory effects for rapid recovery of viral infections and might be effective treatments for AIV infection<sup>[195]</sup>.

#### Other emerging agents

Recently, several active proteases of influenza A viruses such as TMPRSS2 (transmembrane protease serine S1 member 2) and HAT (human airway trypsin-like protease) have been considered potential drug targets. Peptide mimetic protease inhibitors (eg, BAPA) suppressed the cleavage activation of HA and the spread of virus in TMPRSS2- and HAT-expressing cells<sup>[196, 197]</sup>.

Serine proteases, which mediate influenza HA cleavage, are responsible for influenza virus activation. Agents targeted to serine proteases (eg, aprotinin, leupeptin, and camostat) suppressed the cleavage of HA and limited the reproduction of human and avian influenza viruses that have a single arginine in the HA cleavage site. It is anticipated that these agents will be used for the treatment of HPAI viruses (eg, H5 and H7), whose hemagglutinins possess multi-arginine/lysine cleavage sites<sup>[198]</sup>.

There are also several compounds that target the NS1 protein and show considerable anti-influenza potential. For example, JJ3297 inhibits the replication and spread of influenza virus by reversing the NS1-induced inhibition of interferon mRNA production in an RNase L-dependent manner<sup>[199]</sup>. A C-Jun N-terminal kinase (JNK) inhibitor, SP600125, reduced the amplification of influenza virus by indirectly inhibiting the NS1-mediated supportive functions<sup>[200]</sup>.

## Conclusion

Persistent outbreaks of avian influenza in Southeast Asia suggest that avian influenza may be the most likely candidate for the next influenza pandemic. Many AIV patients died from overwhelming viral pneumonia with other serious complications. Thus, rapid, sensitive, and confirmatory diagnoses for early identification and continued monitoring of viral adaptation to humans are essential for the control of AIV infections. It should be noted that AIV infections differ from human influenza infections in humans in many ways, including viral transmission, viral dissemination, clinical features, pathogenesis, and host response. The diagnosis and therapy of AIV infections have unique features as well. For example, sample analysis from the lower respiratory tract may offer a more sensitive diagnosis. Several drugs have been used as prophylactic or therapeutic treatments against AIV infection, including M2 inhibitors, neuraminidase inhibitors, ribavirin, *etc.* As the frequency of drug-resistant influenza increases, the rational use of antivirals, and drug-resistant monitoring should be encouraged. Meanwhile, efforts should be made to design and develop further new antivirals that target the basic steps of viral replication, such as attachment, internalization, and cellular processes, or relatively specific targets, such as the viral RNA polymerase, PB1-F2, NS1, *etc.*

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