

REVIEW

# Roles of the hemagglutinin of influenza A virus in viral entry and development of antiviral therapeutics and vaccines

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## ABSTRACT

Seasonal influenza epidemics and influenza pandemics caused by influenza A virus (IAV) has resulted in millions of deaths in the world. The development of anti-IAV vaccines and therapeutics is urgently needed for prevention and treatment of IAV infection and for controlling future influenza pandemics. Hemagglutinin (HA) of IAV plays a critical role in viral binding, fusion and entry, and contains the major neutralizing epitopes. Therefore, HA is an attractive target for developing anti-IAV drugs and vaccines. Here we have reviewed the recent progress in study of conformational changes of HA during viral fusion process and development of HA-based antiviral therapeutics and vaccines.

**KEYWORDS** influenza A virus, hemagglutinin, viral entry, antiviral drugs, vaccines

## INTRODUCTION

Influenza continues to pose serious threats to public health worldwide since seasonal influenza epidemics can affect up to 15% of the population and result in more than 500,000 deaths worldwide each year ([http://www.who.int/csr/disease/influenza/Research\\_Agenda\\_Document.pdf](http://www.who.int/csr/disease/influenza/Research_Agenda_Document.pdf)), while some influenza pandemics, like the 1918 flu pandemic (the Spanish Flu), may lead to millions of deaths (Taubenberger 2006). In 2009, a new influenza pandemic caused by a novel swine-origin influenza A virus (S-OIV) H1N1 has resulted in millions of infections in more than 213 countries and overseas territories or communities ([http://www.who.int/csr/don/2010\\_03\\_12/en/index.html](http://www.who.int/csr/don/2010_03_12/en/index.html)) (Garten et al., 2009; Miller

et al., 2009; Smith et al., 2009; Itoh et al., 2009). The rapid spread of S-OIV in humans worldwide and the continuous mutations of the S-OIV proteins (Pan and Jiang 2009; Pan et al., 2010) suggests that this newly emerging, animal-origin virus has acquired potent human-to-human transmissibility, which has raised a growing concern about the evolution of the highly pathogenic avian influenza (HPAI) H5N1 viruses that have caused 289 fatal cases among a total of 489 infected individuals, as of March 16, 2010, with a case-fatality rate of ~60% ([http://www.who.int/csr/disease/avian\\_influenza/country/cases\\_table\\_2010\\_03\\_16/en/index.html](http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_03_16/en/index.html)) (the real H5N1 mortality rate should be closer to 14%-33% based on surveillance and seroprevalence studies conducted in several countries) (Li et al., 2008).

The influenza virus consists of three genera: A, B and C. Although all three types of influenza viruses are able to infect humans, only influenza A virus (IAV) may cause influenza pandemics (Watts 2009). Based on the antigenicity of the two surface proteins—hemagglutinin (HA) and neuraminidase (NA), IAV can be further classified into different subtypes including 16 HA (H1–H16) and 9 NA (N1–N9) subtypes (Yamashita et al., 2010). HA is responsible for the viral entry (Russell et al., 2008) and contains the major neutralizing epitopes (Ndifon et al., 2009), thus serving as an attractive target for drug and vaccine development.

## ROLE OF HA IN VIRAL ENTRY

HA plays crucial roles in the early stage of virus infection, including virus binding to host receptors, viral entry, and membrane fusion (Skehel and Wiley, 2000). HA is initially synthesized as a precursor, HA0, which trimerizes in the endoplasmic reticulum in association with chaperones and is

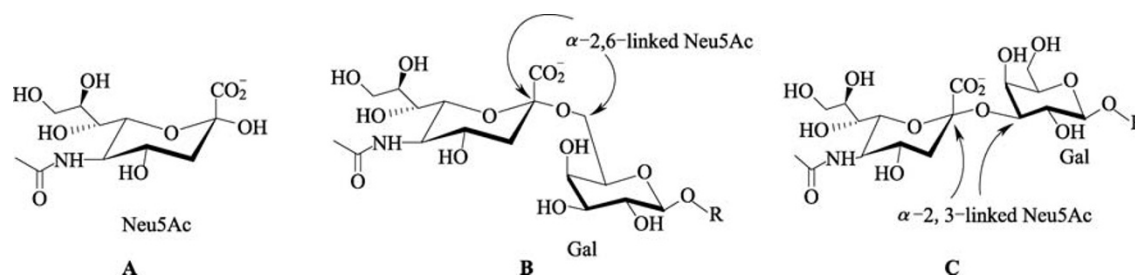
transferred to the cell surface through the Golgi apparatus. HA0 is proteolytically cleaved into the functional HA1 and HA2 subunits linked by a single disulfide bond. Most influenza virus strains contain an HA cleavage site with only a single basic amino acid residue which is cleaved by tissue restricted proteases only, thereby limiting spread in the infected host. Cleavage occurs at the cell surface or on released viruses. For the HA0 of the H1, H2, and H3 subtype viruses that have caused epidemics, cleavage may be mediated by the serine protease, trypsin-like, produced by Clara cells of the bronchiolar epithelium (Kido et al., 1992). This enzyme shows recognition specificity for the sequence Q/E-X-R found at the cleavage sites of these HAs. In contrast, for the H5 and H7 HPAI subtypes, the HA1 and HA2 polypeptide chains are separated by polybasic sequences that are inserted at the cleavage site (Perdue et al., 1997). In these cases, cleavage is intracellular and involves subtilisin-like enzymes that are active in the post-translational processing of hormone and growth factor precursors (Stieneke-Gröber et al., 1992). The furin recognition sequence R-X-R/K-R is a common feature of the inserted polybasic sequences. The wide tissue distribution of furin-like enzymes and the high efficiency of intracellular cleavage, compared with extracellular cleavage, appear to be related to the widespread systemic and virulent infections caused by the H5 and H7 viruses in birds and the localized outbreak of H5N1 infection in humans in Hong Kong in 1997 (Steinhauer, 1999).

The receptors for IAV are sialic acids (Weis et al., 1988; Bullough et al., 1994; Liu et al., 2009). Depending on the species infected, the receptor recognition varies in specificity, which is the nature of the glycosidic linkage between sialic acid and the penultimate sugar of the side-chains (Rogers and Paulson, 1983). For example, the HAs of human IAVs bind with sialic acid in  $\alpha$ -2,6-linkage that is predominant in the upper human respiratory tracts (Matrosovich et al., 2004), whereas the HAs of avian IAVs prefer sialic acid in  $\alpha$ -2,3-linkage to galactose, which predominates in the avian enteric tract (Naeve et al., 1984) (Fig. 1). Therefore, HA is a key component for an animal IAV to become transmissible in humans. For instance, an H5N1 IAV may acquire human-to-human transmissibility if its HA gains the ability to bind to  $\alpha$ -2,6-linked receptors (Stevens et al., 2006; Xu et al., 2010).

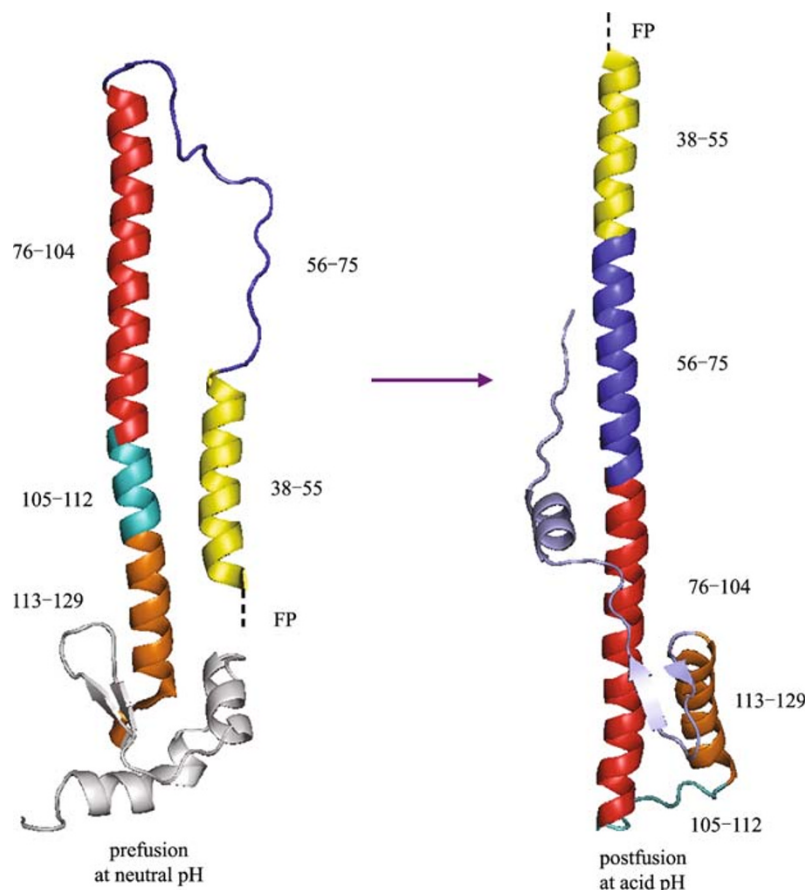
After binding to receptor, virus is taken into cells by endocytosis. Within the endosomal compartment, the virion is exposed to the increasing acid pH 5–6, the HA protein undergoes an irreversible conformation change from its metastable prefusion conformation to a low-pH hairpin structure involving extrusion of the “fusion peptide (FP)” from the interior of the HA2 at the neutral-pH structure toward the endosomal membrane, promoting fusion of the viral and endosomal membranes (Harrison 2008; Reed et al., 2010). X-ray crystallographic studies have demonstrated the extensive rearrangement of residues in HA2 at low pH with respect to their relative orientation and coil-coil formation, loop-to-helix or helix-to-loop transitions (Bullough et al., 1994; Durrer et al., 1996; Plotch et al., 1999; Harrison 2008).

At the initial step, de-trimerization of membrane distal HA1 domain is triggered by low pH, while the domain remain tethered to the inner-core triple-stranded coiled coil structure of HA2 by the region of residues 28–43 (Bizebard et al., 1995). This step is driven by electrostatic forces because the positively charged HA1 favor the electrostatic interaction with the negatively charged HA2 in neutral pH. The short helix (residues 38–55) and the extended loop (residues 56–75) in the N-terminal region of HA2 become an extension of the central triple-stranded coiled coil in the native HA to form a long helix (residues 38–104), relocating the FP over 100 Å from its original buried position. At the same time, the middle portion (residues 105–112) of the long helix of HA2 converts to a loop, allowing the second half of the long helix to jack-knife back to lie antiparallel against the first half (Fig. 2), resulting in formation of the hairpin structure, a common conformation presented in the class I transmembrane proteins of the enveloped viruses (Kielian and Rey 2006; Harrison, 2008).

The interaction between FP and the target membrane leads to an extended intermediate that bridges the viral and cell membranes. Then the intermediate collapses by zipping up of the C-terminal part of the ectodomain alongside the trimer-clustered N-terminal part, which brings the two membranes into close proximity, resulting in formation of a hemifusion stalk. A fusion pore opens up, through which the genetic material of IAV is released into the host cell to generate new virions (Harrison, 2008).



**Figure 1.** The glycosidic linkage between sialic acid and the penultimate sugar of the side-chains. (A) Sialic acid or N-acetylneuraminic acid (Neu5Ac). (B) Sialic acid in  $\alpha$ -2,6-linkage to galactose. (C) Sialic acid in  $\alpha$ -2,3-linkage to galactose.



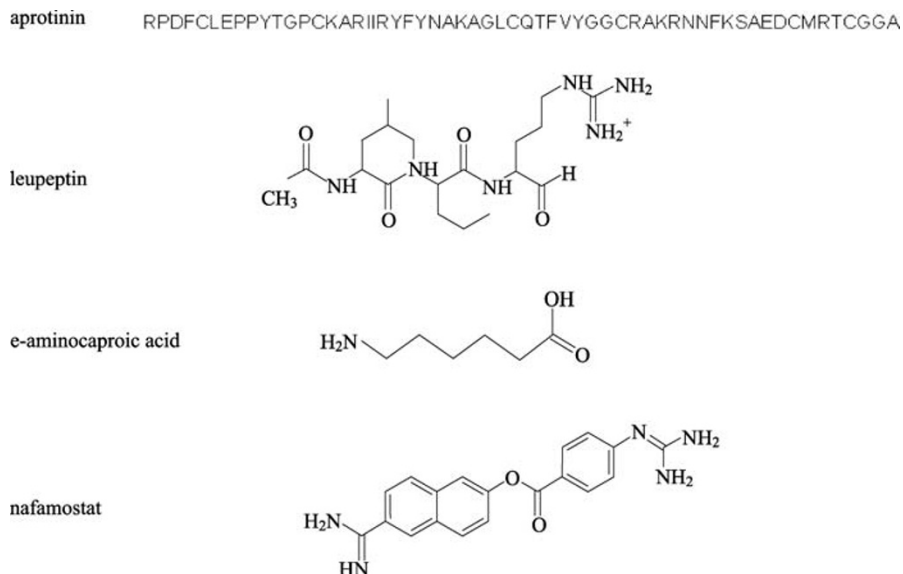
**Figure 2. Diagrams of pH-induced conformational change of HA2 subunit at the prefusion and postfusion states.** In the prefusion state (at the neutral pH), the each of the HA2 monomer in the homotrimer maintains a hairpin structure with the N-terminal helix (residues 38–55) and C-terminal helix (residues 76–129) joined by an extended loop (residues 56–75) in antiparallel fashion. In the postfusion state (at the acid pH), the each of the HA2 monomer still retains the hairpin structure. The middle portion (residues 105–112) of the original long helix unfolds to form a reverse turn and the C-terminal helix (residues 113–129) jack-knives back to lie antiparallel against the newly formed N-terminal long helix (residues 38–104), resulting in the relocation of the FP (dash line) over 100 Å from its previously buried position to the exposed position. The figure was prepared using the PyMol program (Delano, 2005) (<http://www.pymol.org>) and the X-ray crystal structures of HA at neutral pH (PDB ID: 2VIU) (Fleury et al., 1998) and low pH (PDB ID: 1HTM) (Bullough et al., 1994) downloaded from Protein Data Bank (<http://www.rcsb.org/pdb/home/home.do>).

## DEVELOPMENT OF ANTIVIRAL DRUGS TARGETING HA

### IAV fusion inhibitors that suppress HA0 cleavage

Cleavage of the HA precursor HA0 into the HA1 and HA2 subunits by host cell proteases, especially those in the airway, such as trypsin Clara (Kido et al., 1992), mini-plasmin (Murakami et al., 2001), and ectopic pancreatic trypsin (Le et al., 2006), is essential for subsequent steps of viral replication (Liu et al., 2009; Okumura et al., 2010). Therefore, any molecules that can block the cleavage of HA0 may inhibit IAV infection (Kido et al., 2007). Several serine protease inhibitors, such as aprotinin, a 58-mer peptide (Zhirnov et al., 1984), leupeptin (also known as *N*-acetyl-L-leucyl-L-leucyl-L-argininal) (Tashiro et al., 1987; Schröder et al., 1993),

*e*-aminocaproic acid (Kido et al., 1992), nafamostat (Hosoya et al., 1992) (Fig. 3), pulmonary surfactant (a surface-active lipoprotein complex, phospholipoprotein, produced by type II alveolar cells) (Kido et al., 1993), human mucus protease inhibitor (Beppu et al., 1997), have been shown to reduce HA cleavage and IAV infection in cultures and animal models. Aprotinin (Trasylool, Bayer), a bovine pancreatic trypsin inhibitor, has been used as an injection drug for reduction of bleeding during complex surgery. However, Trasylool was withdrawn from the market in May 2008 after studies suggested that its use increased the risk of complications or death ([http://www.trasylool.com/Trasylool\\_11\\_05\\_07.pdf](http://www.trasylool.com/Trasylool_11_05_07.pdf)). There are several FDA approved pulmonary surfactants in the market, including Exosurf, Curosurf, Infasurf, and Survanta, for increasing pulmonary compliance and preventing infant respiratory distress syndrome (IRDS). It has long



**Figure 3.** The protein sequence and chemical structures of IAV inhibitors by suppressing the cleavage of the HA precursor HA0 into the functional HA1 and HA2 subunits.

been known that the peptide substrate analog inhibitor (dec-R-V-K-R-cmk) against furin could block H7 HPAI IAV replication (Stieneke-Gröber et al., 1992). But later, it is observed that dec-R-V-K-R-cmk inhibits not only furin but also the ubiquitous type II transmembrane serine proteases, MSPL and its splice variant TMPRSS13, and other trypsin-type protease such as plasmin (Okumura et al., 1997). Most recently, Kido and colleagues reported that MSPL/TMPRSS13 may also serve as the HA processing proteases of H5 and H7 HPAI IAVs (Okumura et al., 2010). Therefore, the natural inhibitors of MSPL and TMPRSS13 in humans may also be used as leads for development of HA-based anti-IAV drugs.

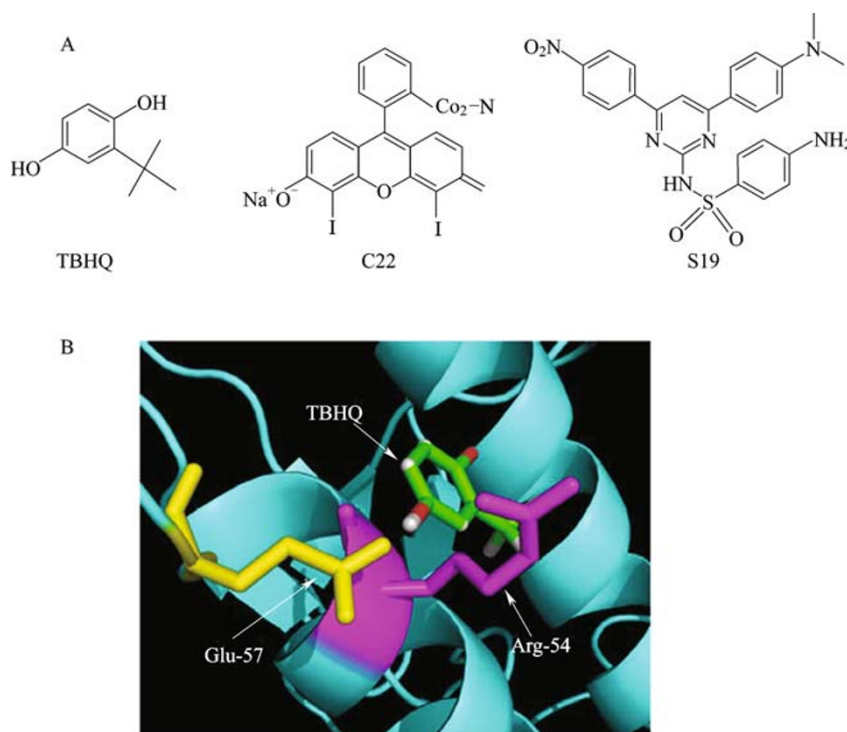
#### IAV fusion inhibitors that block the pH-induced conformation change of HA

During the HA-mediated membrane fusion, HA is activated in endosomes at acidic pH for induction of an irreversible reorganization of HA structure (Wilson et al., 1981; Bullough et al., 1994). Several small molecules that can block the pH-dependent conformational changes of HA, such as benzoquinones and hydroquinones, were shown to inhibit IAV infection (Bodian et al., 1993). The most potent hydroquinone derivative is *tert*-butyl hydroquinone (TBHQ) (Fig. 4A), which inhibits IAV infection at low micromolar level (Bodian et al., 1993). Through an improved molecular docking analysis, the same group identified two new inhibitors with more potent IAV fusion inhibitory activity, S19 ( $IC_{50} = 0.8 \mu\text{M}$ ) and C22 ( $IC_{50} = 8 \mu\text{M}$ ) (Fig. 4A) (Hoffman et al., 1997). TBHQ could induce drug-resistance mutations in HA2 subunit (Hoffman et al., 1997), suggesting that it acts on the HA2

protein. X-ray crystallographic study indicates that TBHQ binds with HA2 and locks the HA2 trimer at the neutral-pH conformation, through the interaction of TBHQ with three ionizable amino acids in this site: Arg-54 and Glu-57 from one HA2 monomer and Glu-97 from another HA2 monomer (Fig. 4B). Notably, TBHQ can inhibit H14 subtype avian influenza virus but has no inhibition on H5 subtype virus because the HA2 of H5 subtype has an intermonomer salt bridge between Lys-58 and Glu-97, while Glu-97 in HA2 of H14 subtype prefer to form salt bridge with Arg-54. As a result, Lys-58 in HA2 of H14 subtype is not well ordered, and the TBHQ site is accessible (Russell et al., 2008).

By screening a chemical library, scientists at Wyeth-Ayerst Research have identified three compounds, CL61917 (an N-substituted piperidine), CL 385319 (the 5-fluoro analog of CL 61917), and CL62554 (Fig. 5A), with inhibitory activity against H1 and H2 subtypes of IAVs with  $IC_{50}$  at low micromolar levels (Plotch et al., 1999). Analysis of HA genes of the mutant viruses resistant to these compounds showed that single amino acid mutations clustered in the stem region of the HA trimer in and near the HA2 FP. Computer-assisted molecular modeling revealed that CL61917 could dock into a pocket in this region. These results suggest that occupation of this pocket by CL61917 may interfere with the low pH-induced structural rearrangements of HA by disrupting the ionic and hydrophobic forces that maintain HA at prefusion state or by blocking the movements of the polypeptide chain during the conformational reorganization (Plotch et al., 1999).

Researchers at the Shionogi Discovery Research Laboratories in Japan have identified several HA-mediated membrane fusion inhibitors, Stachyflin and its derivatives (Fig. 5B), purified from the fermentation broth of *Stachybotrys* sp.



**Figure 4.** The chemical structures of TBHQ and its derivatives (A) and its binding to the HA2 subunit of H14 subtype IAV (B). The figure was prepared using the PyMol program and the X-ray crystal structures of HA in complex with TBHQ downloaded from Protein Data Bank (PDB ID: 3EYK) (Russell et al., 2008).

(Yoshimoto et al., 1999). Stachyflin is effective in inhibiting infection by H1 and H2 subtype IAVs with  $IC_{50}$  at low  $\mu\text{M}$  level. It was shown that Stachyflin interfered with low pH-induced conformational change of HA, thereby blocking HA-mediated virus-cell fusion. Analysis of the HA gene sequences of the IAV variants with resistance to Stachyflin revealed that two amino acid substitutions, K51R and K121E, in the HA2 subunit of the HA protein, were enough to confer a Stachyflin-resistant phenotype of HA protein (Yoshimoto et al., 2000a), suggesting that the binding site of Stachyflin is located in the HA2 subunit. The same group then designed and synthesized several Stachyflin derivatives, such as acetylstachyflin (Fig. 5B), with improved oral availability and antiviral activity (Yoshimoto et al., 2000b; Minagawa et al., 2002). But no further information about development of this class of anti-IAV drugs was available lately.

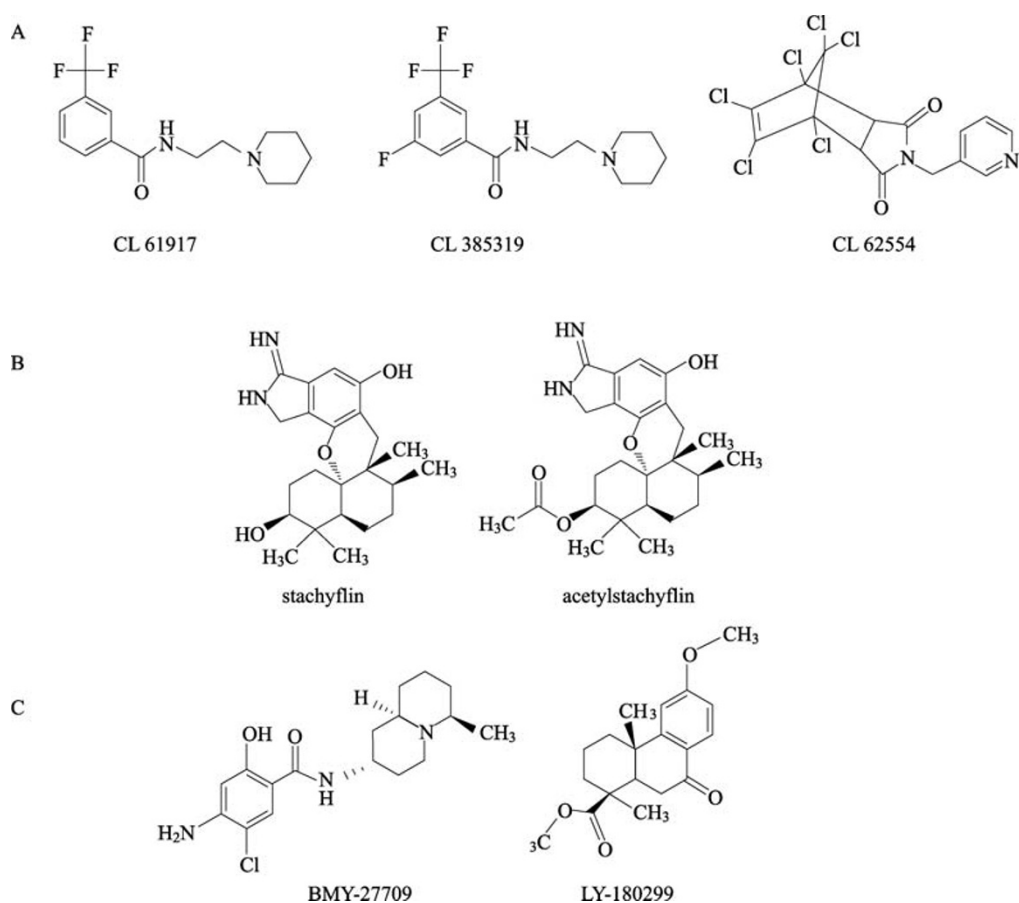
Investigators at Bristol Myers Squibb identified a novel IAV fusion inhibitor, BMY-27709 (Fig. 5C), which inhibited A/WSN/33 virus replication with  $IC_{50}$  at 3–8  $\mu\text{M}$  and was effective against all H1 and H2 subtype viruses tested (Luo et al., 1996). Further studies by this group indicated that BMY-27709 inhibited IAV infection through its specific interaction with the HA protein to repress its conformational change of HA induced by low-pH. Sequence analyses of the HA gene of the variants resistant to BMY-27709 mapped the amino acid substitutions responsible for drug resistance to a region

located near the N terminus of HA2, suggesting that this compound inhibits HA-mediated membrane fusion by targeting the N-terminal domain of HA2 subunit (Luo et al., 1997).

Researchers at Eli Lilly found that a podocarpic acid derivative, methyl-O-methyl-7-ketopodocarpate (LY-180299) (Fig. 5C) could effectively inhibit infection of A/Kawasaki/86 (H1N1) IAV strain by affecting an early step of viral replication (Staschke et al., 1998). Genetic analysis of the LY-180299-sensitive and resistant reassortant viruses indicated that the HA protein conferred the 180299-resistant (180299r) phenotype and the mutations clustered in the interface between HA1 and HA2 and in a region near the FP of HA2. The pH-of-inactivation of the resistant mutants was increased by 0.3–0.6 pH unit, compared with the wild-type viruses. These data suggest that LY-180299 may interact with the neutral pH conformation of HA, resulting in prevention of the low-pH-induced change of HA to its fusogenic conformation.

#### Peptidic IAV fusion/entry inhibitor targeting HA2

In the early 1990s, several peptides derived from the HIV-1 gp41 HR2 domain were found to be highly potent in inhibiting gp41-mediated membrane fusion and HIV-1 replication (Jiang et al., 1993; Wild et al., 1994; Lu et al., 1995). One of the HR2-peptides, T20 (generic name: enfuvirtide, brand name: Fuzeon) was licensed by the US FDA as the first member



**Figure 5.** The chemical structures of IAV inhibitors by interfering with the pH-induced conformation changes of HA2.

of a new class of anti-HIV drugs known as HIV fusion/entry inhibitors for the treatment of HIV-infected patients who fail to respond to the current antiretroviral therapeutics (Kilby and Eron, 2003). It was later proven that the HR2-peptides inhibited viral fusion by binding to the gp41 HR1 domain and interfering with the gp41 fusogenic six-helix bundle core formation (Chan et al., 1997; Weissenhorn et al., 1997; Liu et al., 2007). Discovery of anti-HIV peptides has opened new avenues for developing viral fusion/entry inhibitors against other viruses with class I membrane fusion proteins, such as respiratory syncytial virus (RSV), measles virus (Lambert et al., 1996), Ebola virus (Weissenhorn et al., 1998), Nipah and Hendra viruses (Xu et al., 2004a), and severe acute respiratory syndrome-associated coronavirus (SARS-CoV) (Liu et al., 2004; Xu et al., 2004b; Zhu et al., 2004). Since IAV is also a virus with class I membrane fusion proteins, several groups have attempted to identify T20-like anti-IAV peptides using similar approaches. But no success has been reported so far, possibly because of the limitations in developing anti-IAV peptides. Unlike HIV-1 envelope glycoprotein gp120/gp41 that change their conformation induced by receptor binding and mediate membrane fusion by acting on the cell

surface at neutral pH, the IAV HA mediates membrane fusion in the endosome at acid pH. The peptides derived from the HR2 region of HA2, even they can interact with the HR1 region of HA2 *in vitro* at neutral pH, may not be effective in inhibiting HA-mediated membrane fusion if the peptides cannot enter the cell and endosome or they become inactive at acid pH. A chemical strategy, termed hydrocarbon stapling (Walensky et al., 2004), for designing cell-penetrating helical peptide with anti-HIV-1 activity (Zhang et al., 2008), may be adapted for developing anti-IAV peptides targeting the HA2 subunit of HA. Another strategy is to identify small molecule weight compounds with cell-penetrating ability or oral availability that can interact with the HR1 domain of HA and block the formation of trimer of heterodimer as IAV fusion/entry inhibitors. However, identification of proper "pocket" on the HR1-trimer at the prefusion or intermediate conformation is the prerequisite for screening small molecule anti-IAV compounds targeting HA2. The sequence of the pocket region in the HIV-1 gp41 HR1 domain is highly conserved and this hydrophobic pocket plays a critical role in viral fusion (Chan et al., 1998; Ji et al., 1999). Therefore, the peptidic HIV fusion inhibitor containing the pocket binding sequence, such

as T-2544 (Dwyer et al., 2007) or small molecule HIV fusion inhibitor targeting the conserved pocket region, e.g., ADS-J1 (Wang et al., 2009) are reluctant to induce drug-resistance mutations because the virus may not survive the mutations in the highly conserved pocket region. Therefore, more efforts should be exerted in identification of peptidic or non-peptidic IAV fusion/entry inhibitors targeting sequence-conserved portion of HA, especially the interface of the coiled-coil contacts (Carr and Kim, 1993).

## DEVELOPMENT OF HA-BASED ANTI-IAV VACCINES

Vaccination should be the most effective way to prevent or eliminate the future influenza pandemic. However, the currently licensed seasonal influenza vaccines are ineffective against the newly emerging influenza viruses. Therefore, development of vaccines against IAVs, especially those might induce highly pathogenic, i.e., HPAI H5N1, remains a priority.

Conventional approaches for development of influenza vaccines are mainly based on inactivated or live-attenuated influenza viruses (Treanor et al., 2006; Sasaki et al., 2007). These vaccines have shown promising efficiency, immunogenicity or cost-saving in clinical trials (Ruben 2004; Mitchell et al., 2005), but they have obvious disadvantages, including (i) the reliance on the availability of embryonated chicken eggs, which may be in short supply in a bird-flu outbreak, (ii) the length of vaccine generation time between the vaccine strain selection and the availability of the formulated vaccines, (iii) potential side effects on people who are allergy to egg protein, and (iv) the safety concerns for potential introduction of a live virus into human populations (Ellebedy and Webby, 2009). These limitations of conventional vaccines highlight the need for exploring new approaches for designing and developing influenza vaccines.

HA, one of the most important IAV surface proteins, is the major target for inducing neutralizing antibody responses and thus a main constituent for all influenza vaccine formulations. Different categories of HA-based influenza vaccines have been developed, some of which are evaluated in clinical trials.

### HA-based DNA vaccines

A variety of influenza vaccines have been developed based on the DNA encoding HAs of IAVs. Comparison of the efficacy of DNA vaccine coding HA protein (DNA-HA) with those coding other IAV proteins has demonstrated that DNA-HA is more effective in inducing high level of antibody and T cell responses than the DNA vaccines encoding NA, nucleoprotein (NP) or matrix protein 2 (M2) protein, thus affording better protection than the others in preventing immunized mice from challenge with homologous or heterologous H5N1 viruses (Patel et al., 2009). In addition, the vaccination of mice with DNA-HA could significantly increase survival rate (Chen

et al., 2009a). A clinical trial showed that a trivalent DNA vaccine consisting of three plasmids expressing HA of different seasonal influenza virus strains was safe and could elicit immunological responses that protected human subjects from A/H3 Panama/2007/99 virus challenge (Jones et al., 2009). A monovalent DNA vaccine containing an A/Vietnam/1203/04 H5N1 HA-encoding plasmid was also shown to be safe and effective in Phase I clinical trials (Smith et al., 2010). However, DNA-based vaccines also have some disadvantages, including (i) risk of affecting genes that control cell growth, (ii) potential to induce anti-DNA antibody responses, and (iii) possible toxic effect to the injection sites when repeated doses are used (Du et al., 2008).

### HA-based viral vector vaccines

Viral vectors have been selected as an alternative approach to develop HA-based vaccines. Reported viral vectors expressing HAs of influenza viruses include newcastle disease virus (NDV) (DiNapoli et al., 2010), parainfluenza virus type 5 (PIV5) (Tompkins et al., 2007), vesicular stomatitis virus (VSV) (Barefoot et al., 2009), adenovirus (Gao et al., 2006) and modified vaccinia virus Ankara (MVA) (Kreijtz et al., 2009a, b), and some of them have shown efficacy in preclinical trials. DiNapoli et al. reported that a NDV-vectored vaccine expressing HA of a HPAI H5N1 developed high levels of neutralizing antibodies against homologous and heterologous strains of HPAI IAVs and protected African green monkeys against homologous virus challenge (DiNapoli et al., 2010). According to Kreijtz et al., a recombinant MVA vector expressing the HA of A/Vietnam/1194/04 (H5N1) could induce cross-reactive antibody responses that protected vaccinated cynomolgus macaques from viral infection in the respiratory tract and prevented the development of severe necrotizing bronchiointerstitial pneumonia (Kreijtz et al., 2009a). However, it should be noted that the immunogenicity of viral-vector-based vaccines could be suppressed by the presence of preexisting immunity to the vector, or the potential of causing harmful immune responses to vaccinated subjects (Barouch et al., 2004; Lasaro and Ertl 2009).

### HA-based virus-like particle vaccines

Enveloped virus-like particle (VLP) vaccines containing HA and NA of influenza virus may be produced in insect or mammalian cells via simultaneously expression of the two proteins together with a viral core protein, such as M1 or a retroviral Gag protein (Haynes 2009). This new influenza vaccine approach provides overall advantages over the conventional methods to improve immunogenicity and protection. Previous results have demonstrated that VLPs containing H5N1 HA, NA and M proteins can induce high

titers of H5N1-specific antibodies and/or long-term protection against heterologous as well as homologous H5N1 IAVs (Bright et al., 2008; Kang et al., 2009). A bivalent influenza VLP vaccine containing HA of H1 virus A/PR/8/34 and H3 virus A/Aichi/2/68 (X31) induced neutralizing activities and completely inhibited infection by the homologous strains and the heterologous strains, including H1N1 strains A/PR/8/34 and A/WSN/33 as well as H3N2 strains A/Aichi/2/68 (X31) and A/Hong Kong/68 of virus (Quan et al., 2008). This implies that influenza VLPs, particularly multivalent VLPs, can be used as an alternative method for developing safe and effective vaccines to control the spread of influenza viruses.

### HA-based recombinant subunit vaccines

Recombinant subunit vaccines have been considered attractive for developing vaccines against IAVs (Amorij et al., 2007; Biesova et al., 2009) since this kind of vaccines generally contain no infectious materials, thus being non-infectious and safe to use (Du et al., 2008). In addition, the currently available advanced recombinant technology would benefit the expeditious vaccine production. Furthermore, the high purity of the recombinant protein in the vaccine would enable administration at much higher doses without a significant increase in side effects in human subjects, especially those with immunodeficiency or high-risk medical conditions (Safdar and Cox, 2007). Currently, the HA-based subunit vaccines have been tested in various animal models or evaluated in human clinical trials (Wei et al., 2008; King et al., 2009). Reports have shown that an *E-coli*-expressed recombinant vaccine containing HA of A/Vietnam/1203/2004 (H5N1), formulated with alum, induced high immune responses, particularly HA-specific antibodies in young outbred mice, with the antibody levels consistent with the FDA guidelines for vaccines against epidemic and pandemic influenza (Biesova et al., 2009). Phase I clinical trial of a subunit influenza A/Hong Kong/1073/99 (H9N2) vaccine in healthy adults has shown that this subunit vaccine is safe and is able to induce antibody responses at the level similar to that induced by whole virus particles (Stephenson et al., 2003). Although they are safe and easy to produce, HA-based subunit vaccines might not induce strong immune responses, particularly in young children. This phenomenon is evidenced by a trivalent recombinant baculovirus-expressed HA influenza vaccine, which is shown to induce less immunogenicity than an egg-grown trivalent influenza vaccine in the healthy children aged 6–59 months (King et al., 2009). Therefore, it is crucial to improve the immunogenicity of HA-based subunit vaccines, in which the conformation of HA protein plays an important role. Recent studies have indicated the possibility of inducing stronger neutralizing antibody responses by HA in a high-molecular-weight oligomeric form than HAs in trimeric or monomeric forms (Wei et al., 2008).

### Vaccines based on the conserved HA sequences

With the continuous threat of an influenza pandemic, there is an urgent need to develop vaccines that can provide protection not only against currently identified virus strains but also any future emerging virus strains. The rapid mutation of the protective HA antigen in the circulating IAVs requires reformulation of the influenza vaccines with broad specificity. As such, influenza vaccines directed against the conserved regions of the HA protein would have a great chance to meet these requirements.

The FP region of HA2 subunit contains conserved sequences that maintain high homology across all IAVs (Du et al., 2010). Recent studies have indicated that monoclonal antibody (mAb) CR6261, which recognizes a highly conserved helical region in the membrane-proximal stem of HA1 and HA2, could neutralize the virus by blocking conformational rearrangements associated with viral membrane fusion (Ekiert et al., 2009). Other studies have revealed that mAb 1C9, which binds to GLFGAIAGF of the conserved FP region of HA2, completely protected mice from lethal challenge of two different clades of HPAI H5N1 IAVs (Prabhu et al., 2009). Sui et al. have identified the broad neutralizing activity of an HA2-specific mAb (F10) with the binding sites located in a highly conserved FP-involved pocket in the stem region of HA (Sui et al., 2009).

In addition to conserved FP region, other conserved regions in the HA protein of IAVs may also serve as important vaccine targets. Human mAbs B-1 and D-1, which were recently obtained using the peripheral blood lymphocytes from two influenza-vaccinated volunteers, showed strong global neutralization of H3N2 strains, with the recognized sequences similar to the amino acid residues 173–181 and 227–239 of HA2 proteins, while these two sequences were fairly conserved in clearly separable clusters for H1N1, H3N2 and H5N1 with different host origins (Kubota-Koketsu et al., 2009; Yamashita et al., 2010). Another mAb 13D4, which mapped to at least two conserved sites of H5N1 HA, residues 152 and 182 in HA1, also protected mice against lethal challenge by four H5N1 strains that represent the current major genetic populations, clades 1, 2.1, 2.2 and 2.3 (Chen et al., 2009b). Using large-scale sequence analysis of the HA protein corresponding to H1, H2, H3 and H5 subtypes, Sahini et al. have identified nine conserved regions, five of which have the structural characteristics suitable for an anti-viral/anti-peptide response (Sahini et al., 2010).

### CONCLUSION AND PROSPECTS

HA, the major surface protein of IAV, mediates the viral binding, membrane fusion, and viral entry. Its receptor binding specificity determines the species specificity of IAV infection, e.g., HAs of human and avian IAVs preferably bind with sialic acids in  $\alpha$ -2,6- and  $\alpha$ -2,3-linkage, respectively. Further study



is needed to understand the potential role of HA in the acquisition of human transmissibility by the animal-origin IAVs, such as the 2009 S-OIV H1N1 strain.

Determination of the crystal structures of HA0, HA1/HA2 complex, HA1/antibody complex, HA2/inhibitor complex, and HA2 trimer, provides important information for understanding of the pH-induced conformational changes of HA at the prefusion, intermediate and postfusion states for identification of the targets in the HA-trimers at the prefusion and intermediate conformation, and for development of anti-IAV drugs. Given the fact that the sequences and conformations of the HAs in the H1, H2, and H3 subtype IAVs are much different from those of the H5 and H7 HPAI subtypes, it is worthwhile to explore the key residues which contribute the difference of conformations. Those key residues may be the "hot spots" for anti-IAV drug design and screening, especially for drugs against the high mortality H5N1 IAV.

Although a series of anti-IAV drugs targeting the NA and M2 proteins are currently available, the emergency of drug-resistance viruses due to the widespread use of these drugs has raised the great concern on their ineffectiveness against the newly emerging IAVs, such as the 2009 S-OIV H1N1 strains and the HPAI viruses (Le et al., 2010; Mitrasinovic 2010). Thus, it is essential to develop novel anti-IAV drugs with new targets. A number of protein-based or small molecule anti-IAV agents have been shown to interfere with the HA-mediated membrane fusion by blocking the cleavage of HA0 to the functional HA1 and HA2 subunits mediated by host proteases, or by inhibiting the low pH-mediated conformation changes of HA. However, most of these drugs have low potency (at  $\mu\text{M}$ ) in inhibiting IAV replication. It is still a long way to develop these inhibitors as clinically usable anti-IAV therapeutics. The successful strategy to discover and develop peptidic anti-HIV drug, T20 (enfuvirtide) has been applied for identification of peptidic fusion inhibitors against other viruses bearing the class I transmembrane proteins, including IAV. However, no such anti-IAV peptide has been ever reported, suggesting the difficulty to identify a peptide that can enter into the cell and endosome to block the fusion between viral and endosomal membranes mediated by HA at acid pH environment. There are hopes to design or screen cell-permissible short peptides or small molecular weight organic compounds that can interact with targets on the HR1-trimer and block the formation of the HA fusion-active core. Though the peptidic drugs are expensive, it is worthwhile to develop if they can save lives of the patients during the influenza pandemics.

Because of the antigen shift and drift, it is a great challenge to develop effective and safe vaccines against divergent IAVs, especially those newly emerging HPAI subtypes that may cause future influenza pandemic. Since HA contains the major neutralizing epitopes, it serves an important target for developing anti-IAV vaccines. A number of HA-based DNA vaccines, viral vector vaccines, virus-like particle vaccines,

and recombinant subunit vaccines have been developed under preclinical and clinical studies. The identification of the neutralizing epitopes in the conserved FP and other regions of HA proteins demonstrate the potential of these conserved regions in serving as important vaccine targets, accordingly bringing some hopes for the development of influenza vaccines that may induce cross-protection against divergent IAVs (Ekiert et al., 2009; Prabhu et al., 2009; Sui et al., 2009; Du et al., 2010). More and more effective epitopes are expected to be found in the conserved regions of HA as vaccine targets for future prevention of an influenza pandemic.

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## ABBREVIATIONS

DNA-HA, DNA vaccine coding HA protein; FP, fusion peptide; HA, hemagglutinin; HPAI, highly pathogenic avian influenza; IAV, influenza A virus; IRDS, infant respiratory distress syndrome; mAb, monoclonal antibody; MVA, modified vaccinia virus Ankara; M2, matrix protein 2; NA, neuraminidase; NDV, newcastle disease virus; NP, nucleoprotein; PIV5, parainfluenza virus type 5; RSV, respiratory syncytial virus; SARS-CoV, severe acute respiratory syndrome-associated coronavirus; S-OIV, swine-origin influenza A virus; VLP, enveloped virus-like particle; VSV, vesicular stomatitis virus

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