

Emerging Antiviral Strategies to Interfere with Influenza Virus Entry

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Published online 25 June 2013 in Wiley Online Library (wileyonlinelibrary.com).

DOI 10.1002/med.21289



Abstract: Influenza A and B viruses are highly contagious respiratory pathogens with a considerable medical and socioeconomical burden and known pandemic potential. Current influenza vaccines require annual updating and provide only partial protection in some risk groups. Due to the global spread of viruses with resistance to the M2 proton channel inhibitor amantadine or the neuraminidase inhibitor oseltamivir, novel antiviral agents with an original mode of action are urgently needed. We here focus on emerging options to interfere with the influenza virus entry process, which consists of the following steps: attachment of the viral hemagglutinin to the sialylated host cell receptors, endocytosis, M2-mediated uncoating, low pH-induced membrane fusion, and, finally, import of the viral ribonucleoprotein into the nucleus. We review the current functional and structural insights in the viral and cellular components of this entry process, and the diverse antiviral strategies that are being explored. This encompasses small molecule inhibitors as well as macromolecules such as therapeutic antibodies. There is optimism that at least some of these innovative concepts to block influenza virus entry will proceed from the proof of concept to a more advanced stage. Special attention is therefore given to the challenging issues of influenza virus (sub)type-dependent activity or potential drug resistance. © 2013 Wiley Periodicals, Inc. *Med. Res. Rev.*,

34, No. 2, 301–339, 2014

Key words: influenza virus; antiviral; hemagglutinin; M2 channel; nucleoprotein

1. INTRODUCTION

Human influenza A and B viruses cause significant morbidity and mortality, particularly in infants and elderly people, or those suffering from preexisting pathology or immunodeficiency.^{1,2} The United States Centers for Disease Control and Prevention estimated that, from 1976 to 2000, seasonal influenza epidemics were responsible for >200,000 annual hospitalizations and an annual average of >30,000 influenza-associated deaths in the USA.³ Approximately 90% of the influenza-associated deaths occur among adults aged ≥ 65 years.⁴

To evade the immune response, the circulating influenza H3N2, H1N1, and B viruses continuously change their antigens, and this explains why current influenza vaccines require

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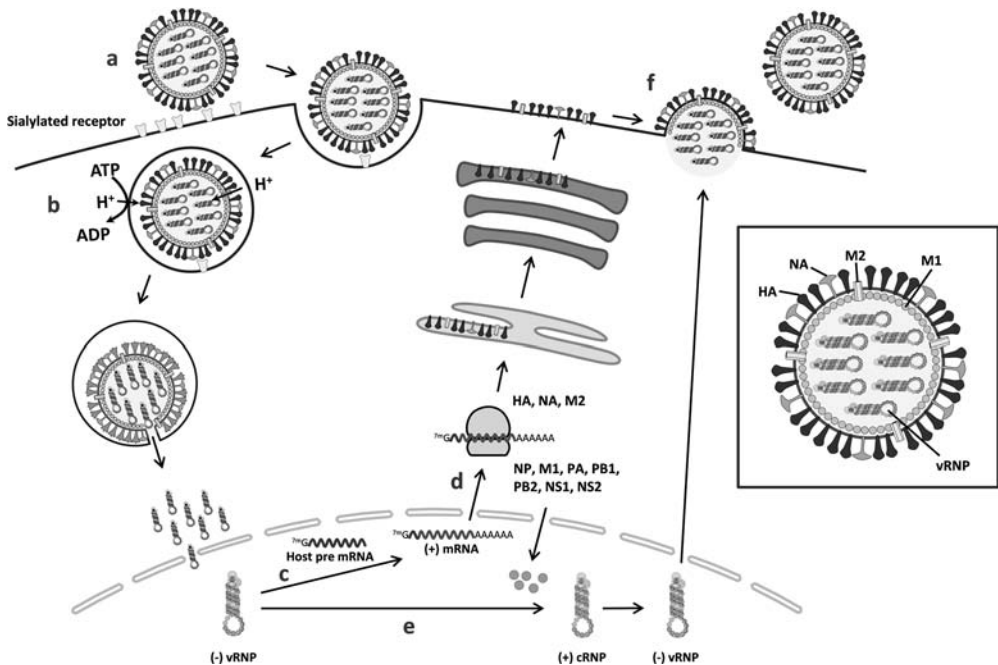


Figure 1. Overview of the influenza virus entry and replication process. In the inset on the right, the different virion components are specified. (a) After binding of the viral HA to sialylated glycans on the host cell surface, the virus is internalized by endocytosis. (b) Acidification of the endosome leads to activation of the M2 proton channel and virion acidification, resulting in virus uncoating (i.e., dissociation of the vRNPs from the M1 capsid protein). The low pH inside the endosome also triggers a conformational change in the HA, leading to fusion of the viral and endosomal membranes. After vRNP release in the cytoplasm and dissociation of residual M1, nuclear localization signals in NP direct the transport of the vRNPs into the nucleus. (c) In the nucleus, the viral polymerase starts mRNA synthesis by cleaving off 5'-capped RNA fragments from host cell pre-mRNAs. Then, viral mRNA transcription is initiated from the 3' end of the cleaved RNA cap. (d) Viral mRNAs are transported to the cytoplasm for translation into viral proteins. HA, M2, and NA are processed in the endoplasmic reticulum and the Golgi apparatus, and subsequently transported to the cell membrane. (e) Besides viral mRNA synthesis, the viral polymerase performs the unprimed replication of vRNAs. The vRNAs are first transcribed into positive-stranded cRNAs, which then function as the template for the synthesis of new vRNAs. During their synthesis, vRNAs and cRNAs are encapsidated by NPs. Export of the newly formed vRNPs into the cytoplasm is mediated by an M1-NS2 complex that is bound to the vRNPs. (f) As they reach the cell membrane, the vRNPs associate with viral glycoproteins at the plasma membrane from which new virions bud off. Finally, the NA cleaves the sialic acid termini on viral and cell membrane glycoproteins, thereby releasing the progeny virions from the host cell.

annual updating. These vaccines provide inadequate protection in some target populations (particularly the elderly).⁵ Besides, there is concern that the widely spread and highly pathogenic avian H5N1 influenza virus may acquire human transmissibility and become a potentially disastrous pandemic virus. The human case-fatality rate of this avian H5N1 is reported to be 59%,⁶ although some investigators have raised the possibility that subclinical cases of H5N1 infections in humans may remain unnoticed.⁷ For comparison, the case-fatality rate of the 1918 influenza virus was estimated >2.5%.⁸

As shown in Figure 1, the influenza virus replication cycle contains several steps amenable to antiviral intervention. This review focuses on the viral entry pathway, which, given the acute onset of influenza virus infection and the inflammation associated with it, is a particularly attractive process to interfere with. We describe the current insights into the structure and functions of the viral and cellular components involved in this entry process, and the antiviral strategies that are being explored (an overview of the described compounds is given in Table I).

Table I. Overview of reported influenza virus entry inhibitors

Compound code or class	General structure	Proposed mode of action ^a	Demonstrated activity ^b or clinical status	Activity spectrum ^c	References
<i>Inhibitors of the HA-receptor interaction</i>					
CH65	Immunoglobulin or Fab	Binding close to HA RBS	In vitro	H1	79,80
C05	Idem	Idem	In vivo	H1, H2, H3, H9, H12	82
S139/1	Idem	Idem	In vivo	H1, H2, H13, H16	83,84
3A2 and 10C4	Idem	Idem	In vitro	B	85
CR8033 and CR8071	Idem	Idem	In vivo	B	86
Nanobodies	Single-domain antibody	Idem	In vivo	HA subtype-specific	88,89
Surfactant protein D	Lectin	Idem	In vitro / In vivo	A and B	90,92,93
CL-43	Lectin	Idem	In vitro / In vivo	A and B	94
Cyanovirin-N	Lectin	Idem	In vitro / In vivo	A and B	96,97
SPG and GM3	Sialylated ganglioside	Binding to HA RBS	In vitro		101,102
LSTc-bearing liposomes	Sialylated liposome	Idem	In vivo		103
Pentadecapeptides	Peptide	Idem	In vitro		104
A22	Aptamer	Idem	In vivo		105
NMSO3	Sulfated sialyl lipid	Not precisely known	In vitro		106,107
c01 and c03	Acylated peptides	Binding to sialic acid	In vitro		109
DAS181	Sialidase enzyme	Receptor destruction	In vivo / Phase II	A and B	112,114
<i>Inhibitors of viral endocytosis</i>					
Fattiviracin	Neutral glycolipid	Membrane fluidity modulator	In vitro		124
Glycyrrhizin	Triterpene glycoside	Inhibits virus internalization; immunomodulator	In vivo	Natural medicine	126–128
LJ001	Small molecule	Intercalates into viral membranes	In vitro		129
Bafilomycin A1	Small molecule	V-ATPase inhibitor	In vitro		135
Diphyllin	Small molecule	Idem	In vitro		135
Chloroquine	4-Aminoquinoline	Raises endosomal pH	Not active in humans		137–139
SA-19	Aglycoristocetin lipoglycopeptide	Intra-cytoplasmic virus trapping	In vitro	A and B	107,131

Table 1. Continued

Compound code or class	General structure	Proposed mode of action ^a	Demonstrated activity ^b or clinical status	Activity spectrum ^c	References
<i>Inhibitors of M2-mediated proton transport</i>					
Amantadine	Adamantane	Block the M2 channel	Approved	A	146,155,159
Rimantadine	Adamantane	Idem	Approved	A	148
+analogues	Adamantane	Idem	In vitro	A	161,166–169
<i>Inhibitors of HA-mediated fusion at low pH</i>					
TBHQ	Small molecule	Binds to HA stem and inhibits HA refolding	In vitro	H3- specific	55,175
4c	Small molecule	Idem	In vitro	H3- specific	176
BMY-27709	Small molecule	Idem	In vitro	H1- and H2-specific	177,182
CL 61917	Small molecule	Idem	In vitro	H1- and H2-specific	179
Stachyfin	Small molecule	Idem	In vitro	H1- and H2-specific	180
RO5464466	Small molecule	Idem	In vitro	H1- and H2-specific	178
Dextran sulfate	Sulfated polysaccharide	Inhibits membrane mixing?	In vitro	A	184,187–189
Retrocyclin-2	Circular peptide	Immobilizes surface glycoproteins	In vitro		194
Arbidol	Small molecule	Inhibitor of HA refolding; membrane fluidity modulator	Approved (Russia and China)	A and B	196,197,199
F10; CR6261	Immunoglobulin or Fab	Bind to conserved HA-stem epitope; inhibit HA refolding	In vivo	Group 1 HAs	206–208
CR8020	Immunoglobulin or Fab	Idem	In vivo	Group 2 HAs	209
F16v3	Immunoglobulin or Fab	Idem	In vivo	All A HAs	210
CR9114	Immunoglobulin or Fab	Idem	In vivo	A and B	86
<i>Inhibitors of NP-mediated viral nuclear import</i>					
Nucleozin + analogues	Small molecule	Aggregates NP; also inhibits vRNP activity	In vivo	A	236–239
<i>Inhibitors of entry-related cellular protein kinases</i>					
Bisindolylmaleimide I	Small molecule	Broad protein kinase C inhibitor	In vitro		245

^aFor compounds with broader antiviral activity, only the mode of action relevant for influenza virus is given.

^bThe most advanced level for demonstration of anti-influenza virus activity is given, in the sequence: *in vitro* (i.e. in cell culture), *in vivo* (i.e. animal studies), and in humans.

^cFor most compounds, literature reports are limited to a number of influenza A or B viruses tested, and hence, the activity spectrum was not (yet) specified. The information in this column indicates the clearly defined activity spectrum.

For antiviral approaches affecting other stages in the viral life cycle, the reader is referred to other recent review articles.^{9–12}

2. CURRENTLY AVAILABLE ANTI-INFLUENZA VIRUS DRUGS

Effective antiviral drugs to prevent or treat influenza infections should at all times be available. Today, two classes of anti-influenza virus drugs exist: the M2 proton channel blockers (i.e., the adamantane compounds, amantadine and rimantadine), and the neuraminidase inhibitors (NAIs) (oseltamivir and zanamivir).¹³ The first two compounds have limited utility, since they are associated with neurological side effects, have no activity against influenza B virus, and the vast majority of circulating strains are adamantane-resistant.¹³ A detailed description of their mode of action and resistance mechanisms will be given below. The obviously superior class of anti-influenza virus drugs are the NAIs oseltamivir and zanamivir that are active against all influenza A and B viruses. These structural analogues of sialic acid bind to the catalytic pocket of the viral NA and inhibit its function in releasing the newly produced virus from the host cells.^{14,15} There is a critical difference in the NA binding mode of oseltamivir compared to that of zanamivir, which explains their significantly different resistance profile. Due to its larger hydrophobic side chain, oseltamivir requires rotation of the noncatalytic Glu276 residue within NA to create a binding space for oseltamivir.¹⁶ By contrast, the smaller size of zanamivir enables direct binding of this compound to NA. In a mutant N1 NA containing a His to Tyr substitution at position 274, this rotation can no longer occur, rendering the NA resistant to oseltamivir binding. During the 2008–2009 season, oseltamivir-resistant H1N1 viruses were isolated all over the globe, even from untreated patients.^{17,18} In a Japanese study in 2004, nine out of 50 children treated with oseltamivir carried oseltamivir-resistant H3N2 viruses.¹⁹ Fortunately, oseltamivir-resistant viruses are still sensitive to zanamivir, for which resistance has only scarcely been reported.^{20,21} On the other hand, the patient-unfriendly administration route for zanamivir (i.e., by powder inhalation device) explains why oseltamivir (which is given by oral capsules) is generally preferred in the clinical setting. Inhalation of zanamivir is a priori excluded in patients suffering from severe influenza symptoms with acute respiratory distress, such as patients infected with the highly pathogenic avian H5N1 virus, or severe cases of the 2009 pandemic H1N1 virus. To address this issue, an intravenous formulation of zanamivir is under consideration.^{22,23} Besides, new NAIs are being developed. Peramivir, which has to be administered intravenously, has been licensed in Japan and South Korea, while, in the United States, its use was temporarily allowed during the 2009 H1N1 pandemic.²⁴ Unfortunately, the widespread oseltamivir-resistant H1N1 His274Tyr mutants show intermediate cross-resistance to peramivir.²⁵ Another NAI, laninamivir (CS-8958), was approved in Japan in 2010 and is currently in Phase III trials in the United States.^{26,27} This promising compound requires only one single intranasal administration (based on its long half-life), and has a similar NA binding mode and favorable resistance profile as zanamivir.²⁸ Finally, novel NAIs with a sialic acid-related or unrelated structure have been developed by rational design, but are still in the early experimental stage.^{29–31}

To face the emerging resistance to NAIs (in particular, oseltamivir), entirely novel anti-influenza virus drugs are urgently needed. The two products that are most advanced in clinical development are the nucleobase analogue T-705 (favipiravir) and the receptor destroying protein DAS181. For T-705, Phase III trials in the United States are pending. Its active ribose-triphosphate metabolite is recognized by the influenza virus polymerase, causing competitive inhibition of viral RNA synthesis and/or lethal viral mutagenesis.³² T-705 has broad anti-RNA virus activity beyond influenza virus and is presumed (based on cell culture data) to have a

high barrier for viral resistance.³³ The second agent, DAS181, is currently in Phase II trials. This recombinant protein is a sialidase that cleaves the influenza virus receptors in the airway epithelia. More details on DAS181 are provided in Section 3.

3. INHIBITORS OF THE HEMAGGLUTININ-RECEPTOR INTERACTION

A. Structure of the Viral Hemagglutinin

Within the influenza virus particle, the single-stranded, negative-oriented RNA genome is divided over eight viral ribonucleoprotein (vRNP) segments, which are protected by the capsid shell formed by the M1 protein, further surrounded by the viral envelope. Two viral spike proteins protrude from the virion: the hemagglutinin (HA) and NA, which have a leading role in viral entry and release, respectively. The HA and NA glycoproteins are the main antigens against which the host immune response is raised. In the case of influenza A virus, 17 HA and 10 NA subtypes are known, which are all present in aquatic birds, the natural reservoir for influenza A viruses.³⁴ The only exception is H17, which was isolated only recently from bats.^{35,36} The emergence of a new pandemic virus is explained by the reassortment of genome segments, which occasionally occurs upon dual infection of an animal species (such as a pig) that carries the avian- as well as the human-type influenza virus receptors.³⁷

The influenza virus HA (Fig. 2A) is a homotrimeric type 1 membrane glycoprotein. Its membrane-distal globular head domain contains the receptor binding site (RBS), whereas the HA stem structure (which contains the fusion peptide) is responsible for intraendosomal membrane fusion.³⁴ In influenza virus-infected cells, HA is first synthesized as its precursor protein HA0, which assembles into a noncovalently linked homotrimer³⁸ and is cleaved into two polypeptides (HA1 and HA2 containing, in the case of H3, 328 and 221 amino acids, respectively), which remain covalently attached by a disulfide bond.³⁹ For most HAs, HA0 cleavage occurs at a single arginine residue and is performed by a membrane-bound or secreted serine protease that is restricted to bronchiolar epithelium, such as tryptase Clara, the human airway trypsin-like protease or TMPRSS2.^{40,41} The HAs from highly pathogenic avian viruses contain a series of basic residues at their cleavage site,⁴² allowing recognition by furin-like intracellular proteases that are widely distributed in avian tissues, thus explaining their systemic spread and high virulence.⁴³ Inhibition of the cellular proteases performing HA0 cleavage is an original antiviral strategy, and peptidomimetic furin inhibitors have proven to inhibit the replication of an avian influenza virus in cell culture.⁴⁴ After HA0 cleavage, minor rearrangements lead to insertion of the fusion peptide (located at the N-terminus of HA2) into a negatively charged cavity, thus priming the HA for pH-dependent fusion.⁴⁰ Posttranslational modifications of HA comprise the addition of acyl chains to the short cytoplasmic tail,⁴⁵ and N-glycosylation at several asparagine residues in the ectodomain.³⁹ Besides masking the antigenic epitopes by sterically hindering antibody recognition,^{46,47} the N-linked glycans also function in the correct folding of HA in the endoplasmic reticulum,^{48,49} modulation of receptor binding,⁵⁰ controlling HA0 cleavage,⁵¹ and maintaining the HA in its metastable conformation required for fusion activity.⁵² The N-glycans that are most conserved among various influenza HAs are located at the N-terminus of HA0 (or after cleavage, HA1)⁴⁸ and in the HA stem region.⁵³

The 17 influenza HA subtypes are classified into two phylogenetic groups (Fig. 2B). The H1 and H5 HAs belong to the same clade within group 1, whereas H3 HA belongs to group 2.^{35,54,55} Although this phylogenetic classification was primarily based on HA protein sequence, comparison of available HA crystal structures indicates that the regions involved in membrane fusion show striking similarities on a group-specific basis.⁵⁴

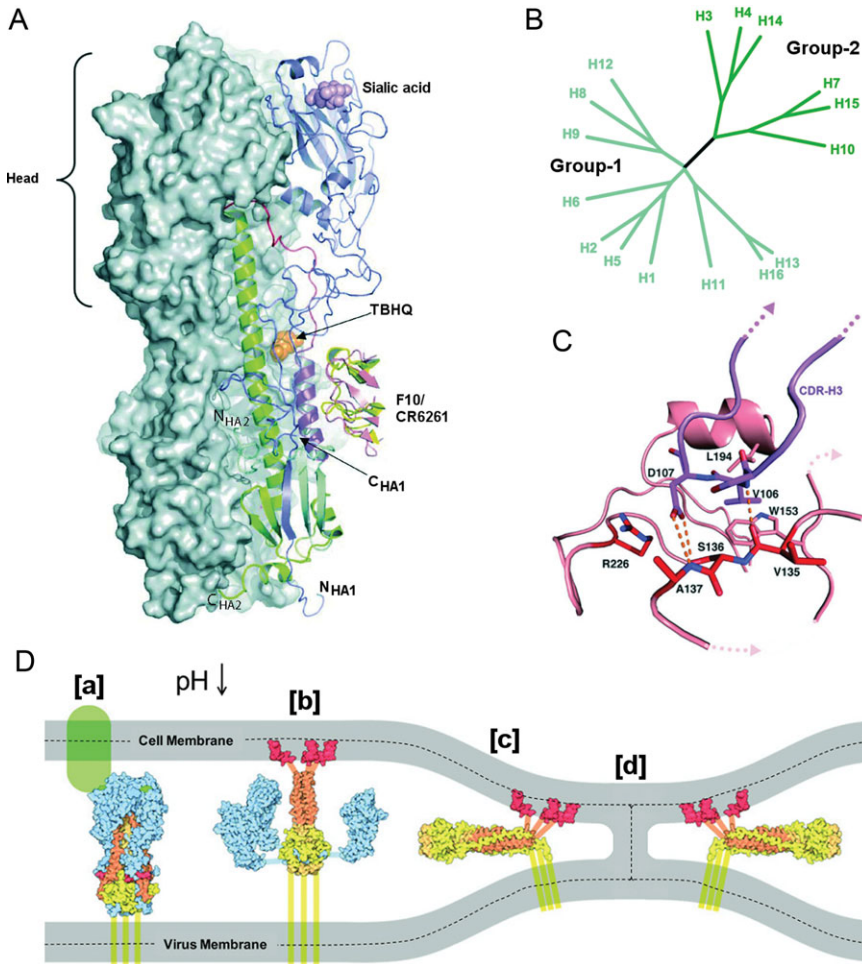


Figure 2. Structure and classification of influenza A HAs. (A) Structure of the viral hemagglutinin, showing the binding site for sialic acid (violet) in the globular head domain (blue ribbon structure), as well as the binding pockets in the HA stem structure for fusion inhibitors reported to prevent the HA conformational change, that is, the small-molecule inhibitor TBHQ (orange) and the broad-acting antibodies F10 (pink) and CR6261 (yellow). Two HA subunits are represented by their combined molecular surface, while the third one is shown in a ribbon diagram. [Reprinted by permission from Macmillan Publishers Ltd: Nature Structural & Molecular Biology Ref. Das et al.¹⁰ © (2010).] (B) Phylogenetic tree of influenza A HAs. Group 1 (cyan) can be subdivided into three clades (H8, H9, and H12; H1, H2, H5, and H6; H11, H13, and H16). Group 2 (green) is subdivided in two clades (H3, H4, and H14; H7, H10, and H15). The newly identified H17 is classified in the H1 clade of group 1.³⁵ [Taken from Russell et al.,⁵⁵ Copyright (2008) National Academy of Sciences, USA.] (C) Detail of the HA RBS indicating the binding mode of the CDR-H3 loop (heavy-chain complementarity determining region 3) of antibody CH65, which acts as a sialic acid mimic. The HA RBS is colored pink and the CDR-H3 loop is shown in blue. The residues relevant for the antibody-HA interaction are labeled; some of these are conserved HA1 residues involved in sialic acid binding (Ser136₁, Trp153₁, and Leu194₁). [Taken, with permission, from Whittle et al.⁷⁹] (D) Cartoon of the structural changes in HA during the HA-mediated membrane fusion process. [a] The HA RBS binds to the sialylated cell receptor (in green). [b] The acidic pH in the endosome induces HA refolding, which leads to the exposure of the fusion peptide (in red) and its insertion in the endosomal membrane. [c] As a result of further conformational changes in HA, the viral and endosomal membranes are pulled together. [d] Mixing of the outer membrane leaflets generates the prefusion stalk intermediate. The dashed lines separate the inner and outer membrane leaflets. [Taken from Hamilton et al.,²⁵¹ with permission]

B. Species-Specific Virus Binding to Sialylated Glycan Receptors

In the first step of the infection cycle, the HA attaches, via the RBS in its globular head, to sialylated glycoproteins or glycolipids on the host epithelial cells.⁵⁶ This HA-receptor interaction is highly specific for sialylglycoconjugates and plays an essential part in the species recognition of avian versus human influenza viruses.⁵⁷ The HAs from human-adapted viruses, including the pandemic viruses of the H1N1, H2N2, or H3N2 subtype, preferentially bind to cell-surface glycans terminating in α 2-6-linked sialyl-galactosyl residues [Neu5Ac(α 2-6)Gal], whereas avian influenza A viruses have a preference for α 2-3-linked sialyl-galactosyl termini.^{58–62} The HAs of influenza B viruses which, in nature, are only detected in humans and seals, show a binding preference for α 2-6-linked glycans.^{63–65} Thus, it is important to underline that the species specificity of the HA–glycan interaction is not based on recognition of the terminal sialic acid itself, but, rather, its linkage to the vicinal galactose and the sugars beyond galactose.^{66,67} A correlation between glycan topology and species specificity was established from HA–glycan cocrystal structures as well as glycan array data.⁵⁸ With regard to the HA residues that are directly involved in sialic acid binding, these are highly conserved across different HA subtypes. These amino acids (Tyr98₁, Ser136₁, Trp153₁, His183₁, Leu194₁) [amino acid numbering based on the H3 HA sequence; the suffixes 1 and 2 denote location in the HA1 and HA2 subunit, respectively] lead to a fixed orientation of the sialic acid relative to the HA RBS.⁶⁸

Although sialic acid is generally considered to be the primary attachment receptor, influenza virus is able to bind and enter (though considerably less efficiently) into cells of which all surface sialic acids, whether attached to glycolipids or glycoproteins, were removed by treatment with exogenous *Micromonospora viridifaciens* sialidase.⁶⁹ Hence, it has been proposed that, besides sialic acid, other receptors may be involved in influenza virus entry, which can work either independently or via a multistep process.^{69,70}

Which specific amino acid residues in HA govern its avian versus human receptor preference, varies among the different HAs, and is still incompletely understood, although α 2-6 tropism is generally linked to residues Asp190₁ and Asp225₁ in H1 and Leu226₁ in H2 and H3 HAs.⁷¹ To cross the avian–human species barrier, acquisition of the human receptor binding preference is not sufficient, since additional amino acid changes are required, particularly in the influenza virus polymerase complex.⁷¹ In a recent study in which the avian H5N1 virus was passed in ferrets, four mutations in the head domain of H5 HA, combined with the Glu627Lys hallmark mutation in the PB2 subunit of the polymerase complex, were able to lead to airborne transmission of this virus in ferrets.⁷² A similar study with a reassortant virus carrying the HA of avian H5N1 also concluded that its avian-to-mammalian adaptation requires a combination of HA mutations to not only switch its receptor preference from α 2-3 to α 2-6, but also increase the stability of the HA protein.⁷³

C. Antiviral Strategies to Interfere with HA-Receptor Binding

When considering the HA-receptor binding as an antiviral target, the multivalent nature of this interaction may present as a challenge. This binding is highly dynamic and involves an ensemble of sialylated glycans making contact with multiple HA trimers.⁷⁴ In this manner, the avidity effects of the multivalent interaction compensate for the intrinsically low glycan binding affinity for a single binding site on HA [with a dissociation constant (K_d) in the millimolar range].⁷⁵

Thus, to develop inhibitors that block the receptor binding of HA, at least three factors need to be taken into account: large sequence variation among HA subtypes and antigenic drift of HA; avian versus human-specific receptor use; and multivalent nature of the HA-receptor interaction. An ideal inhibitor would be species- and HA subtype-independent. There are

three conceivable strategies for inhibiting attachment of influenza virus to its target cell: (i) an antiviral compound binding to the HA RBS; (ii) an inhibitor blocking the sialic acid-containing receptors on the epithelial cell membrane; or (iii) a receptor-destroying agent.

1. HA-Binding Agents

Virus-neutralizing antibodies

The first and natural types of binding inhibitors are the virus-neutralizing antibodies raised during the course of an influenza virus infection. These neutralizing antibodies are predominantly directed toward the surface of the membrane-distal globular head domain of HA.⁷⁶ During the 1918 pandemic, some patients were treated with human blood products from recovering influenza patients.⁷⁷ Eight controlled studies reported between 1918 and 1925 were recently reviewed, and it was concluded that the overall case-fatality rate was reduced from 37% among control patients to 16% among treated patients. Treatment was most effective when initiated early (i.e., less than 4 days after pneumonia became apparent).⁷⁷ These historical data demonstrate that passive immunization with anti-HA antibodies can be considered in case a pandemic occurs. Obviously, safety considerations about the use of patient-derived materials need to be addressed. An elegant method for the isolation of human antibodies was reported by Simmons et al.,⁷⁸ who prepared H5N1 neutralizing monoclonal antibodies from the memory B-cells of patients recovered from an H5N1 infection. Two monoclonal antibodies were effective in a mouse influenza model when administered no later than 72 hr after infection.⁷⁸ An attractive new concept is the development of monoclonal antibodies that bind to the conserved RBS of HA and, hence, are endowed with heterosubtypic HA neutralizing activity. A first human monoclonal antibody directed against H1 HA, encoded **CH65**, was derived from plasma cells of a person immunized with the 2007 trivalent influenza vaccine. Cocrystallization of its Fab fragment with H1 HA revealed that this antibody acts as a sialic acid mimic since the tip of its heavy-chain complementarity determining region 3 (HCDR3) inserts in the RBS of H1 HA (Fig. 2C).⁷⁹ Since CH65 was shown to neutralize 31 out of 36 H1N1 isolates covering a period of more than 30 years, and to interact with the conserved RBS itself, resistance selection by CH65 may be expected to be rare, unless associated with reduced viral fitness.^{79,80} It should however be noted that the RBS of HA is smaller than the interaction site of an antibody⁸¹ and, therefore, CH65 forms additional interactions with RBS surrounding residues that are less conserved among the different HAs. The more broadly acting monoclonal antibody **C05** binds to H1, H2, H3, H9, and H12 HAs and was isolated from a phage-display library constructed from bone marrow donated after seasonal influenza infection. Cocrystallization studies demonstrated that the HCDR3 part of C05 forms a loop that inserts into the conserved RBS of HA, while its HCDR1 region makes only minimal contact with RBS surrounding and more variable residues.⁸² A third cross-reactive monoclonal antibody, **S139/1**, neutralizes H1, H2, H3, H13, and H16 virus strains.⁸³ The HCDR2 region of S139/1 was shown to form multiple hydrophobic interactions within the RBS of H3 HA. The rather low affinity of this binding interaction is compensated in the bivalent IgG molecule, and this avidity effect is required to broaden the neutralizing activity of S139/1 to strains of the H1, H2, H13, and H16 subtypes.⁸⁴ Regarding influenza B viruses, the human monoclonal antibodies **3A2** and **10C4**, reactive against B viruses of the Yamagata lineage, recognize the 190-helix (residues 190–198 in HA1) near the RBS.⁸⁵ The human monoclonal antibodies **CR8033** and **CR8071** were shown to neutralize both Yamagata and Victoria lineage B viruses and protect mice after challenge with a lethal dose of influenza virus.⁸⁶ Although the therapeutic use of an anti-influenza antibody may appear complicated, some parallel can be seen with the palivizumab antibody that is already in use for the prophylaxis of another respiratory virus, that is, respiratory syncytial virus (RSV).⁸⁷ An innovative strategy to improve the pharmacokinetics and reduce the production cost of therapeutic

antibodies consists of single-domain antibody fragments (also referred to as Nanobodies) derived from camelid immunoglobulins.⁸⁸ A Nanobody directed to the globular head of H5 HA was shown to be effective in H5N1-infected mice. The activity of the monovalent Nanobody was increased by a factor 60 when using a bivalent format, consisting of two paratope containing domains connected by a flexible linker.⁸⁹

Lectins

Another type of immune proteins capable of catching viruses is the collagenous C-type lectins (referred to as collectins) such as the lung surfactant proteins. The role of **surfactant protein D** (SP-D) in the innate immune response to influenza virus is explained by its capacity to cause virus particle aggregation, thereby preventing virus attachment to the host cells.⁹⁰ Besides, SP-D has various immunological effects that account for its ability to limit lung inflammation by respiratory pathogens.⁹¹ Regarding potential antiviral use, design of modified forms of the porcine SP-D lectin (which has higher anti-influenza virus activity than its human counterpart) is aided by the growing insight into how its carbohydrate recognition domain (CRD) precisely interacts with the high-mannose glycans attached near the RBS of HA.^{92,93} In addition, *N*-linked sialoglycans attached to the CRD of SP-D are considered important, since they may cause additional interactions between the SP-D and the HA RBS and enhance the antiviral effect.⁹⁰ A similar action principle, that is, binding to high-mannose carbohydrates on the viral HA, accounts for the anti-influenza virus activity of the bovine serum lectin **CL-43**.⁹⁴ Likewise, **cyanovirin-N**, a lectin isolated from *Escherichia coli*, recognizes high-mannose oligosaccharide structures on diverse viral glycoproteins, explaining its broad activity against unrelated viruses such as influenza virus and HIV.⁹⁵ Cyanovirin-N was shown to inhibit influenza virus replication in cell culture as well as mouse and ferret infection models.^{96,97} Although SP-D and cyanovirin-N manifest broad anti-influenza A and B virus activity, some virus strains (such as the A/PR/8/34 H1N1 strain) are known to be insensitive, due to the lack of particular Asn-linked oligosaccharides on the head of their HA.⁹⁸ The location and number of glycans attached to the head of HA is quite variable, since acquisition of epitope shielding oligosaccharides is part of the viral immune escape.⁴⁶ In contrast, the glycans attached to the HA stem have a structural function in protein refolding, and the corresponding glycosylation sites are therefore more conserved.^{52,53} This implies that antiviral use of lectin compounds directed toward HA head glycans might lead to escape mutants devoid of specific glycans, although the newly exposed antigenic sites might also render the mutated virus susceptible to immunological control.⁹⁹

Sialyl-containing macromolecules and sialomimetics

An alternative approach to block the HA RBS makes use of receptor mimics, such as sialyl-containing macromolecules. The gangliosides sialylparagloboside (SPG) and **GM3** (Neu5Ac α 2-3Gal β 1-4Glc β 1-1'ceramide) were proven to bind to HA and inhibit the virus-induced cytopathic effect,¹⁰⁰⁻¹⁰² and their antiviral activity correlated with their HA binding affinities.¹⁰¹ The hydrophobic ceramide moiety of SPG and GM3 was found essential, since the uncoupled trisaccharides 3'-sialyllactosamine and 3'-sialyllactose (which constitute the termini of SPG and GM3, respectively) produced no effect. Micelle formation of these gangliosides in aqueous solution likely causes protrusion of their sialic acid parts toward the outside of the micelles, resulting in high sialic acid density and, hence, a multivalent binding interaction with HA.¹⁰¹

In a recent report, Hendricks et al. described that liposomes bearing sialylneolacto-*N*-tetraose c (**LSTc**) can form multivalent interactions with influenza virus.¹⁰³ In contrast to

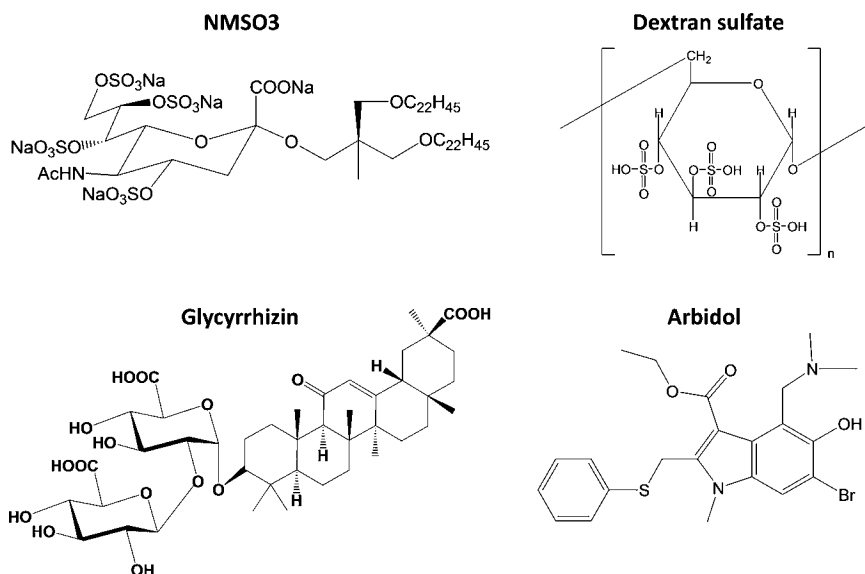


Figure 3. Chemical structures of diverse antiviral agents reported to inhibit the entry of, among others, influenza viruses. The sulfated sialyl lipid NMSO₃ may act upon influenza virus binding¹⁰⁷; glycyrrhizin may reduce membrane fluidity^{125,126}; and dextran sulfate¹⁸⁴ and arbidol¹⁹⁷ probably interfere with the low pH-induced fusion process (see the text for all details).

monovalent LSTc, these decoy liposomes are able to competitively bind influenza virus in a hemagglutination inhibition assay, and suppress influenza virus replication in cell culture and mouse models.

Pentadecapeptides binding to H1 and H3 HAs were obtained from phage-displayed random peptide libraries by serially repeated affinity selection. A docking simulation indicated that these peptides act as sialomimetics. Some showed inhibitory activity against H1 and H3 influenza viruses in cell culture.¹⁰⁴ Jeon et al. used a peptide with a sequence derived from the globular head region of HA to screen a DNA library for HA-binding aptamers. The selected aptamer, A22, was proven to block the RBS of HA and inhibit influenza A viruses in vitro (i.e., cell culture) and in vivo (i.e., animal studies).¹⁰⁵

Another macromolecule, the sulfated sialyl lipid NMSO₃ (Fig. 3) showed antiviral activity against influenza H3N2, but not against B viruses.¹⁰⁶ We recently found that NMSO₃ inhibits influenza virus binding to cells at 4°C.¹⁰⁷ Although NMSO₃ has a strong negative charge and, hence, a direct interaction of NMSO₃ with the sialic acid binding residues of the HA RBS can be anticipated, the precise mode of action of this antiviral compound remains to be determined. NMSO₃ has broad activity against diverse viruses (in cell culture as well as animal models)^{106,108} and presents as a relevant antiviral lead compound.

2. Receptor-Binding Agents

The opposite strategy to block binding of influenza virus to its cell receptor, is the development of sialic acid binding compounds. The HA-binding and Neu5Acα2-3Gal-containing ganglioside GM3 was used to select potential inhibitors from a phage-displayed random peptide library.¹⁰⁹ Two pentadecapeptides, c01 (GWYKGRARPVSAVA) and c03 (RAVWRHS-VATPSHSV), were picked out and acylated to a C18 group, in order to form a molecular assembly and promote multivalent binding. Both C18-peptides provided inhibition of influenza

virus infection in cell culture. Their anti-influenza virus activity was comparable to that of the wheat-germ agglutinin lectin,¹⁰⁹ which is known to interact with sialoglycoconjugates.¹¹⁰

3. Receptor Destroying Agents

Finally, a third strategy for inhibition of virus binding is destruction of the sialylated glycan receptors. Years ago, it was observed that cells are less susceptible to influenza virus infection after enzymatic removal of sialic acid from the cell surface.¹¹¹ The new anti-influenza virus agent **DAS181** is a recombinant fusion protein, consisting of a sialidase catalytic domain derived from *Actinomyces viscosus* and an epithelium-anchoring domain. DAS181 efficiently removes α 2-3- and α 2-6-linked sialic acids and displays broad activity against influenza A and B viruses as well as parainfluenza viruses.^{112,113} Since DAS181 acts on the host cell rather than the virus, it is assumed to have a reduced potential for generating drug resistance. After more than 30 passages in the presence of DAS181, influenza virus mutants were selected with low to moderate resistance to the compound (i.e., three- to 18-fold increase in antiviral EC₅₀ value). The resistant viruses showed an attenuated phenotype compared to the wild-type virus, yet unchanged virulence in mice. When further passaged in the absence of compound, the viruses quickly regained the wild-type sensitivity. Sequencing revealed that the resistant mutants contained substitutions in the HA near its RBS, as well as in the NA, leading to altered HA and NA functionality.¹¹⁴ The concern that desialylation of the airway epithelium might unmask certain cryptic receptors and increase the susceptibility to *Streptococcus pneumoniae*, was contradicted by mouse experiments showing that DAS181 treatment does not lead to an increased incidence of secondary pneumonia.¹¹⁵ DAS181 requires topical delivery as an inhalant. It is currently in Phase II clinical trials (at once daily dosing of 10 mg during 3 days) for the treatment and prophylaxis of influenza-like illness.¹¹⁶

4. INHIBITION OF ENDOCYTIC UPTAKE OR VIRUS TRAFFICKING

A. Different Endocytic Routes Exploited by Influenza Virus

After binding to the sialylated glycans on the cell surface, influenza virions are internalized by endocytosis. In general, viruses can be internalized by clathrin-mediated endocytosis (CME); caveolin-mediated endocytosis; macropinocytosis; or other less characterized mechanisms.¹¹⁷

Early electron microscopic analysis of influenza virus-infected cells showed the presence of virions in clathrin-coated pits and vesicles, providing direct evidence that influenza virus can enter the cell by CME. However, since virions were also found in smooth pits, the virus is able to follow an alternative clathrin-independent pathway.¹¹⁸ Further support came from investigations in which dominant negative forms of cellular endocytic regulators were expressed, or by using pharmacological inhibitors, that is, the CME inhibitor chlorpromazine, the cholesterol-depleting agents nystatin or methyl- β -cyclodextrin; or genistein, an inhibitor of caveola formation.¹¹⁹

Additional evidence that influenza virus exploits CME and a clathrin- and caveolin-independent route in parallel, was provided by real-time imaging. Both routes appear to be equally efficient in supporting the infection once the virus is internalized.¹²⁰ Only recently, the clathrin- and caveolae-independent influenza virus uptake was shown to have the characteristics of macropinocytosis.¹²¹ Influenza virus entry was completely inhibited when cells were simultaneously treated with dynasore and the amiloride derivative EIPA, which inhibit dynamin-dependent CME and macropinocytosis, respectively.

The sialic acid attachment sites for influenza virus possess no host cell signaling capacity and, hence, additional postattachment factors may be required for efficient viral entry.¹²² Since

the virus fails to enter Lec1 cells, a mutant CHO cell line that is totally deficient in N-terminal glycosylation, it was suggested that *N*-linked glycoproteins may be required for efficient endocytosis of the virus.¹²² Besides, binding of influenza A virus to cells was found to induce lipid raft rearrangement and activation of signaling molecules, such as the epidermal growth factor receptor (EGFR) or other receptor tyrosine kinases. Also, it was observed that the activated EGFR kinase is involved in promoting the initial virus uptake, and that virus internalization was considerably reduced in the presence of genistein, a broad inhibitor of receptor tyrosine kinases.¹²³

Thus, the precise mechanisms for endocytic uptake of influenza virus are still not fully understood. Whether any of these insights may be translated into a relevant antiviral concept is unsure. Influenza virus appears to exploit endocytic routes and signaling platforms that are intimately linked to normal cell functioning and thus not readily amenable to selective antiviral intervention. For instance, the above-mentioned pharmacological agents, which were very useful to demonstrate the role of CME or macropinocytosis, only affect the viral uptake at subtoxic concentrations.

B. Antiviral Strategies to Interfere with Endocytic Uptake and Virus Trafficking

One potential approach is the use of membrane fluidity modulators, which restrict the movement of membrane molecules. The neutral glycolipid fattiviracin (FV-8; isolated from *Streptomyces*) interferes with cell–cell fusion in HIV-infected cells and was also reported to have anti-influenza virus activity.¹²⁴ Interference with cell membrane fluidity may also be the principal mode of action of **glycyrrhizin** (Fig. 3), the main active constituent of licorice root. Glycyrrhizin has broad antiviral activity against diverse enveloped viruses, including influenza virus, herpes simplex virus (HSV), varicella-zoster virus (VZV), vaccinia virus, vesicular stomatitis virus, measles virus, HIV-1, and the SARS coronavirus.^{125,126} Its anti-influenza virus activity was already demonstrated in 1983.¹²⁷ More recently, a flow cytometric internalization assay was used to show that glycyrrhizin inhibits the endocytic uptake of influenza virus.¹²⁶ Glycyrrhizin was further proven to decrease the fluidity of the cell membrane, an effect that was attributed to its cholesterol-related chemical structure¹²⁵ (Fig. 3). Besides its antiviral effect, glycyrrhizin displays anti-inflammatory and immunomodulatory effects.¹²⁸ These combined pharmacological effects may be advantageous in the treatment of virus infections with a strong inflammatory component, such as the severe airway inflammation (cytokine storm) caused by the avian H5N1 virus.¹²⁸ In Japan, glycyrrhizin is already in clinical use since many years, and based on this the compound is considered to have favorable safety with no serious side effects.^{125,126}

Another broad-spectrum antiviral agent interfering with membrane fusion is the aryl methyldiene rhodamine derivative **LJ001**. This compound displays activity against a wide range of unrelated enveloped viruses, including influenza A virus, HIV-1, yellow fever virus, hepatitis C virus (HCV), vesicular stomatitis virus, and vaccinia virus.¹²⁹ Time-of-addition experiments demonstrated that LJ001 acts upon virus entry, since inhibition was only achieved when the compound was added before or during infection. LJ001 was shown to intercalate into viral as well as cellular membranes. Its potent antiviral activity and low cytotoxicity was explained by the capacity of the host cell for active and rapid biogenic repair, while disruption of the virion envelope is irreversible.

Lipoglycopeptides are lipophilic derivatives of glycopeptide antibiotics such as the widely used antibiotic vancomycin. Some lipoglycopeptides are not only endowed with increased antibacterial activity, but also display activity against diverse viruses such as HIV, herpes viruses, or flaviviruses.¹³⁰ Regarding influenza virus, we recently described the structure–activity relationship of a series of aglycoristocetin derivatives containing an aryl-substituted cyclobutenedione.¹³¹ The lead compound **SA-19**, which carries a phenylbenzyl substituent,

displayed strong and consistent activity against all influenza A and B viruses tested.¹⁰⁷ No resistance to SA-19 was observed after 15 virus passages in cell culture. This compound was shown to cause intracytoplasmic trapping of influenza virus prior to its nuclear entry, presumably by disturbing the endocytic uptake of the virus at the site of the plasma membrane. It would be relevant to see whether kistamicin A and B, two ristocetin-related glycopeptides that were reported to have anti-influenza virus activity several years ago,¹³² display a similar mode of action as SA-19. This is somewhat suggested by the fact that the antiviral activity is higher for kistamicin B, which contains a lipophilic substituent analogous to that of SA-19.

An alternative strategy would be to interfere with endosome acidification. Upon internalization and entry into early endosomes, influenza viruses undergo an initial acidification step to pH ~ 6. They then traffic to late endosomes, where further acidification to pH ~ 5 provides the trigger for fusion of the endosomal and viral membranes.¹³³ Acidification of the endosomes is accomplished by the cellular vacuolar proton ATPase (V-ATPase), which is potently and selectively inhibited by the macrolide antibiotics **bafilomycin A1** and **concanamycin A**. Both compounds block influenza virus entry when added within the first 10 min after infection.¹³⁴ A different type of V-ATPase inhibitor, the natural compound diphyllin, produced surprisingly potent and selective inhibition of influenza virus replication in cell culture.¹³⁵ Likewise, lysosomotropic weak bases such as ammonium chloride and chloroquine inhibit influenza virus entry by elevating the endosomal pH.^{118,136} **Chloroquine** shows in vitro anti-influenza virus activity at concentrations that can, based on data from its use for malaria prophylaxis, be reached in humans.¹³⁷ However, a double-blind, placebo-controlled efficacy trial concluded that chloroquine is unable to prevent influenza virus infection,¹³⁸ and this agrees with its failure to prevent influenza virus infection in mouse and ferret models.¹³⁹ Possibly, the chloroquine dose used in the clinical study may have been too low. This dose was estimated to produce blood concentrations in the range of the 50% antiviral concentrations in cell culture, and was selected so as to avoid any serious side effects.¹³⁸ Thus, although bafilomycin A1 and chloroquine represent excellent tools to examine the precise mechanism of influenza virus entry, their relevance for influenza therapy is limited.

As explained in the next part, the adamantane compounds amantadine and rimantadine block influenza A virus entry mainly by inhibiting the M2 proton channel. At higher (~100 μ M) concentrations, they raise the endosomal pH due to their basic character, thereby affecting HA-mediated fusion at low pH.¹⁴⁰ Hence, amantadine-resistant viruses selected in vitro can contain mutations in either the M2 or HA protein. Most of these HA substitutions render the HA less stable, and thus increase the pH at which fusion occurs. We recently observed that some H1N1 viruses such as the A/PR/8/34 strain are particularly sensitive to a subtle increase in the endosomal pH, as caused by newly synthesized amantadine analogues bearing different scaffold structures (Torres, unpublished data).

5. INHIBITION OF THE VIRAL M2 PROTON CHANNEL

A. Structure of the M2 Ion Channel

The low pH inside the endosomes activates the viral M2 proton channel that is embedded in the viral membrane, leading to transport of proton ions into the interior of the endosomally entrapped virus. As a result, the vRNPs become dissociated from the M1 matrix protein (the so-called “uncoating” event), and the viral genome is released.¹⁴¹

The M2 of influenza A virus (A/M2) is a short polypeptide of only 97 residues, assembled into a homotetrameric, integral membrane channel protein consisting of (i) a short unstructured N-terminal ectodomain (residues 1–24); (ii) a pore-forming transmembrane helix (residues

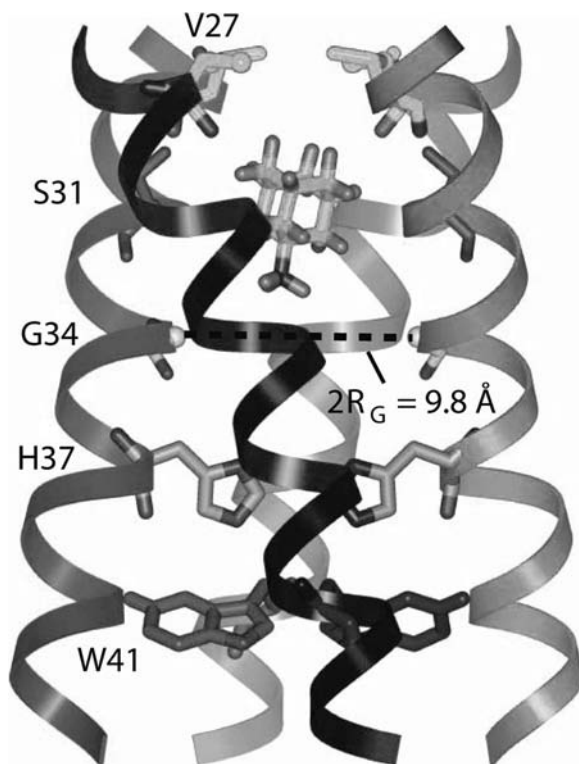


Figure 4. Solid-state NMR structure of amantadine-bound A/M2 proton channel in lipid bilayers. Side view showing the luminal site. His37 and Trp41 function as pH sensor and gate, respectively, while Val27 acts as a gatekeeper controlling the entrance of protons. The amantadine binding pocket is formed by Val27, Ala30, Ser31, and Gly34. Substitution of these residues causes amantadine resistance. [Reprinted by permission from Macmillan Publishers Ltd: Nature Ref. Cady et al.¹⁵⁵ © (2010).]

25–46) responsible for tetramerization and proton translocation; (iii) a cytoplasmic amphipathic helix (residues 47–61), involved in virus assembly and budding; and (iv) a disordered tail (residues 61–97) that interacts with the M1 matrix protein¹⁴¹ (Fig. 4). Activation of the M2 ion channel below pH 6 is caused by protonation of the third His37 residue in the M2 tetramer.^{142, 143} The protonated imidazole ring of His37 is involved in a cation– π interaction with the indole ring of Trp41.¹⁴² These two residues, His37 and Trp41, functioning as a pH sensor and gate, respectively, are critical for M2 proton channel function, and hence invariable among influenza A and B viruses.^{144, 145} Besides, Val27 forms a valve that controls the entrance of protons, while Asp44 is indirectly hydrogen bonded to the indole nitrogen of Trp41 via a water cluster at the exit of the channel. Thus, Asp44 and Val27 act as gatekeepers at opposite ends of the channel.^{146, 147} Comparison of the NMR and crystal structures of the A/M2 transmembrane domain obtained at neutral (pH 7.5–8), intermediate (pH 6.5), or acidic (pH 5) pH, provided a detailed insight into the low pH-induced changes in A/M2 protein conformation.^{146–148} At neutral pH, the Val27 valve is open, whereas the Trp basket, formed by the Trp41 residues at the opposing end, has a small hydrophobic opening. When the pH is reduced, the Val27 valve constricts, while the Trp basket opens.¹⁴⁷ Two mechanisms for proton transport through the aqueous pore of A/M2 have been proposed. In the wire model, protons are conducted via a continuous column of water molecules. Opening of the pore is achieved by electrostatic repulsion of the protonated His37 residues, which, according to this model, play only a passive role.^{142, 149} In contrast, in the shuttle model, His37 plays an active role in proton transport

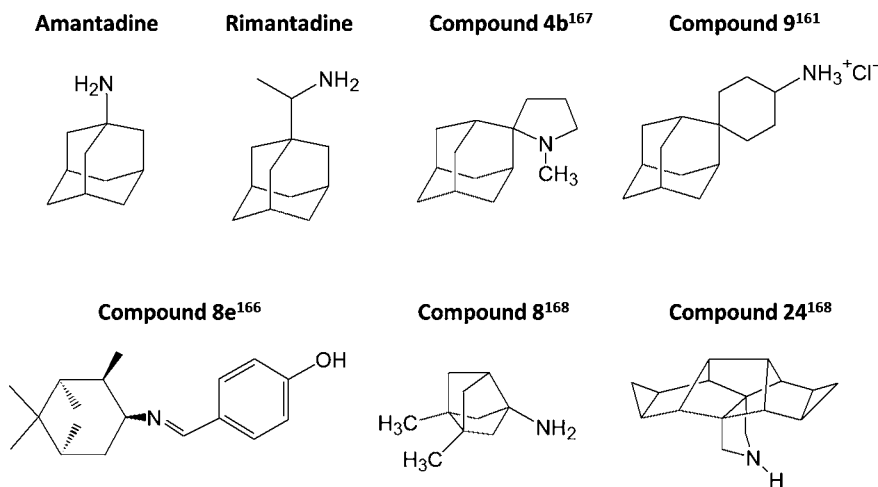


Figure 5. Chemical structures of amantadine, rimantadine, and a selection of published analogues. The codes shown are those used in the original reports. The spiro-adamantane compound 9¹⁶¹ possesses activity against mutant A/M2 ion channels. The imine compound 8e¹⁶⁶ and spiro compound 4b¹⁶⁷ are both ~200-fold more potent than amantadine. Compounds 8¹⁶⁸ and 24¹⁶⁸ are ring-contracted and ring-expanded polycyclic analogues, respectively.

by protonation and deprotonation, which is facilitated by imidazole ring reorientations and small-amplitude backbone fluctuations.^{150, 151}

In analogy to the A/M2 protein, BM2 (the M2 protein from influenza B virus) forms a homotetrameric integral membrane protein, with characteristic proton channel activity and a pH profile similar to that of its functional homolog A/M2. Due to its coiled-coil structure, the transmembrane region of BM2 is able to form a stable tetramer by itself, without the C-terminal amphipathic helix that is necessary for tetramerization of A/M2.¹⁵² Except for the HXXXW motif in the transmembrane domain, with the His and Trp acting as key residues for proton channel activation and gating, A/M2 and BM2 share little sequence homology. Furthermore, the BM2 proton channel activity is higher than that of A/M2.¹⁴⁴ This higher conductance may in part be explained by two extra serine residues in the channel pore of BM2, which can facilitate proton relay.¹⁵²

B. Inhibitors of the M2 Ion Channel

The discovery that the adamantane derivatives **amantadine** and **rimantadine** (Fig. 5) inhibit influenza A virus replication was made decades ago¹⁵³ and, in fact, was instrumental in elucidating the function of M2.¹⁵⁴ Both amantadine and rimantadine are inactive against influenza B viruses. Cocrystallization of amantadine with the transmembrane domain of A/M2 identified a drug binding site in the N-terminal channel lumen, that is surrounded by residues that are mutated in amantadine-resistant viruses (in particular, Val27, Ala30, Ser31, and Gly34). Binding of amantadine apparently leads to occlusion of the channel pore, but may also affect protonation of the critical His37 residue.¹⁴⁶ On the other hand, a solution NMR of the A/M2 channel in complex with rimantadine revealed four equivalent binding sites, located on the lipid-facing side of the channel, between adjacent helices near the Trp41 gate. In this way, binding of rimantadine could stabilize the closed state of the A/M2 tetramer.¹⁴⁸ Finally, solid-state NMR spectroscopy of A/M2 in phospholipid bilayers showed the existence of two amantadine binding sites: a high-affinity site in the N-terminal lumen, which is occupied by a single

amantadine molecule, and a low-affinity site at the C-terminal protein surface, which only becomes occupied at higher amantadine concentrations.¹⁵⁵ The presence of both binding sites was confirmed by molecular dynamics simulations, which further indicated that amantadine can bind inside the N-terminal lumen under low- and high-pH conditions.¹⁵⁶ Importantly, the identification of the A/M2 binding sites for amantadine and rimantadine provided an explanation why both compounds lack activity against influenza B viruses. Compared to A/M2, the BM2 pore has two more serine residues, which probably disable binding of the hydrophobic adamantane moiety within the BM2 channel.^{149,152} Also, the residues that make up the low-affinity binding site for amantadine in A/M2 have uncorrelated counterparts in the BM2 protein.¹⁵²

During many years, amantadine and rimantadine have been successfully used for both prophylaxis and therapy of influenza A virus infections, though amantadine is associated with neurological side effects.¹³ Nowadays, their clinical utility is limited since most circulating strains are adamantane-resistant.^{157–159} Thirty percent of treated patients shed adamantane-resistant mutants, which replicate equally well as wild-type virus, are cross-resistant to amantadine and rimantadine, and are readily transmitted to contact persons.^{13,159} During the 2009–2010 season, 99.9% of H1N1 virus isolates were adamantane-resistant.¹⁵⁷ The resistance mutations are mostly located in the transmembrane region of the A/M2 protein, the most common changes being Leu26Phe, Val27Ala, Ala30Thr, Ser31Asn, Gly34Glu, and Leu38Phe.¹⁶⁰

Attempts were made to develop new adamantane derivatives, which are able to interfere with the A/M2 ion channel activity of amantadine-resistant viruses. Guided by the novel structural insights into the A/M2 binding interaction of amantadine, Wang et al. recently developed spiro-adamantane inhibitors with potent activity against Val27Ala and Leu26Phe mutant A/M2 proteins.¹⁶¹ These molecules have a larger size than amantadine and are therefore able to fill the upper pore of A/M2, even when its volume is increased by the Val27Ala or Leu26Phe substitution. One compound (**9**¹⁶¹; Fig. 5) showed antiviral activity against the wild type as well as the A/M2-Val27Ala and A/M2-Leu26Phe mutant viruses, and its EC₅₀ values were similar to that of amantadine against the wild-type virus.¹⁶¹ An imidazole derivative of pinamine, synthesized by Zhao et al., showed moderate inhibition of an A/M2-Ser31Asn mutant virus.¹⁶²

Several research groups have developed polycyclic amine compounds to achieve more potent inhibitors of A/M2.^{163–165} Two fine examples are the imine compound **8e**¹⁶⁶ (Fig. 5) and the spiro compound **4b**¹⁶⁷ (Fig. 5), which were both reported to be ~200-fold more potent than amantadine. Although compound **8e**¹⁶⁶ was found to be cross-resistant to amantadine when evaluated against an A/M2 mutant virus, it can serve as a novel scaffold for the design of superior M2 blockers. Another study explored the size limits of polycyclic amine derivatives as potential A/M2 inhibitors.¹⁶⁸ Surprisingly, both ring-contracted (**8**¹⁶⁸ in Fig. 5) and ring-expanded (**24**¹⁶⁸ in Fig. 5) polycyclic compounds were able to bind to wild-type A/M2, and some analogues showed increased binding affinity compared to amantadine itself. Biochemical studies with mutant A/M2 proteins and molecular docking indicated that compared to amantadine, one of the ring-expanded derivatives showed a different binding mode to the high-affinity A/M2 binding site (i.e., the inner channel pore region delineated by Val27, Ala30, and Ser31).

Amantadine not only targets the A/M2 channel, but, as a weak base, also indirectly inhibits HA-mediated fusion at concentrations at least 100-fold higher. Thus, an alternative approach is to develop a compound reacting with both targets at similar concentrations. In this case, viral resistance would require the appearance of amino acid changes in two separate viral proteins, which may be expected to be a rare event. Replacement of the primary amino group of amantadine by a more basic secondary or tertiary amino group, and addition of side groups on the adamantane ring system, resulted in compounds interfering with HA at lower

concentrations, while the concentration affecting M2 proton channel activity was increased. However, during passage of the virus in the presence of these compounds, the escape rate was still high, yielding drug-resistant mutants with amino acid substitutions in both the HA and A/M2 proteins.¹⁶⁹ A reason for this high escape rate may be that the resistance mutations selected by these compounds can be located at different sites in HA or M2, without any reduction in viral fitness.

6. HA-MEDIATED MEMBRANE FUSION: AN EMERGING ANTIVIRAL TARGET

A. Low pH-Induced Fusion Mechanism

The low pH inside the late endosome leads to an extensive and irreversible conformational change of the viral HA, resulting in fusion of the viral and endosomal membranes (Fig. 2D). A key role is played by the fusion peptide (defined as the 23 N-terminal residues of HA2), which is the most conserved region of HA and contains a series of hydrophobic residues.^{170,171} At neutral pH, the fusion peptide is sequestered in a pocket of ionizable residues, but upon acidification, to pH 5–6 for most influenza viruses, the fusion peptide is extruded toward the target membrane. By comparing the X-ray crystallographic structures of the ectodomain portion of HA, obtained at either neutral or acidic pH, the following rearrangements were noted to occur at low pH: (i) the globular head domain containing the RBS detrimers; (ii) the N-terminus of the central triple-stranded coiled coil is extended by the interhelical chain and the short α -helix, hereby releasing the fusion peptide from its buried position; and (iii) in the middle of the long α -helix two turns undergo a helix-to-loop transition to form a 180° reverse turn, positioning the fusion peptide and viral membrane anchor at the same end.^{55,172} The actual membrane fusion proceeds through a hemifusion intermediate¹⁷³ (Fig. 2D). According to the stalk-pore model, the extruded fusion peptide inserts into the endosomal membrane. At the same time, the C-terminus of HA2, which is anchored in the viral membrane, is reoriented thereby drawing the endosomal and viral membranes together. After mixing of the outer membrane leaflets (prefusion stalk intermediate), a hemifusion diaphragm is formed. Mixing of the inner and outer membrane leaflets results in the formation of a fusion pore, allowing release of the vRNPs into the cytoplasm.¹⁷⁴

B. Inhibitors of HA-Mediated Membrane Fusion

1. Small Molecules Binding to the HA Stem

A first approach to interfere with the HA-mediated fusion process is to inhibit the acid-induced conformational change of HA, using small molecules that bind to and stabilize the neutral pH conformation. One of the first influenza virus fusion inhibitors to be reported was *tert*-butyl hydroquinone (TBHQ; Fig. 6), which specifically inhibits H3 viruses.¹⁷⁵ Several years later, the binding site of TBHQ within the H3 HA stem structure was identified by crystallization of the TBHQ-HA complex, and was shown to lie within a hydrophobic pocket, formed at an interface between HA subunits.⁵⁵ Besides several hydrophobic interactions, TBHQ is hydrogen bonded with the side chain carbonyl of Glu57₂ and the main chain carbonyl of Arg54₂ of one monomer, and the main chain carbonyl of Leu98₂ of another monomer, hereby stabilizing the nonfusogenic HA conformation.⁵⁵ During the conformational change of HA, a critical role is played by the adjacent Lys58₂, located at the C-terminus of the short α -helix and involved in the loop-to-helix transition.⁵⁵ The relevance of the hydrophobic pocket around Glu57₂ for the development of fusion inhibitors active against group 2 HAs was further confirmed by our studies with the novel anti-influenza virus agent **4c** (Fig. 6).¹⁷⁶ Although

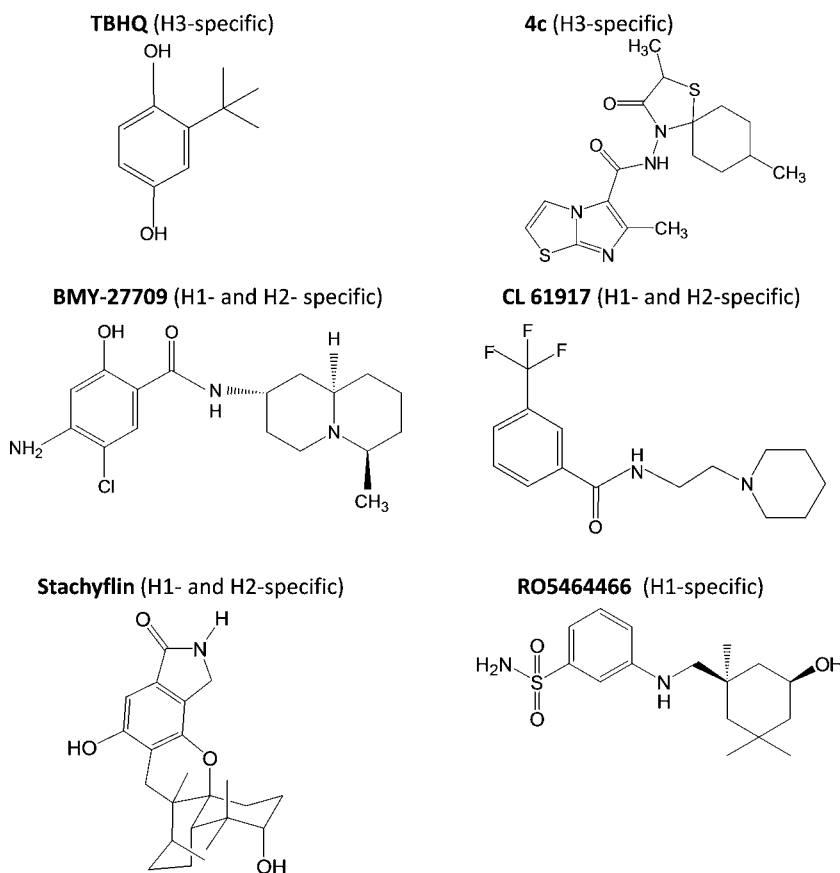


Figure 6. Chemical structures of small-molecule inhibitors of the HA conformational change. For each compound, the subtype specificity, as far as tested, is given in brackets. See the text for references on individual compounds.

4c and TBHQ have very different chemical structures, we noticed a clear similarity between the HA binding mode of TBHQ and that predicted for the *N*-(1-thia-4-azaspiro[4.5]decan-4-yl)carboxamide part of 4c. However, the aromatic imidazo[2,1-b]thiazole ring of 4c allows the formation of several additional hydrophobic interactions within this cavity. The inactivity of the two compounds against group 1 viruses can be explained by analysis of HA crystal structures, which revealed that residues 56₂–58₂ in group 1 HAs form an extra turn, resulting in blockage of the TBHQ/4c binding site.⁵⁵ Unfortunately, the antiviral activity of 4c is restricted to H3N2 viruses, since an H7N2 virus was shown to be insensitive, despite the fact that the H3 and H7 HAs belong to the same phylogenetic group 2. Also, resistance to 4c emerged within only three passages in cell culture.¹⁷⁶ Conversely, several fusion inhibitors targeting group 1 HAs have been reported in the literature, that is, **BMY-27709**, **CL-385319**, **RO5464466**, and **stachyflin** (see Fig. 6 for chemical structures), which inhibit the conformational change of H1 (and, when tested, H2) HA but, unfortunately, have no activity against H3 viruses.^{177–180} Attempts to override this subtype dependency by synthesizing novel derivatives proved unsuccessful.¹⁸¹ Also, initial predictions of their HA binding pocket using *in silico* docking did not fully correlate with subsequent data obtained after cocrystallization of the compound with HA or photoaffinity labeling.^{55,175,177,182} Whatever their virus specificity, these small molecule fusion inhibitors were all found to readily select for resistance, at least in cell culture. Two types of resistance mutations

have been identified. The first are amino acid substitutions within the binding pocket, which affect the inhibitor binding to HA. Alternatively, the HA stabilizing effect of the inhibitors can be counteracted by HA mutations that elevate the fusion pH, meaning that the mutant HA acquires its fusogenic conformation at less acidic pH.^{55,176}

Thus, further development of this type of small molecule fusion inhibitors has been hindered by their subtype-dependent anti-influenza virus activities and low barrier for resistance selection. There may, however, be other ways to inhibit the HA-mediated membrane fusion. Instead of preventing HA refolding, diiodofluorescein induces the irreversible conformational change of HA. These premature rearrangements, resulting in virions with fusion-inactive HAs, also lead to inhibition of the fusion process.¹⁸³

2. Antivirals Interfering with Membrane Fusion

Furthermore, it may be possible to interfere with membrane fusion following the HA refolding event. This mode of action has been proposed for **dextran sulfate**, a sulfated polysaccharide (Fig. 3) with broad-spectrum antiviral activity. This agent has been reported to inhibit not only influenza A virus, but also HIV, RSV, HSV, and cytomegalovirus.^{184–186} The anti-influenza virus activity of dextran sulfate, which appears to be restricted to influenza A viruses, correlates with its molecular weight, and levels off when the molecular weight increases above 10,000.¹⁸⁴ The anionic dextran sulfate can be assumed to form electrostatic interactions with the viral HA, which has a net positive charge at pH 7 or less.¹⁸⁷ This is consistent with fluorescence microscopy studies, showing the binding of fluorescein-labeled dextran sulfate to HA-expressing cells.¹⁸⁸ While dextran sulfate had no effect on virus binding at 4°C,¹⁸⁴ it was found to inhibit the low pH-induced fusion process using a fusion assay based on octadecyl-rhodamine fluorescence dequenching.¹⁸⁷ No direct inhibition of the acid-induced refolding of HA was noticed.¹⁸⁸ However, in order to be active, the compound needed to be present during the fusion process at low pH.^{187,188} These combined data suggest that the dextran sulfate binding site might be inaccessible in the low-pH HA-membrane complex and that dextran sulfate may interfere with a step subsequent to the conformational rearrangement of HA, for instance by causing steric hindrance of the membrane mixing event.^{188,189} It remains to be investigated whether other polysulfated polysaccharides with anti-influenza virus activity (such as compound pKG-03 that was isolated from a microalga¹⁹⁰) have a similar mode of action as dextran sulfate.

Another high molecular weight molecule, **retrocyclin 2**, also acts against a wide range of viruses, including influenza virus, HIV, and HSV.^{191–194} Retrocyclin 2 is a circular octadecapeptide belonging to the θ -defensins, which are antimicrobial peptides of the innate immune system.¹⁹⁵ A detailed mechanistic study showed that its inhibitory effect on influenza virus replication was based on prevention of the HA-mediated membrane fusion at low pH.¹⁹⁴ However, retrocyclin 2 remained effective when added after the conversion of HA to its fusogenic conformation or after hemifusion, an intermediate state in which the outer membrane leaflets have merged while the inner leaflets are still separated. Thus, retrocyclin 2 was proposed to prevent the subsequent membrane rearrangements by causing cross-linking and immobilization of surface glycoproteins.¹⁹⁴

A similar interference with the membrane fusion process probably accounts for the broad anti-influenza virus activity of **arbidol**. This small molecule (Fig. 3) has been licensed in Russia and China for influenza virus prophylaxis and therapy. Besides influenza A and B viruses, its antiviral spectrum encompasses RSV, parainfluenza virus, rhinovirus, hepatitis B virus, and HCV.¹⁹⁶ It is well tolerated as a drug and arbidol-resistant influenza viruses have not (yet) been isolated in the clinic.¹⁹⁷ However, arbidol-resistant viruses, obtained after 14 virus passages in cell culture, were shown to carry mutations in the HA2 subunit associated with an increased fusion pH.¹⁹⁷ Arbidol may thus act by stabilizing the prefusogenic HA protein

in a similar manner as TBHQ and the other small molecule fusion inhibitors described above, but, unlike the latter compounds, arbidol is less subtype-specific.¹⁹⁷ An alternative mode of action was proposed from biochemical studies with various model membranes, showing that arbidol has membranotropic properties, particularly due to its interaction with negatively charged membrane phospholipids.^{198,199} Since this membrane interaction is most pronounced at acidic (fusion) pH, arbidol could alter the membrane fluidity during the fusion process and make the bilayer less fusogenic.¹⁹⁶ Likewise, the inhibitory effect of arbidol toward HCV entry was explained by its capacity to dually interact with cell membrane phospholipids and aromatic residues (such as tryptophan) that are present in fusion-mediating glycoproteins of HCV. This complexation would prevent the conformational changes in the viral glycoprotein required for membrane fusion.^{196,198} At this time, a dual interaction of arbidol with membrane phospholipids and the influenza virus HA is merely speculative, but this mode of action would reconcile the biochemical and virological *in vitro* data outlined above. In the context of *in vivo* studies, arbidol may also have immunostimulatory properties by inducing interferon- α , activating phagocytic macrophages, or stimulating the humoral and cell-mediated immune response.²⁰⁰

3. Broad-Neutralizing Antibodies

As already explained, several reported fusion inhibitors suffer from subtype-dependent anti-influenza virus activity and rapid emergence of resistance. These drawbacks could be avoided by targeting the fusion peptide, which is highly conserved among all HAs and contains the 23 N-terminal residues of HA2. A monoclonal antibody directed against the first 15 residues of HA2 was selected from mice immunized with an H5N1 virus.²⁰¹ *In vitro*, this MAb **1C9** antibody inhibits syncytium formation in HA-expressing cells, indicating inhibition of the fusion process. When administered to mice, MAb 1C9 provided protection against H5N1, both prophylactically and therapeutically.²⁰¹ Though highly relevant, cross-reactivity with other HAs was not yet investigated.

A recent strategy with high clinical relevance comes from the discovery of broad neutralizing antibodies directed against relatively conserved pockets in the HA stem structure.²⁰² Already in 1993, the first antibody reacting with different HA subtypes was selected.²⁰³ This mouse monoclonal antibody, designated **C179**, was shown to neutralize the H1, H2, and H5 HAs, all belonging to group 1.^{203,204} Identification of the resistance mutations in C179-resistant viruses, obtained by virus passaging in the presence of this antibody, allowed to locate its binding site in the middle of the HA stem. C179 was proven to inhibit HA-mediated fusion in a polykaryon assay in influenza virus-infected cells,²⁰³ and was shown to be effective in H1N1- or H2N2-infected mice.²⁰⁵ The first human antibodies to be identified were specific for either group 1 or group 2 HAs, and were obtained by systematic screening of a wide array of B-cells from influenza-vaccinated or influenza-infected individuals, or by constructing combinatorial libraries. The antibodies **F10** and **CR6261** show broad neutralizing activity against group 1 HAs, and a partially overlapping binding pocket within the HA stem.^{206–208} Crystallization of F10 and CR6261 in complex with H1 or H5 HA revealed that a conserved hydrophobic tip on their HCDR2 region inserts into a hydrophobic pocket adjacent to the short α -helix in the HA stem, thereby allowing interactions of the antibody with the fusion peptide.^{206,207} Another monoclonal antibody, encoded **CR8020**, interacts with H3 and H7 HAs, which belong to group 2. Cocrystallization of CR8020 with H3 HA identified its binding pocket lower down the HA stalk, thus in closer proximity to the viral membrane compared to CR6261.²⁰⁹ Stabilization of the HA prefusogenic conformation by CR6261 and CR8020 was corroborated by the finding that both antibodies prevented exposure of protease-susceptible sites in HA when the virus was incubated at low pH.^{206,209}

Broad coverage of all influenza A viruses could be achieved by combining a group 1 and group 2 specific antibody. Further significant progress was made by Corti et al., who successfully isolated a pan-influenza A neutralizing antibody that recognizes all group 1 and group 2 HAs, by interrogating a large number (about 100,000) of donor plasma cells.²¹⁰ Cocrystallization of this **FI6** antibody with an H1 or H3 HA protein revealed its interaction with a conserved epitope in the F (fusion) subdomain.²¹⁰ Hence, binding of FI6 or the optimized FI6v3 antibody is assumed to increase the stability of the F subdomain, thus preventing the conformational change of HA that is required for membrane fusion. This mode of action accords with the inhibitory effect of FI6 on syncytium formation in HA-positive cells. Alternatively, prevention of HA0 cleavage (at least for viruses requiring extracellular cleavage) or cross-linking of HA subunits, have been implicated in the virus-neutralizing activity of FI6. Passive immunization with FI6 was shown to confer prophylactic and therapeutic protection to influenza virus-infected mice and ferrets.²¹⁰ Recently, Dreyfus et al. isolated the human monoclonal antibody **CR9114**, which neutralizes influenza A and B viruses.⁸⁶ CR9114 recognizes an epitope in the HA stem that is nearly identical to that of the group 1-specific antibody CR6261. However, subtle conformational differences explain why the CR9114 antibody has a much broader anti-HA reactivity.

Another HA stem-binding antibody, **PN-SIA28**, showed antiviral activity against all group 1 viruses tested (i.e., H1N1, H2N2, H5N1, and H9N2 viruses), as well as some isolates of the H3N2 virus, which belongs to group 2.²¹¹ However, H3N2 strains isolated after 1982 and H7N2 viruses were not inhibited. In order to localize the binding epitope for PN-SIA28 in the HA stem, the authors selected escape mutants by repeated passaging of the virus in the presence of the antibody. Similar attempts to generate escape mutants with some of the other broad neutralizing antibodies suggest that viruses with mutations in the corresponding HA stem regions do not readily emerge in cell culture. For instance, with the CR6261 antibody, ten virus passages were required,²⁰⁸ while in other studies, no escape mutants were detected.²¹² These observations seem to indicate that the conserved HA stem epitopes targeted by these broad acting antibodies are less prone to mutations due to fitness constraints. This hypothesis, however, still remains to be verified by mutational analysis. It is clear that the discovery of these broad neutralizing anti-HA stem antibodies offers entirely new perspectives for passive or active immunization against influenza A viruses. Also, peptides directed against the conserved epitopes in the HA stem region have been developed, such as the HB36 peptide that interacts with the CR6261-binding epitope and recognizes several group 1 HAs (i.e., H1, H2, H5, and H6).²¹³ The concept of a therapeutic peptide used to inhibit virus fusion is validated in the HIV field by the clinical use of enfuvirtide, a 36 amino acid peptide that binds to the HIV gp41 protein.²¹⁴

7. INHIBITION OF NP-MEDIATED VIRAL NUCLEAR IMPORT

After disruption of the vRNPs from the matrix M1 protein and fusion pore formation, the vRNPs are released in the cytoplasm and transported into the nucleus.^{215,216} How the vRNPs are released from M1 is only partially understood. In the intact virion, the M1 protein forms the capsid shell located underneath the envelope, and is tightly associated with the vRNPs.^{217,218} The current insights into the protein structure of M1 and its crucial role in organizing virion structure were recently reviewed.²¹⁹ Once inside the endosome, the M2-mediated acidification of the virion interior leads to vRNP uncoating, possibly by inducing a conformational change in M1.^{220,221} Recent studies indicate that the oligomerization state of M1 is pH-dependent and that oligomers of intact M1 dissociate into stable dimers at acidic pH.²²² The disappearance of a visible M1 layer in virions exposed to pH 5 was imaged by cryoelectron tomography.²²³ After their transport through the fusion pore, the vRNPs appear to be associated with some residual M1

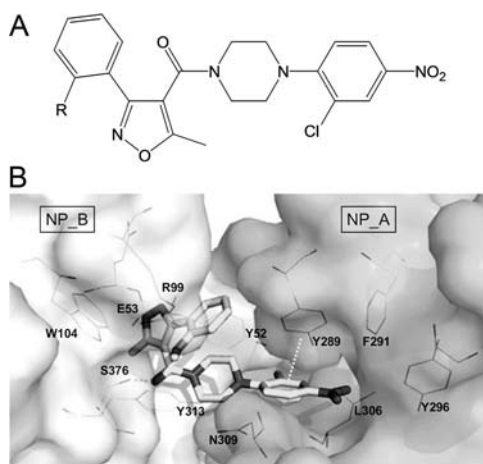


Figure 7. Chemical structure and NP-binding site of nucleozin. (A) Chemical structure of nucleozin (R = H),²³⁶ 3061 (R = Cl),²³⁸ and compound 3 (R = OMe).²³⁹ (B) X-ray structure of the oligomeric complex of compound 3 with influenza virus NP. Six molecules of compound 3 bridge two NP trimers (NP trimer A and NP trimer B) to form a hexamer. [Taken from Gerritz et al.,²³⁹ with permission.] Critical interactions made by compound 3 include a hydrogen bond with Ser376 and a π -stacking interaction with Tyr289.

protein, which, inside the cytoplasm, dissociates from the vRNPs to finally allow their nuclear entry.^{215,224} This second dissociation process may depend on cytosolic M1 modifications, such as phosphorylation or zinc binding.^{220,225} A peptide derived from the zinc finger domain of M1 was reported to display broad and potent anti-influenza virus activity in cell culture when added within 1 hr after infection, classifying this “peptide 6” as an entry inhibitor.²²⁶ As far as we know, no other attempts have been reported in which M1 was explored as an antiviral target. Development of a potent M1 inhibitor might be challenging, due to the abundant presence of this protein in the virion.

At last, the free vRNPs are imported in the nucleus via the nuclear pores. Each vRNP contains one of the eight vRNA genome segments, which is associated with a single copy of the viral polymerase (the heterotrimer of PB1, PB2, and PA), and multiple copies of the nucleoprotein (NP).²²⁷ Although these four viral proteins all contain at least one nuclear localization signal (NLS), the vRNP nuclear import appears to be primarily dependent on the NLS in the N-terminus of NP.^{228–230} Due to this NLS, the vRNP is recognized as a cargo by the cellular importin- α protein, and after formation of a ternary complex with importin- β , is transported into the nucleus.²³¹ The specificity of NP (and PB2) for the different isoforms of importin- α differs for avian and human viruses, implicating a role in influenza virus adaptation.^{231,232}

The viral NP has both structural and regulatory functions in influenza virus replication. Besides being the main structural component of the vRNPs, NP has a crucial role in the consecutive replicative stages, by regulating vRNP nuclear import; viral RNA transcription; and nuclear export (via interaction of NP with M1).²³³ The conserved protein sequence of NP further adds to its attractiveness as an antiviral target, since this implicates that NP inhibitors could be broadly active across the different virus subtypes.²³³ This is illustrated by the small molecule ingavirin, which inhibits influenza A and B viruses *in vitro* and *in vivo*.²³⁴ Ingavirin was reported to inhibit NP oligomerization and subsequent nuclear import of newly synthesized NP.²³⁵ This mechanism of action is distinct from that of the NP aggregating agents **nucleozin** and its structural analogues **3061** and “**compound 3**”, which were independently identified by several groups^{236–239} (Fig. 7A).

These NP binding agents display anti-influenza virus activity against all influenza A viruses tested, including H1N1, H3N2, and H5N1 viruses.^{238,239} In the first report, nucleozin was proven, by fluorescence microscopy, to cause NP aggregation and trap the vRNPs in a perinuclear halo.²³⁶ Nucleozin was also active in the cellular vRNP reconstitution assay, which directly measures the transcriptional activity of vRNP.²³⁶ This indicates that nucleozin not only interferes with nuclear entry, but also with other replicative processes in which NP is involved. The NP aggregating activity of nucleozin was confirmed by experiments in which NP was cocrystallized with the related “compound 3”.²³⁹ Formation of higher order NP oligomers was observed, in which two NP trimers are linked to each other through six molecules of “compound 3”, each interacting with two antiparallel binding pockets.²³⁹ The nitrophenyl moiety of “compound 3” interacts with one binding pocket (close to the NP residues Tyr289 and Asn309) in an NP from one trimer, while the isoxazole heterocycle binds to the other binding pocket (around the Tyr52 residue) of an NP in the other trimer, and vice versa²³⁹ (Fig. 7B). These data nicely agree with the resistance mutations identified in NP (i.e., Tyr52Cys/His, Tyr289His, and Asn309Lys) after virus passaging in the presence of these NP binding agents.^{236,238,239} Nucleozin showed a rather modest *in vivo* activity, protecting 50% of H5N1-infected mice.²³⁶ However, full protection of H5N1-infected mice was obtained with “compound 5”, a derivative of “compound 3” with improved solubility and metabolic stability.²³⁹

8. INTERFERING WITH CELLULAR FACTORS INVOLVED IN INFLUENZA VIRUS ENTRY

Although most available antiviral strategies are directed toward a viral protein, the possibility to block a cellular component with a critical role in virus replication receives increasing attention.²⁴⁰ An antiviral targeting a host cell factor can be assumed to have reduced selectivity (i.e., window between cytotoxicity and antiviral efficacy). On the other hand, its resistance barrier could (in theory) be higher when compared to a direct antiviral compound.²⁴¹ For a virus with a high mutation rate such as influenza virus, this appears a considerable advantage.²⁴²

Two studies using genome-wide RNA interference screening identified several host cell factors necessary for influenza virus replication.^{135,243} Further analyses, based on a pseudotyped particle entry assay, allowed the selection of cellular factors that regulate the low pH-dependent and HA-mediated entry. Among them are proteins involved in the IP3-protein kinase C (PKC) or phosphatidylinositol-3-kinase (PI3K)-Akt signaling pathways; COPI components (involved in endosomal trafficking); vacuolar ATPases; fibroblast growth factor receptor¹³⁵; and SON DNA binding protein (important for influenza virus trafficking to late endosomes).²⁴³ These intriguing data create new opportunities for designing antiviral concepts toward host cell factors.²⁴² In a proof-of-concept study, compounds such as sirolimus, podophyllotoxin, or other inhibitors of any of these host cell factors, were found to inhibit virus replication with quite remarkable selectivity.¹³⁵

The bisindolylmaleimide compounds specifically inhibit all PKC isoenzymes with a similar potency, by blocking the ATP-binding site on the catalytic domain of PKC.²⁴⁴ Bisindolylmaleimide I has been shown to interfere with influenza A and B virus replication early in infection, probably by affecting endocytosis or vesicle transport.²⁴⁵ The PKC β II isoform was proven to be critical for influenza virus entry, since accumulation of the virus in late endosomes was observed in cells expressing a phosphorylation-deficient form of PKC β II.²⁴⁶

The PI3K and its downstream effector Akt/protein kinase B are signaling mediators induced by influenza A virus, and their role in virus replication seems to be multifaceted.²⁴⁷ Upon virus attachment to the cell, PI3K is activated in a short and transient manner, promoting a step during virus entry that precedes early and late endosomal transport.^{123,247} At later

stages of infection, the influenza A virus nonstructural NS1 protein induces a second phase of sustained PI3K activation to prevent premature apoptosis during viral propagation. The influenza B virus NS1 protein apparently lacks this function.²⁴⁸ In contrast, accumulation of vRNA leads to PI3K activation in cells infected with influenza A or B viruses. Finally, PI3K signaling is also essential for efficient IRF-3 activation during type I interferon (IFN) induction.²⁴⁹ The antiviral effect of IFN is mediated by the interferon inducible transmembrane (IFITM) protein 3 and results in inhibition of viral genome release into the cytoplasm.²⁵⁰ Notwithstanding this complex function of PI3K, it was shown that inhibition of PI3K results in decreased influenza virus replication,²⁴⁷ which makes this pathway a potential antiviral target.

9. PERSPECTIVES

During the past years, significant advances have been made in unraveling the structure of the influenza virus proteins involved in virus entry, from its initial attachment to the sialylated receptors until its nuclear entry. The available crystal structures of HA, M2, and NP enable a rational and computer-aided design of directly acting antivirals. A few M2 inhibitors with activity against amantadine-resistant viruses have already been reported. The old paradigm that the HA appears too variable to be a valid antiviral target is challenged by broad-acting macromolecule inhibitors, such as lectins or antibodies interacting with a conserved site in the HA RBS or stem region. Whereas small molecules inhibiting the conformational change of HA have a restricting subtype dependency, this is not the case for compounds (such as arbidol), which interfere with the membrane fusion event itself. The recently discovered NP aggregating agent nucleozin represents an entirely novel class of anti-influenza agents with clinical relevance.

Complementary to these structural studies, much attention is currently given to the complex cell biology of the influenza virus entry pathway, with a particular interest in the host cell factors involved. Diverse compounds interfering with any of these cellular factors have been reported, setting the stage for a new type of indirectly acting antivirals with a higher barrier for resistance selection. In times of increasing resistance to oseltamivir, agents with a more favorable resistance profile are indeed urgently required.

ACKNOWLEDGMENTS

The authors acknowledge a grant from the Geconcerteerde Onderzoeksacties (GOA/10/014). They wish to thank W. van Dam, S. Stevens, L. Persoons, and F. De Meyer for their dedicated contribution to their research work.

REFERENCES

1. Monto AS. Epidemiology of influenza. *Vaccine* 2008;26(Suppl 4):D45–D48.
2. Schanzer DL, Langley JM, Tam TW. Hospitalization attributable to influenza and other viral respiratory illnesses in Canadian children. *Pediatr Infect Dis J* 2006;25:795–800.
3. Thompson WW, Comanor L, Shay DK. Epidemiology of seasonal influenza: use of surveillance data and statistical models to estimate the burden of disease. *J Infect Dis* 2006;194(Suppl 2):S82–S91.
4. Cocoros N, Hernandez R, Harrington N, Rausch-Phung E, Schulte CR, Blog D, Gallagher K, Klevos A, Kim C, Marano N, Alvarado-Ramy F. Estimates of deaths associated with seasonal influenza—United States, 1976–2007. *MMWR Morb Mortal Wkly Rep* 2010;59:1057–1062.

5. Osterhaus A, Fouchier R, Rimmelzwaan G. Towards universal influenza vaccines? *Philos Trans R Soc Lond B Biol Sci* 2011;366:2766–2773.
6. WHO. Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003–2012. http://www.who.int/influenza/human_animal_interface/consulted on 7-6-2012.
7. Palese P, Wang TT. H5N1 influenza viruses: facts, not fear. *Proc Natl Acad Sci USA* 2012;109:2211–2213.
8. Taubenberger JK, Morens DM. 1918 Influenza: the mother of all pandemics. *Emerg Infect Dis* 2006;12:15–22.
9. von Itzstein M. The war against influenza: discovery and development of sialidase inhibitors. *Nat Rev Drug Discov* 2007;6:967–974.
10. Das K, Aramini JM, Ma LC, Krug RM, Arnold E. Structures of influenza A proteins and insights into antiviral drug targets. *Nat Struct Mol Biol* 2010;17:530–538.
11. Krug RM, Aramini JM. Emerging antiviral targets for influenza A virus. *Trends Pharmacol Sci* 2009;30:269–277.
12. Lee SM, Yen HL. Targeting the host or the virus: current and novel concepts for antiviral approaches against influenza virus infection. *Antiviral Res* 2012;96:391–404.
13. Moscona A. Medical management of influenza infection. *Annu Rev Med* 2008;59:397–413.
14. von Itzstein M, Wu WY, Kok GB, Pegg MS, Dyason JC, Jin B, Van Phan T, Smythe ML, White HF, Oliver SW. Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature* 1993;363:418–423.
15. Eisenberg EJ, Bidgood A, Cundy KC. Penetration of GS4071, a novel influenza neuraminidase inhibitor, into rat bronchoalveolar lining fluid following oral administration of the prodrug GS4104. *Antimicrob Agents Chemother* 1997;41:1949–1952.
16. Collins PJ, Haire LF, Lin YP, Liu J, Russell RJ, Walker PA, Skehel JJ, Martin SR, Hay AJ, Gamblin SJ. Crystal structures of oseltamivir-resistant influenza virus neuraminidase mutants. *Nature* 2008;453:1258–1261.
17. Moscona A. Global transmission of oseltamivir-resistant influenza. *N Engl J Med* 2009;360:953–956.
18. van der Vries E, van den Berg B, Schutten M. Fatal oseltamivir-resistant influenza virus infection. *N Engl J Med* 2008;359:1074–1076.
19. Kiso M, Mitamura K, Sakai-Tagawa Y, Shiraishi K, Kawakami C, Kimura K, Hayden FG, Sugaya N, Kawaoka Y. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. *Lancet* 2004;364:759–765.
20. van der Vries E, Stelma FF, Boucher CA. Emergence of a multidrug-resistant pandemic influenza A (H1N1) virus. *N Engl J Med* 2010;363:1381–1382.
21. Thorlund K, Awad T, Boivin G, Thabane L. Systematic review of influenza resistance to the neuraminidase inhibitors. *BMC Infect Dis* 2011;11:134.
22. Shelton MJ, Lovern M, Ng-Cashin J, Jones L, Gould E, Gauvin J, Rodvold KA. Zanamivir pharmacokinetics and pulmonary penetration into epithelial lining fluid following intravenous or oral inhaled administration to healthy adult subjects. *Antimicrob Agents Chemother* 2011;55:5178–5184.
23. Fraaij PL, van der Vries E, Beersma MF, Riezebos-Brilman A, Niesters HG, van der Eijk AA, de Jong MD, Reis MD, Horrevorts AM, Ridwan BU, Wolfhagen MJ, Houmes RJ, van Dissel JT, Fouchier RA, Kroes AC, Koopmans MP, Osterhaus AD, Boucher CA. Evaluation of the antiviral response to zanamivir administered intravenously for treatment of critically ill patients with pandemic influenza A (H1N1) infection. *J Infect Dis* 2011;204:777–782.
24. Birnkrant D, Cox E. The Emergency Use Authorization of peramivir for treatment of 2009 H1N1 influenza. *N Engl J Med* 2009;361:2204–2207.
25. Chairat K, Tarning J, White NJ, Lindegardh N. Pharmacokinetic properties of anti-influenza neuraminidase inhibitors. *J Clin Pharmacol* 2013;53:119–139.

26. Watanabe A, Chang SC, Kim MJ, Chu DW, Ohashi Y. Long-acting neuraminidase inhibitor laninamivir octanoate versus oseltamivir for treatment of influenza: A double-blind, randomized, noninferiority clinical trial. *Clin Infect Dis* 2010;51:1167–1175.
27. Katsumi Y, Otabe O, Matsui F, Kidowaki S, Mibayashi A, Tsuma Y, Ito H. Effect of a single inhalation of laninamivir octanoate in children with influenza. *Pediatrics* 2012;129:e1431–e1436.
28. Vavricka CJ, Li Q, Wu Y, Qi J, Wang M, Liu Y, Gao F, Liu J, Feng E, He J, Wang J, Liu H, Jiang H, Gao GF. Structural and functional analysis of laninamivir and its octanoate prodrug reveals group specific mechanisms for influenza NA inhibition. *PLoS Pathog* 2011;7:e1002249.
29. Mohan S, McAtamney S, Haselhorst T, von Itzstein M, Pinto BM. Carbocycles related to oseltamivir as influenza virus group-1-specific neuraminidase inhibitors. Binding to N1 enzymes in the context of virus-like particles. *J Med Chem* 2010;53:7377–7391.
30. Kim JH, Resende R, Wennekes T, Chen HM, Bance N, Buchini S, Watts AG, Pilling P, Streltsov VA, Petric M, Liggins R, Barrett S, McKimm-Breschkin JL, Niikura M, Withers SG. Mechanism-based covalent neuraminidase inhibitors with broad spectrum influenza antiviral activity. *Science* 2013;340:71–75.
31. An J, Lee DC, Law AH, Yang CL, Poon LL, Lau AS, Jones SJ. A novel small-molecule inhibitor of the avian influenza H5N1 virus determined through computational screening against the neuraminidase. *J Med Chem* 2009;52:2667–2672.
32. Baranovich T, Wong SS, Armstrong J, Marjuki H, Webby RJ, Webster RG, Govorkova EA. T-705 (favipiravir) induces lethal mutagenesis in influenza A H1N1 viruses in vitro. *J Virol* 2013;87:3741–3751.
33. Furuta Y, Takahashi K, Shiraki K, Sakamoto K, Smee DF, Barnard DL, Gowen BB, Julander JG, Morrey JD. T-705 (favipiravir) and related compounds: Novel broad-spectrum inhibitors of RNA viral infections. *Antiviral Res* 2009;82:95–102.
34. Gamblin SJ, Skehel JJ. Influenza hemagglutinin and neuraminidase membrane glycoproteins. *J Biol Chem* 2010;285:28403–28409.
35. Tong S, Li Y, Rivaille P, Conrardy C, Castillo DA, Chen LM, Recuenco S, Ellison JA, Davis CT, York IA, Turmelle AS, Moran D, Rogers S, Shi M, Tao Y, Weil MR, Tang K, Rowe LA, Sammons S, Xu X, Frace M, Lindblade KA, Cox NJ, Anderson LJ, Rupprecht CE, Donis RO. A distinct lineage of influenza A virus from bats. *Proc Natl Acad Sci USA* 2012;109:4269–4274.
36. Zhu X, Yang H, Guo Z, Yu W, Carney PJ, Li Y, Chen LM, Paulson JC, Donis RO, Tong S, Stevens J, Wilson IA. Crystal structures of two subtype N10 neuraminidase-like proteins from bat influenza A viruses reveal a diverged putative active site. *Proc Natl Acad Sci USA* 2012;109:18903–18908.
37. Salomon R, Webster RG. The influenza virus enigma. *Cell* 2009;136:402–410.
38. Copeland CS, Doms RW, Bolzau EM, Webster RG, Helenius A. Assembly of influenza hemagglutinin trimers and its role in intracellular transport. *J Cell Biol* 1986;103:1179–1191.
39. Wilson IA, Skehel JJ, Wiley DC. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature* 1981;289:366–373.
40. Chen J, Lee KH, Steinhauer DA, Stevens DJ, Skehel JJ, Wiley DC. Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. *Cell* 1998;95:409–417.
41. Bertram S, Glowacka I, Steffen I, Kuhl A, Pohlmann S. Novel insights into proteolytic cleavage of influenza virus hemagglutinin. *Rev Med Virol* 2010;20:298–310.
42. Perdue ML, Garcia M, Senne D, Fraire M. Virulence-associated sequence duplication at the hemagglutinin cleavage site of avian influenza viruses. *Virus Res* 1997;49:173–186.
43. Stieneke-Grober A, Vey M, Angliker H, Shaw E, Thomas G, Roberts C, Klenk HD, Garten W. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. *EMBO J* 1992;11:2407–2414.
44. Becker GL, Lu Y, Hards K, Strehlow B, Levesque C, Lindberg I, Sandvig K, Bakowsky U, Day R, Garten W, Steinmetzer T. Highly potent inhibitors of proprotein convertase furin as potential drugs for treatment of infectious diseases. *J Biol Chem* 2012;287:21992–22003.

45. Kordyukova LV, Serebryakova MV, Baratova LA, Veit M. S acylation of the hemagglutinin of influenza viruses: mass spectrometry reveals site-specific attachment of stearic acid to a transmembrane cysteine. *J Virol* 2008;82:9288–9292.
46. Kobayashi Y, Suzuki Y. Evidence for N-glycan shielding of antigenic sites during evolution of human influenza A virus hemagglutinin. *J Virol* 2012;86:3446–3451.
47. Das SR, Puigbo P, Hensley SE, Hurt DE, Bennink JR, Yewdell JW. Glycosylation focuses sequence variation in the influenza A virus H1 hemagglutinin globular domain. *PLoS Pathog* 2010;6:e1001211.
48. Daniels R, Kurowski B, Johnson AE, Hebert DN. N-linked glycans direct the cotranslational folding pathway of influenza hemagglutinin. *Mol Cell* 2003;11:79–90.
49. Gallagher PJ, Henneberry JM, Sambrook JF, Gething MJ. Glycosylation requirements for intracellular transport and function of the hemagglutinin of influenza virus. *J Virol* 1992;66:7136–7145.
50. Ohuchi M, Ohuchi R, Feldmann A, Klenk HD. Regulation of receptor binding affinity of influenza virus hemagglutinin by its carbohydrate moiety. *J Virol* 1997;71:8377–8384.
51. Deshpande KL, Fried VA, Ando M, Webster RG. Glycosylation affects cleavage of an H5N2 influenza virus hemagglutinin and regulates virulence. *Proc Natl Acad Sci USA* 1987;84:36–40.
52. Ohuchi R, Ohuchi M, Garten W, Klenk HD. Oligosaccharides in the stem region maintain the influenza virus hemagglutinin in the metastable form required for fusion activity. *J Virol* 1997;71:3719–3725.
53. Wagner R, Heuer D, Wolff T, Herwig A, Klenk HD. N-Glycans attached to the stem domain of haemagglutinin efficiently regulate influenza A virus replication. *J Gen Virol* 2002;83:601–609.
54. Russell RJ, Gamblin SJ, Haire LF, Stevens DJ, Xiao B, Ha Y, Skehel JJ. H1 and H7 influenza haemagglutinin structures extend a structural classification of haemagglutinin subtypes. *Virology* 2004;325:287–296.
55. Russell RJ, Kerry PS, Stevens DJ, Steinhauer DA, Martin SR, Gamblin SJ, Skehel JJ. Structure of influenza hemagglutinin in complex with an inhibitor of membrane fusion. *Proc Natl Acad Sci USA* 2008;105:17736–17741.
56. Rogers GN, Paulson JC, Daniels RS, Skehel JJ, Wilson IA, Wiley DC. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. *Nature* 1983;304:76–78.
57. Imai M, Kawaoka Y. The role of receptor binding specificity in interspecies transmission of influenza viruses. *Curr Opin Virol* 2012;2:160–167.
58. Wilks S, de Graaf M, Smith DJ, Burke DF. A review of influenza haemagglutinin receptor binding as it relates to pandemic properties. *Vaccine* 2012;19;30:4369–4376.
59. Matrosovich MN, Gambaryan AS, Teneberg S, Piskarev VE, Yamnikova SS, Lvov DK, Robertson JS, Karlsson KA. Avian influenza A viruses differ from human viruses by recognition of sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binding site. *Virology* 1997;233:224–234.
60. Xu R, McBride R, Nycholat CM, Paulson JC, Wilson IA. Structural characterization of the hemagglutinin receptor specificity from the 2009 H1N1 influenza pandemic. *J Virol* 2012;86:982–990.
61. Stevens J, Blixt O, Glaser L, Taubenberger JK, Palese P, Paulson JC, Wilson IA. Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities. *J Mol Biol* 2006;355:1143–1155.
62. Xu R, McBride R, Paulson JC, Basler CF, Wilson IA. Structure, receptor binding, and antigenicity of influenza virus hemagglutinins from the 1957 H2N2 pandemic. *J Virol* 2010;84:1715–1721.
63. Gambaryan AS, Robertson JS, Matrosovich MN. Effects of egg-adaptation on the receptor-binding properties of human influenza A and B viruses. *Virology* 1999;258:232–239.
64. Wang Q, Tian X, Chen X, Ma J. Structural basis for receptor specificity of influenza B virus hemagglutinin. *Proc Natl Acad Sci USA* 2007;104:16874–16879.
65. Wang Q, Cheng F, Lu M, Tian X, Ma J. Crystal structure of unliganded influenza B virus hemagglutinin. *J Virol* 2008;82:3011–3020.

66. Chandrasekaran A, Srinivasan A, Raman R, Viswanathan K, Raguram S, Tumpey TM, Sasisekharan V, Sasisekharan R. Glycan topology determines human adaptation of avian H5N1 virus hemagglutinin. *Nat Biotechnol* 2008;26:107–113.
67. Gambaryan AS, Tuzikov AB, Piskarev VE, Yamnikova SS, Lvov DK, Robertson JS, Bovin NV, Matrosovich MN. Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6'-sialyl(N-acetyl)lactosamine. *Virology* 1997;232:345–350.
68. Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem* 2000;69:531–569.
69. Stray SJ, Cummings RD, Air GM. Influenza virus infection of desialylated cells. *Glycobiology* 2000;10:649–658.
70. Nicholls JM, Chan RW, Russell RJ, Air GM, Peiris JS. Evolving complexities of influenza virus and its receptors. *Trends Microbiol* 2008;16:149–157.
71. Taubenberger JK, Kash JC. Influenza virus evolution, host adaptation, and pandemic formation. *Cell Host Microbe* 2010;7:440–451.
72. Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, de Wit E, Munster VJ, Sorrell EM, Bestebroer TM, Burke DF, Smith DJ, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* 2012;336:1534–1541.
73. Kawaoka Y, Neumann G. Influenza viruses: an introduction. *Methods Mol Biol* 2012;865:1–9.
74. Sieben C, Kappel C, Zhu R, Wozniak A, Rankl C, Hinterdorfer P, Grubmuller H, Herrmann A. Influenza virus binds its host cell using multiple dynamic interactions. *Proc Natl Acad Sci USA* 2012;109:13626–13631.
75. Takemoto DK, Skehel JJ, Wiley DC. A surface plasmon resonance assay for the binding of influenza virus hemagglutinin to its sialic acid receptor. *Virology* 1996;217:452–458.
76. Knossow M, Skehel JJ. Variation and infectivity neutralization in influenza. *Immunology* 2006;119:1–7.
77. Luke TC, Kilbane EM, Jackson JL, Hoffman SL. Meta-analysis: convalescent blood products for Spanish influenza pneumonia: a future H5N1 treatment? *Ann Intern Med* 2006;145:599–609.
78. Simmons CP, Bernasconi NL, Suguitan AL, Mills K, Ward JM, Chau NV, Hien TT, Sallusto F, Ha do Q, Farrar J, de Jong MD, Lanzavecchia A, Subbarao K. Prophylactic and therapeutic efficacy of human monoclonal antibodies against H5N1 influenza. *PLoS Med* 2007;4:e178.
79. Whittle JR, Zhang R, Khurana S, King LR, Manischewitz J, Golding H, Dormitzer PR, Haynes BF, Walter EB, Moody MA, Kepler TB, Liao HX, Harrison SC. Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin. *Proc Natl Acad Sci USA* 2011;108:14216–14221.
80. Schmidt AG, Xu H, Khan AR, O'Donnell T, Khurana S, King LR, Manischewitz J, Golding H, Suphaphiphat P, Carfi A, Settembre EC, Dormitzer PR, Kepler TB, Zhang R, Moody MA, Haynes BF, Liao HX, Shaw DE, Harrison SC. Preconfiguration of the antigen-binding site during affinity maturation of a broadly neutralizing influenza virus antibody. *Proc Natl Acad Sci USA* 2012;110:264–269.
81. Bizebard T, Gigant B, Rigolet P, Rasmussen B, Diat O, Bosecke P, Wharton SA, Skehel JJ, Knossow M. Structure of influenza virus haemagglutinin complexed with a neutralizing antibody. *Nature* 1995;376:92–94.
82. Ekiert DC, Kashyap AK, Steel J, Rubrum A, Bhabha G, Khayat R, Lee JH, Dillon MA, O'Neil RE, Faynboym AM, Horowitz M, Horowitz L, Ward AB, Palese P, Webby R, Lerner RA, Bhatt RR, Wilson IA. Cross-neutralization of influenza A viruses mediated by a single antibody loop. *Nature* 2012;489:526–532.
83. Yoshida R, Igarashi M, Ozaki H, Kishida N, Tomabechi D, Kida H, Ito K, Takada A. Cross-protective potential of a novel monoclonal antibody directed against antigenic site B of the hemagglutinin of influenza A viruses. *PLoS Pathog* 2009;5:e1000350.

84. Lee PS, Yoshida R, Ekiert DC, Sakai N, Suzuki Y, Takada A, Wilson IA. Heterosubtypic antibody recognition of the influenza virus hemagglutinin receptor binding site enhanced by avidity. *Proc Natl Acad Sci USA* 2012;109:17040–17045.
85. Yasugi M, Kubota-Koketsu R, Yamashita A, Kawashita N, Du A, Sasaki T, Nishimura M, Misaki R, Kuhara M, Boonsathorn N, Fujiyama K, Okuno Y, Nakaya T, Ikuta K. Human monoclonal antibodies broadly neutralizing against influenza B virus. *PLoS Pathog* 2013;9:e1003150.
86. Dreyfus C, Laursen NS, Kwaks T, Zuijdgheest D, Khayat R, Ekiert DC, Lee JH, Metlagel Z, Bujny MV, Jongeneelen M, van der Vlugt R, Lamrani M, Korse HJ, Geelen E, Sahin O, Sieuwerts M, Brakenhoff JP, Vogels R, Li OT, Poon LL, Peiris M, Koudstaal W, Ward AB, Wilson IA, Goudsmit J, Friesen RH. Highly conserved protective epitopes on influenza B viruses. *Science* 2012;337:1343–1348.
87. Shadman KA, Wald ER. A review of palivizumab and emerging therapies for respiratory syncytial virus. *Expert Opin Biol Ther* 2011;11:1455–1467.
88. Vanlandschoot P, Stortelers C, Beirnaert E, Ibanez LI, Schepens B, Depla E, Saelens X. Nanobodies®: new ammunition to battle viruses. *Antiviral Res* 2011;92:389–407.
89. Ibanez LI, De Filette M, Hultberg A, Verrips T, Temperton N, Weiss RA, Vandeveldel W, Schepens B, Vanlandschoot P, Saelens X. Nanobodies with in vitro neutralizing activity protect mice against H5N1 influenza virus infection. *J Infect Dis* 2011;203:1063–1072.
90. van Eijk M, Bruinsma L, Hartshorn KL, White MR, Rynkiewicz MJ, Seaton BA, Hemrika W, Romijn RA, van Balkom BW, Haagsman HP. Introduction of N-linked glycans in the lectin domain of surfactant protein D: impact on interactions with influenza A viruses. *J Biol Chem* 2011;286:20137–20151.
91. Nayak A, Dodagatta-Marri E, Tsolaki AG, Kishore U. An insight into the diverse roles of surfactant proteins, SP-A and SP-D in innate and adaptive immunity. *Front Immunol* 2012;3:131.
92. Crouch E, Nikolaidis N, McCormack FX, McDonald B, Allen K, Rynkiewicz MJ, Cafarella TM, White M, Lewnard K, Leymarie N, Zaia J, Seaton BA, Hartshorn KL. Mutagenesis of surfactant protein D informed by evolution and x-ray crystallography enhances defenses against influenza A virus in vivo. *J Biol Chem* 2011;286:40681–40692.
93. van Eijk M, Rynkiewicz MJ, White MR, Hartshorn KL, Zou X, Schulten K, Luo D, Crouch EC, Cafarella TR, Head JF, Haagsman HP, Seaton BA. A unique sugar-binding site mediates the distinct anti-influenza activity of pig surfactant protein D. *J Biol Chem* 2012;287:26666–26677.
94. Hartshorn KL, Holmskov U, Hansen S, Zhang P, Meschi J, Mogues T, White MR, Crouch EC. Distinctive anti-influenza properties of recombinant collectin 43. *Biochem J* 2002;366:87–96.
95. Francois KO, Balzarini J. Potential of carbohydrate-binding agents as therapeutics against enveloped viruses. *Med Res Rev* 2012;32:349–387.
96. O’Keefe BR, Smee DF, Turpin JA, Saucedo CJ, Gustafson KR, Mori T, Blakeslee D, Buckheit R, Boyd MR. Potent anti-influenza activity of cyanovirin-N and interactions with viral hemagglutinin. *Antimicrob Agents Chemother* 2003;47:2518–2525.
97. Smee DF, Bailey KW, Wong MH, O’Keefe BR, Gustafson KR, Mishin VP, Gubareva LV. Treatment of influenza A (H1N1) virus infections in mice and ferrets with cyanovirin-N. *Antiviral Res* 2008;80:266–271.
98. Tate MD, Brooks AG, Reading PC. Specific sites of N-linked glycosylation on the hemagglutinin of H1N1 subtype influenza A virus determine sensitivity to inhibitors of the innate immune system and virulence in mice. *J Immunol* 2011;187:1884–1894.
99. Balzarini J. Targeting the glycans of glycoproteins: a novel paradigm for antiviral therapy. *Nat Rev Microbiol* 2007;5:583–597.
100. Sato T, Ishii M, Ohtake F, Nagata K, Terabayashi T, Kawanishi Y, Okahata Y. Binding affinity of GM3 lactone for influenza virus. *Glycoconj J* 1999;16:223–227.
101. Terabayashi T, Morita M, Ueno M, Nakamura T, Urashima T. Inhibition of influenza-virus-induced cytopathy by sialylglycoconjugates. *Carbohydr Res* 2006;341:2246–2253.

102. Suzuki Y, Nakao T, Ito T, Watanabe N, Toda Y, Xu G, Suzuki T, Kobayashi T, Kimura Y, Yamada A. Structural determination of gangliosides that bind to influenza A, B, and C viruses by an improved binding assay: strain-specific receptor epitopes in sialo-sugar chains. *Virology* 1992;189:121–131.
103. Hendricks GL, Weirich KL, Viswanathan K, Li J, Shriver ZH, Ashour J, Ploegh HL, Kurt-Jones EA, Fygenon DK, Finberg RW, Comolli JC, Wang JP. Sialylneolacto-N-tetraose c (LSTc)-bearing liposomal decoys capture influenza A virus. *J Biol Chem* 2013;288:8061–8073.
104. Matsubara T, Onishi A, Saito T, Shimada A, Inoue H, Taki T, Nagata K, Okahata Y, Sato T. Sialic acid-mimic peptides as hemagglutinin inhibitors for anti-influenza therapy. *J Med Chem* 2010;53:4441–4449.
105. Jeon SH, Kayhan B, Ben-Yedidia T, Arnon R. A DNA aptamer prevents influenza infection by blocking the receptor binding region of the viral hemagglutinin. *J Biol Chem* 2004;279:48410–48419.
106. Kimura K, Mori S, Tomita K, Ohno K, Takahashi K, Shigeta S, Terada M. Antiviral activity of NMSO3 against respiratory syncytial virus infection in vitro and in vivo. *Antiviral Res* 2000;47:41–51.
107. Vanderlinden E, Vanstreels E, Boons E, Ter Veer W, Huckriede A, Daelemans D, Van Lommel A, Roth E, Sztaricskai F, Herczegh P, Naesens L. Intracytoplasmic trapping of influenza virus by a lipophilic derivative of aglycoristocetin. *J Virol* 2012;86:9416–9431.
108. Terada M, Fujita S, Suda I, Mastico R. Polysulfated sialic acid derivatives as anti-human immunodeficiency virus. *Biomed Pharmacother* 2005;59:423–429.
109. Matsubara T, Sumi M, Kubota H, Taki T, Okahata Y, Sato T. Inhibition of influenza virus infections by sialylgalactose-binding peptides selected from a phage library. *J Med Chem* 2009;52:4247–4256.
110. Bhavanandan VP, Katlic AW. The interaction of wheat germ agglutinin with sialoglycoproteins. The role of sialic acid. *J Biol Chem* 1979;254:4000–4008.
111. Gottschalk A. On the mechanism underlying initiation of influenza virus infection. *Ergeb Mikrobiol Immunitätsforsch Exp Ther* 1959;32:1–22.
112. Malakhov MP, Aschenbrenner LM, Smee DF, Wandersee MK, Sidwell RW, Gubareva LV, Mishin VP, Hayden FG, Kim DH, Ing A, Campbell ER, Yu M, Fang F. Sialidase fusion protein as a novel broad-spectrum inhibitor of influenza virus infection. *Antimicrob Agents Chemother* 2006;50:1470–1479.
113. Moscona A, Porotto M, Palmer S, Tai C, Aschenbrenner L, Triana-Baltzer G, Li QX, Wurtman D, Niewiesk S, Fang F. A recombinant sialidase fusion protein effectively inhibits human parainfluenza viral infection in vitro and in vivo. *J Infect Dis* 2010;202:234–241.
114. Triana-Baltzer GB, Sanders RL, Hedlund M, Jensen KA, Aschenbrenner LM, Larson JL, Fang F. Phenotypic and genotypic characterization of influenza virus mutants selected with the sialidase fusion protein DAS181. *J Antimicrob Chemother* 2011;66:15–28.
115. Hedlund M, Aschenbrenner LM, Jensen K, Larson JL, Fang F. Sialidase-based anti-influenza virus therapy protects against secondary pneumococcal infection. *J Infect Dis* 2010;201:1007–1015.
116. Ison MG. Expanding the armamentarium against respiratory viral infections: DAS181. *J Infect Dis* 2012;206:1806–1808.
117. Mercer J, Schelhaas M, Helenius A. Virus entry by endocytosis. *Annu Rev Biochem* 2010;79:803–833.
118. Matlin KS, Reggio H, Helenius A, Simons K. Infectious entry pathway of influenza virus in a canine kidney cell line. *J Cell Biol* 1981;91:601–613.
119. Siczekarski SB, Whittaker GR. Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. *J Virol* 2002;76:10455–10464.
120. Rust MJ, Lakadamyali M, Zhang F, Zhuang X. Assembly of endocytic machinery around individual influenza viruses during viral entry. *Nat Struct Mol Biol* 2004;11:567–573.
121. de Vries E, Tscherne DM, Wienholts MJ, Cobos-Jimenez V, Scholte F, Garcia-Sastre A, Rottier PJ, de Haan CA. Dissection of the influenza A virus endocytic routes reveals macropinocytosis as an alternative entry pathway. *PLoS Pathog* 2011;7:e1001329.

122. Chu VC, Whittaker GR. Influenza virus entry and infection require host cell N-linked glycoprotein. *Proc Natl Acad Sci USA* 2004;101:18153–18158.
123. Eierhoff T, Hrincius ER, Rescher U, Ludwig S, Ehrhardt C. The epidermal growth factor receptor (EGFR) promotes uptake of influenza A viruses (IAV) into host cells. *PLoS Pathog* 2010;6:e1001099.
124. Harada S, Yokomizo K, Monde K, Maeda Y, Yusa K. A broad antiviral neutral glycolipid, fattiviracin FV-8, is a membrane fluidity modulator. *Cell Microbiol* 2007;9:196–203.
125. Harada S. The broad anti-viral agent glycyrrhizin directly modulates the fluidity of plasma membrane and HIV-1 envelope. *Biochem J* 2005;392:191–199.
126. Wolkerstorfer A, Kurz H, Bachhofner N, Szolar OH. Glycyrrhizin inhibits influenza A virus uptake into the cell. *Antiviral Res* 2009;83:171–178.
127. Pompei R, Paghi L, Ingianni A, Uccheddu P. Glycyrrhizic acid inhibits influenza virus growth in embryonated eggs. *Microbiologica* 1983;6:247–250.
128. Michaelis M, Geiler J, Naczk P, Sithisarn P, Ogbomo H, Altenbrandt B, Leutz A, Doerr HW, Cinatl J Jr. Glycyrrhizin inhibits highly pathogenic H5N1 influenza A virus-induced pro-inflammatory cytokine and chemokine expression in human macrophages. *Med Microbiol Immunol* 2010;199:291–297.
129. Wolf MC, Freiberg AN, Zhang T, Akyol-Ataman Z, Grock A, Hong PW, Li J, Watson NF, Fang AQ, Aguilar HC, Porotto M, Honko AN, Damoiseaux R, Miller JP, Woodson SE, Chantasirivivisai S, Fontanes V, Negrete OA, Krogstad P, Dasgupta A, Moscona A, Hensley LE, Whelan SP, Faull KF, Holbrook MR, Jung ME, Lee B. A broad-spectrum antiviral targeting entry of enveloped viruses. *Proc Natl Acad Sci USA* 2010;107:3157–3162.
130. Preobrazhenskaya MN, Olsufyeva EN. Polycyclic peptide and glycopeptide antibiotics and their derivatives as inhibitors of HIV entry. *Antiviral Res* 2006;71:227–236.
131. Naensens L, Vanderlinden E, Roth E, Jeko J, Andrei G, Snoeck R, Pannecouque C, Illyes E, Batta G, Herczegh P, Sztaricskai F. Anti-influenza virus activity and structure-activity relationship of aglycoristocetin derivatives with cyclobutenedione carrying hydrophobic chains. *Antiviral Res* 2009;82:89–94.
132. Naruse N, Tenmyo O, Kobaru S, Hatori M, Tomita K, Hamagishi Y, Oki T. New antiviral antibiotics, kistamicins A and B. I. Taxonomy, production, isolation, physico-chemical properties and biological activities. *J Antibiot (Tokyo)* 1993;46:1804–1811.
133. Lakadamyali M, Rust MJ, Zhuang X. Endocytosis of influenza viruses. *Microbes Infect* 2004;6:929–936.
134. Guinea R, Carrasco L. Requirement for vacuolar proton-ATPase activity during entry of influenza virus into cells. *J Virol* 1995;69:2306–2312.
135. Konig R, Stertz S, Zhou Y, Inoue A, Hoffmann HH, Bhattacharyya S, Alamares JG, Tscherne DM, Ortigoza MB, Liang Y, Gao Q, Andrews SE, Bandyopadhyay S, De Jesus P, Tu BP, Pache L, Shih C, Orth A, Bonamy G, Miraglia L, Ideker T, Garcia-Sastre A, Young JA, Palese P, Shaw ML, Chanda SK. Human host factors required for influenza virus replication. *Nature* 2010;463:813–817.
136. Yoshimura A, Kuroda K, Kawasaki K, Yamashina S, Maeda T, Ohnishi S. Infectious cell entry mechanism of influenza virus. *J Virol* 1982;43:284–293.
137. Ooi EE, Chew JS, Loh JP, Chua RC. In vitro inhibition of human influenza A virus replication by chloroquine. *Virol J* 2006;3:39.
138. Paton NI, Lee L, Xu Y, Ooi EE, Cheung YB, Archuleta S, Wong G, Wilder-Smith A. Chloroquine for influenza prevention: a randomised, double-blind, placebo controlled trial. *Lancet Infect Dis* 2011;11:677–683.
139. Vigerust DJ, McCullers JA. Chloroquine is effective against influenza A virus in vitro but not in vivo. *Influenza Other Respi Viruses* 2007;1:189–192.
140. Daniels RS, Downie JC, Hay AJ, Knossow M, Skehel JJ, Wang ML, Wiley DC. Fusion mutants of the influenza virus hemagglutinin glycoprotein. *Cell* 1985;40:431–439.
141. Wang J, Qiu JX, Soto C, DeGrado WF. Structural and dynamic mechanisms for the function and inhibition of the M2 proton channel from influenza A virus. *Curr Opin Struct Biol* 2011;21:68–80.

142. Okada A, Miura T, Takeuchi H. Protonation of histidine and histidine-tryptophan interaction in the activation of the M2 ion channel from influenza A virus. *Biochemistry* 2001;40:6053–6060.
143. Hu J, Fu R, Nishimura K, Zhang L, Zhou HX, Busath DD, Vijayvergiya V, Cross TA. Histidines, heart of the hydrogen ion channel from influenza A virus: toward an understanding of conductance and proton selectivity. *Proc Natl Acad Sci USA* 2006;103:6865–6870.
144. Mould JA, Paterson RG, Takeda M, Ohigashi Y, Venkataraman P, Lamb RA, Pinto LH. Influenza B virus BM2 protein has ion channel activity that conducts protons across membranes. *Dev Cell* 2003;5:175–184.
145. Balannik V, Carnevale V, Fiorin G, Levine BG, Lamb RA, Klein ML, DeGrado WF, Pinto LH. Functional studies and modeling of pore-lining residue mutants of the influenza A virus M2 ion channel. *Biochemistry* 2010;49:696–708.
146. Stouffer AL, Acharya R, Salom D, Levine AS, Di Costanzo L, Soto CS, Tereshko V, Nanda V, Stayrook S, DeGrado WF. Structural basis for the function and inhibition of an influenza virus proton channel. *Nature* 2008;451:596–599.
147. Acharya R, Carnevale V, Fiorin G, Levine BG, Polishchuk AL, Balannik V, Samish I, Lamb RA, Pinto LH, DeGrado WF, Klein ML. Structure and mechanism of proton transport through the transmembrane tetrameric M2 protein bundle of the influenza A virus. *Proc Natl Acad Sci USA* 2010;107:15075–15080.
148. Schnell JR, Chou JJ. Structure and mechanism of the M2 proton channel of influenza A virus. *Nature* 2008;451:591–595.
149. Pinto LH, Lamb RA. The M2 proton channels of influenza A and B viruses. *J Biol Chem* 2006;281:8997–9000.
150. Hu F, Schmidt-Rohr K, Hong M. NMR detection of pH-dependent histidine-water proton exchange reveals the conduction mechanism of a transmembrane proton channel. *J Am Chem Soc* 2012;134:3703–3713.
151. Hu F, Luo W, Hong M. Mechanisms of proton conduction and gating in influenza M2 proton channels from solid-state NMR. *Science* 2010;330:505–508.
152. Wang J, Pielak RM, McClintock MA, Chou JJ. Solution structure and functional analysis of the influenza B proton channel. *Nat Struct Mol Biol* 2009;16:1267–1271.
153. Davies WL, Grunert RR, Haff RF, McGahan JW, Neumayer EM, Paulshock M, Watts JC, Wood TR, Hermann EC, Hoffmann CE. Antiviral activity of 1-adamantanamine (amantadine). *Science* 1964;144:862–863.
154. Pinto LH, Holsinger LJ, Lamb RA. Influenza virus M2 protein has ion channel activity. *Cell* 1992;69:517–528.
155. Cady SD, Schmidt-Rohr K, Wang J, Soto CS, DeGrado WF, Hong M. Structure of the amantadine binding site of influenza M2 proton channels in lipid bilayers. *Nature* 2010;463:689–692.
156. Khurana E, Devane RH, Dal Peraro M, Klein ML. Computational study of drug binding to the membrane-bound tetrameric M2 peptide bundle from influenza A virus. *Biochim Biophys Acta* 2011;1808:530–537.
157. Gubareva LV, Trujillo AA, Okomo-Adhiambo M, Mishin VP, Deyde VM, Sleeman K, Nguyen HT, Sheu TG, Garten RJ, Shaw MW, Fry AM, Klimov AI. Comprehensive assessment of 2009 pandemic influenza A (H1N1) virus drug susceptibility in vitro. *Antivir Ther* 2010;15:1151–1159.
158. Belshe RB, Burk B, Newman F, Cerruti RL, Sim IS. Resistance of influenza A virus to amantadine and rimantadine: results of one decade of surveillance. *J Infect Dis* 1989;159:430–435.
159. Deyde VM, Xu X, Bright RA, Shaw M, Smith CB, Zhang Y, Shu Y, Gubareva LV, Cox NJ, Klimov AI. Surveillance of resistance to adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide. *J Infect Dis* 2007;196:249–257.
160. Pielak RM, Chou JJ. Flu channel drug resistance: a tale of two sites. *Protein Cell* 2010;1:246–258.

161. Wang J, Ma C, Fiorin G, Carnevale V, Wang T, Hu F, Lamb RA, Pinto LH, Hong M, Klein ML, DeGrado WF. Molecular dynamics simulation directed rational design of inhibitors targeting drug-resistant mutants of influenza A virus M2. *J Am Chem Soc* 2011;133:12834–12841.
162. Zhao X, Jie Y, Rosenberg MR, Wan J, Zeng S, Cui W, Xiao Y, Li Z, Tu Z, Casarotto MG, Hu, W. Design and synthesis of pinanamine derivatives as anti-influenza A M2 ion channel inhibitors. *Antiviral Res* 2012;96:91–99.
163. Hu W, Zeng S, Li C, Jie Y, Li Z, Chen L. Identification of hits as matrix-2 protein inhibitors through the focused screening of a small primary amine library. *J Med Chem* 2010;53:3831–3834.
164. Torres E, Duque MD, Lopez-Querol M, Taylor MC, Naesens L, Ma C, Pinto LH, Sureda FX, Kelly JM, Vazquez S. Synthesis of benzopolycyclic cage amines: NMDA receptor antagonist, trypanocidal and antiviral activities. *Bioorg Med Chem* 2012;20:942–948.
165. Zoidis G, Kolocouris N, Kelly JM, Prathalingam SR, Naesens L, De Clercq E. Design and synthesis of bioactive adamantanoinoalcohols and adamantanamines. *Eur J Med Chem* 2010;45:5022–5030.
166. Zhao X, Li C, Zeng S, Hu W. Discovery of highly potent agents against influenza A virus. *Eur J Med Chem* 2011;46:52–57.
167. Kolocouris N, Kolocouris A, Foscolos GB, Fytas G, Neyts J, Padalko E, Balzarini J, Snoeck R, Andrei G, De Clercq E. Synthesis and antiviral activity evaluation of some new aminoadamantane derivatives. 2. *J Med Chem* 1996;39:3307–3318.
168. Duque MD, Ma C, Torres E, Wang J, Naesens L, Juarez-Jimenez J, Camps P, Luque FJ, DeGrado WF, Lamb RA, Pinto LH, Vazquez S. Exploring the size limit of templates for inhibitors of the M2 ion channel of influenza A virus. *J Med Chem* 2011;54:2646–2657.
169. Scholtissek C, Quack G, Klenk HD, Webster RG. How to overcome resistance of influenza A viruses against adamantane derivatives. *Antiviral Res* 1998;37:83–95.
170. Skehel JJ, Bayley PM, Brown EB, Martin SR, Waterfield MD, White JM, Wilson IA, Wiley DC. Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. *Proc Natl Acad Sci USA*. 1982;79:968–972.
171. Carr CM, Kim PS. A spring-loaded mechanism for the conformational change of influenza hemagglutinin. *Cell* 1993;73:823–832.
172. Bullough PA, Hughson FM, Skehel JJ, Wiley DC. Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature* 1994;371:37–43.
173. Kemble GW, Danieli T, White JM. Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. *Cell* 1994;76:383–391.
174. Cross KJ, Langley WA, Russell RJ, Skehel JJ, Steinhauer DA. Composition and functions of the influenza fusion peptide. *Protein Pept Lett* 2009;16:766–778.
175. Bodian DL, Yamasaki RB, Buswell RL, Stearns JF, White JM, Kuntz ID. Inhibition of the fusion-inducing conformational change of influenza hemagglutinin by benzoquinones and hydroquinones. *Biochemistry* 1993;32:2967–2978.
176. Vanderlinden E, Goktas F, Cesur Z, Froeyen M, Reed ML, Russell CJ, Cesur N, Naesens L. Novel inhibitors of influenza virus fusion: structure-activity relationship and interaction with the viral hemagglutinin. *J Virol* 2010;84:4277–4288.
177. Luo G, Torri A, Harte WE, Danetz S, Cianci C, Tiley L, Day S, Mullaney D, Yu KL, Ouellet C, Dextraze P, Meanwell N, Colonna R, Krystal M. Molecular mechanism underlying the action of a novel fusion inhibitor of influenza A virus. *J Virol* 1997;71:4062–4070.
178. Zhu L, Li Y, Li S, Li H, Qiu Z, Lee C, Lu H, Lin X, Zhao R, Chen L, Wu JZ, Tang G, Yang W. Inhibition of influenza A virus (H1N1) fusion by benzenesulfonamide derivatives targeting viral hemagglutinin. *PLoS One* 2011;6:e29120.
179. Plotch SJ, O'Hara B, Morin J, Palant O, LaRocque J, Bloom JD, Lang SA Jr, DiGrandi MJ, Bradley M, Nilakantan R, Gluzman Y. Inhibition of influenza A virus replication by compounds interfering with the fusogenic function of the viral hemagglutinin. *J Virol* 1999;73:140–151.
180. Yoshimoto J, Kakui M, Iwasaki H, Fujiwara T, Sugimoto H, Hattori N. Identification of a novel HA conformational change inhibitor of human influenza virus. *Arch Virol* 1999;144:865–878.

181. Deshpande MS, Wei J, Luo G, Cianci C, Danetz S, Torri A, Tiley L, Krystal M, Yu KL, Huang S, Gao Q, Meanwell NA. An approach to the identification of potent inhibitors of influenza virus fusion using parallel synthesis methodology. *Bioorg Med Chem Lett* 2001;11:2393–2396.
182. Cianci C, Yu KL, Dischino DD, Harte W, Deshpande M, Luo G, Colonna RJ, Meanwell NA, Krystal M. pH-dependent changes in photoaffinity labeling patterns of the H1 influenza virus hemagglutinin by using an inhibitor of viral fusion. *J Virol* 1999;73:1785–1794.
183. Hoffman LR, Kuntz ID, White JM. Structure-based identification of an inducer of the low-pH conformational change in the influenza virus hemagglutinin: irreversible inhibition of infectivity. *J Virol* 1997;71:8808–8820.
184. Hosoya M, Balzarini J, Shigeta S, De Clercq E. Differential inhibitory effects of sulfated polysaccharides and polymers on the replication of various myxoviruses and retroviruses, depending on the composition of the target amino acid sequences of the viral envelope glycoproteins. *Antimicrob Agents Chemother* 1991;35:2515–2520.
185. Baba M, Snoeck R, Pauwels R, De Clercq E. Sulfated polysaccharides are potent and selective inhibitors of various enveloped viruses, including herpes simplex virus, cytomegalovirus, vesicular stomatitis virus, and human immunodeficiency virus. *Antimicrob Agents Chemother* 1988;32:1742–1745.
186. Ito M, Baba M, Sato A, Pauwels R, De Clercq E, Shigeta S. Inhibitory effect of dextran sulfate and heparin on the replication of human immunodeficiency virus (HIV) in vitro. *Antiviral Res* 1987;7:361–367.
187. Luscher-Mattli M, Gluck R. Dextran sulfate inhibits the fusion of influenza virus with model membranes, and suppresses influenza virus replication in vivo. *Antiviral Res* 1990;14:39–50.
188. Krumbiegel M, Dimitrov DS, Puri A, Blumenthal R. Dextran sulfate inhibits fusion of influenza virus and cells expressing influenza hemagglutinin with red blood cells. *Biochim Biophys Acta* 1992;1110:158–164.
189. Ramalho-Santos J, de Lima MC. Fusion and infection of influenza and Sendai viruses as modulated by dextran sulfate: a comparative study. *Biosci Rep* 2001;21:293–304.
190. Kim M, Yim JH, Kim SY, Kim HS, Lee WG, Kim SJ, Kang PS, Lee CK. In vitro inhibition of influenza A virus infection by marine microalga-derived sulfated polysaccharide p-KG03. *Antiviral Res* 2012;93:253–259.
191. Yasin B, Wang W, Pang M, Cheshenko N, Hong T, Waring AJ, Herold BC, Wagar EA, Lehrer RI. Theta defensins protect cells from infection by herpes simplex virus by inhibiting viral adhesion and entry. *J Virol* 2004;78:5147–5156.
192. Liang QL, Zhou K, He HX. Retrocyclin 2: a new therapy against avian influenza H5N1 virus in vivo and vitro. *Biotechnol Lett* 2010;32:387–392.
193. Cole AM, Hong T, Boo LM, Nguyen T, Zhao C, Bristol G, Zack JA, Waring AJ, Yang OO, Lehrer RI. Retrocyclin: a primate peptide that protects cells from infection by T- and M-tropic strains of HIV-1. *Proc Natl Acad Sci USA* 2002;99:1813–1818.
194. Leikina E, Delanoe-Ayari H, Melikov K, Cho MS, Chen A, Waring AJ, Wang W, Xie Y, Loo JA, Lehrer RI, Chernomordik LV. Carbohydrate-binding molecules inhibit viral fusion and entry by crosslinking membrane glycoproteins. *Nat Immunol* 2005;6:995–1001.
195. Selsted ME, Ouellette AJ. Mammalian defensins in the antimicrobial immune response. *Nat Immunol* 2005;6:551–557.
196. Teissier E, Zandomenighi G, Loquet A, Lavillette D, Lavergne JP, Montserret R, Cosset FL, Bockmann A, Meier BH, Penin F, Pecheur EI. Mechanism of inhibition of enveloped virus membrane fusion by the antiviral drug arbidol. *PLoS One* 2011;6:e15874.
197. Leneva IA, Russell RJ, Boriskin YS, Hay AJ. Characteristics of arbidol-resistant mutants of influenza virus: implications for the mechanism of anti-influenza action of arbidol. *Antiviral Res* 2009;81:132–140.
198. Pecheur EI, Lavillette D, Alcaras F, Molle J, Boriskin YS, Roberts M, Cosset FL, Polyak SJ. Biochemical mechanism of hepatitis C virus inhibition by the broad-spectrum antiviral arbidol. *Biochemistry* 2007;46:6050–6059.

199. Villalain J. Membranotropic effects of arbidol, a broad anti-viral molecule, on phospholipid model membranes. *J Phys Chem B* 2010;114:8544–8554.
200. Liu MY, Wang S, Yao WF, Wu HZ, Meng SN, Wei MJ. Pharmacokinetic properties and bioequivalence of two formulations of arbidol: an open-label, single-dose, randomized-sequence, two-period crossover study in healthy Chinese male volunteers. *Clin Ther* 2009;31:784–792.
201. Prabhu N, Prabakaran M, Ho HT, Velumani S, Qiang J, Goutama M, Kwang J. Monoclonal antibodies against the fusion peptide of hemagglutinin protect mice from lethal influenza A virus H5N1 infection. *J Virol* 2009;83:2553–2562.
202. Mancini N, Solforosi L, Clementi N, De Marco D, Clementi M, Burioni R. A potential role for monoclonal antibodies in prophylactic and therapeutic treatment of influenza. *Antiviral Res* 2011;92:15–26.
203. Okuno Y, Isegawa Y, Sasao F, Ueda S. A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. *J Virol* 1993;67:2552–2558.
204. Smirnov YA, Lipatov AS, Gitelman AK, Okuno Y, van Beek R, Osterhaus AD, Claas EC. An epitope shared by the hemagglutinins of H1, H2, H5, and H6 subtypes of influenza A virus. *Acta Virol* 1999;43:237–244.
205. Okuno Y, Matsumoto K, Isegawa Y, Ueda S. Protection against the mouse-adapted A/FM/1/47 strain of influenza A virus in mice by a monoclonal antibody with cross-neutralizing activity among H1 and H2 strains. *J Virol* 1994;68:517–520.
206. Ekiert DC, Bhabha G, Elsliger MA, Friesen RH, Jongeneelen M, Throsby M, Goudsmit J, Wilson IA. Antibody recognition of a highly conserved influenza virus epitope. *Science* 2009;324:246–251.
207. Sui J, Hwang WC, Perez S, Wei G, Aird D, Chen LM, Santelli E, Stec B, Cadwell G, Ali M, Wan H, Murakami A, Yammanuru A, Han T, Cox NJ, Bankston LA, Donis RO, Liddington RC, Marasco WA. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat Struct Mol Biol* 2009;16:265–273.
208. Throsby M, van den Brink E, Jongeneelen M, Poon LL, Alard P, Cornelissen L, Bakker A, Cox F, van Deventer E, Guan Y, Cinatl J, ter Meulen J, Lasters I, Carsetti R, Peiris M, de Kruif J, Goudsmit J. Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. *PLoS One* 2008;3(12):e3942.
209. Ekiert DC, Friesen RH, Bhabha G, Kwaks T, Jongeneelen M, Yu W, Ophorst C, Cox F, Korse HJ, Brandenburg B, Vogels R, Brakenhoff JP, Kompier R, Koldijk MH, Cornelissen LA, Poon LL, Peiris M, Koudstaal W, Wilson IA, Goudsmit J. A highly conserved neutralizing epitope on group 2 influenza A viruses. *Science* 2011;333:843–850.
210. Corti D, Voss J, Gamblin SJ, Codoni G, Macagno A, Jarrossay D, Vachieri SG, Pinna D, Minola A, Vanzetta F, Silacci C, Fernandez-Rodriguez BM, Agatic G, Bianchi S, Giacchetto-Sasselli I, Calder L, Sallusto F, Collins P, Haire LF, Temperton N, Langedijk JP, Skehel JJ, Lanzavecchia A. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science* 2011;333:850–856.
211. Clementi N, De Marco D, Mancini N, Solforosi L, Moreno GJ, Gubareva LV, Mishin V, Di Pietro A, Vicenzi E, Siccardi AG, Clementi M, Burioni R. A human monoclonal antibody with neutralizing activity against highly divergent influenza subtypes. *PLoS One* 2011;6:e28001.
212. Han T, Marasco WA. Structural basis of influenza virus neutralization. *Ann N Y Acad Sci* 2011;1217:178–190.
213. Fleishman SJ, Whitehead TA, Ekiert DC, Dreyfus C, Corn JE, Strauch EM, Wilson IA, Baker D. Computational design of proteins targeting the conserved stem region of influenza hemagglutinin. *Science* 2011;332:816–821.
214. Yu F, Lu L, Du L, Zhu X, Debnath AK, Jiang S. Approaches for identification of HIV-1 entry inhibitors targeting gp41 pocket. *Viruses* 2013;5:127–149.
215. Martin K, Helenius A. Transport of incoming influenza virus nucleocapsids into the nucleus. *J Virol* 1991;65:232–244.

216. Hutchinson EC, Fodor E. Nuclear import of the influenza A virus transcriptional machinery. *Vaccine* 2012;30:7353–7358.
217. Noton SL, Medcalf E, Fisher D, Mullin AE, Elton D, Digard P. Identification of the domains of the influenza A virus M1 matrix protein required for NP binding, oligomerization and incorporation into virions. *J Gen Virol* 2007;88:2280–2290.
218. Whittaker G, Bui M, Helenius A. The role of nuclear import and export in influenza virus infection. *Trends Cell Biol* 1996;6:67–71.
219. Rossman JS, Lamb RA. Influenza virus assembly and budding. *Virology* 2011;411:229–236.
220. Bui M, Whittaker G, Helenius A. Effect of M1 protein and low pH on nuclear transport of influenza virus ribonucleoproteins. *J Virol* 1996;70:8391–8401.
221. Helenius A. Unpacking the incoming influenza virus. *Cell* 1992;69:577–578.
222. Zhang K, Wang Z, Liu X, Yin C, Basit Z, Xia B, Liu W. Dissection of influenza A virus M1 protein: pH-dependent oligomerization of N-terminal domain and dimerization of C-terminal domain. *PLoS One* 2012;7:e37786.
223. Fontana J, Cardone G, Heymann JB, Winkler DC, Steven AC. Structural changes in Influenza virus at low pH characterized by cryo-electron tomography. *J Virol* 2012;86:2919–2929.
224. Bukrinskaya AG, Vorkunova NK, Kornilayeva GV, Narmanbetova RA, Vorkunova GK. Influenza virus uncoating in infected cells and effect of rimantadine. *J Gen Virol* 1982;60:49–59.
225. Elster C, Fourest E, Baudin F, Larsen K, Cusack S, Ruigrok RW. A small percentage of influenza virus M1 protein contains zinc but zinc does not influence in vitro M1-RNA interaction. *J Gen Virol* 1994;75:37–42.
226. Nasser EH, Judd AK, Sanchez A, Anastasiou D, Bucher DJ. Antiviral activity of influenza virus M1 zinc finger peptides. *J Virol* 1996;70:8639–8644.
227. Noda T, Sagara H, Yen A, Takada A, Kida H, Cheng RH, Kawaoka Y. Architecture of ribonucleoprotein complexes in influenza A virus particles. *Nature* 2006;439:490–492.
228. Ye Q, Krug RM, Tao YJ. The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA. *Nature* 2006;444:1078–1082.
229. Wu WW, Weaver LL, Pante N. Ultrastructural analysis of the nuclear localization sequences on influenza A ribonucleoprotein complexes. *J Mol Biol* 2007;374:910–916.
230. Cros JF, Garcia-Sastre A, Palese P. An unconventional NLS is critical for the nuclear import of the influenza A virus nucleoprotein and ribonucleoprotein. *Traffic* 2005;6:205–213.
231. O'Neill RE, Jaskunas R, Blobel G, Palese P, Moroiianu J. Nuclear import of influenza virus RNA can be mediated by viral nucleoprotein and transport factors required for protein import. *J Biol Chem* 1995;270:22701–22704.
232. Gabriel G, Klingel K, Otte A, Thiele S, Hudjetz B, Arman-Kalcek G, Sauter M, Shmidt T, Rother F, Baumgarte S, Keiner B, Hartmann E, Bader M, Brownlee GG, Fodor E, Klenk HD. Differential use of importin-alpha isoforms governs cell tropism and host adaptation of influenza virus. *Nat Commun* 2011;2:156.:156.
233. Portela A, Digard P. The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. *J Gen Virol* 2002;83:723–734.
234. Zarubaev V, Garshinina A, Kalinina N, Shtro A, Belyaevskaya S, Slita A, Nebolsin V, Kiselev O. Activity of Ingavirin (6-[2-(1H-Imidazol-4-yl)ethylamino]-5-oxohexanoic Acid) against human respiratory viruses in in vivo experiments. *Pharmaceuticals* 2011;4:1518–1534.
235. Semenova NP, Prokudina EN, Livov DK, Nebol'sin VE. Effect of the antiviral drug Ingavirin on intracellular transformations and import into the nucleus of influenza A virus nucleocapsid protein. *Vopr Virusol* 2010;55:17–20.
236. Kao RY, Yang D, Lau LS, Tsui WH, Hu L, Dai J, Chan MP, Chan CM, Wang P, Zheng BJ, Sun J, Huang JD, Madar J, Chen G, Chen H, Guan Y, Yuen KY. Identification of influenza A nucleoprotein as an antiviral target. *Nat Biotechnol* 2010;28:600–605.

237. Cheng H, Wan J, Lin MI, Liu Y, Lu X, Liu J, Xu Y, Chen J, Tu Z, Cheng YS, Ding K. Design, synthesis, and in vitro biological evaluation of 1H-1,2,3-triazole-4-carboxamide derivatives as new anti-influenza A agents targeting virus nucleoprotein. *J Med Chem* 2012;55:2144–2153.
238. Su CY, Cheng TJ, Lin MI, Wang SY, Huang WI, Lin-Chu SY, Chen YH, Wu CY, Lai MM, Cheng WC, Wu YT, Tsai MD, Cheng YS, Wong CH. High-throughput identification of compounds targeting influenza RNA-dependent RNA polymerase activity. *Proc Natl Acad Sci USA* 2010;107:19151–19156.
239. Gerritz SW, Cianci C, Kim S, Pearce BC, Deminie C, Discotto L, McAuliffe B, Minassian BF, Shi S, Zhu S, Zhai W, Pendri A, Li G, Poss MA, Edavettal S, McDonnell PA, Lewis HA, Maskos K, Mortl M, Kiefersauer R, Steinbacher S, Baldwin ET, Metzler W, Bryson J, Healy MD, Philip T, Zoeckler M, Schartman R, Sinz M, Leyva-Grado VH, Hoffmann HH, Langley DR, Meanwell NA, Krystal M. Inhibition of influenza virus replication via small molecules that induce the formation of higher-order nucleoprotein oligomers. *Proc Natl Acad Sci USA* 2011;108:15366–15371.
240. Watanabe T, Watanabe S, Kawaoka Y. Cellular networks involved in the influenza virus life cycle. *Cell Host Microbe* 2010;7:427–439.
241. Ludwig S. Targeting cell signalling pathways to fight the flu: towards a paradigm change in anti-influenza therapy. *J Antimicrob Chemother* 2009;64:1–4.
242. Shaw ML. The host interactome of influenza virus presents new potential targets for antiviral drugs. *Rev Med Virol* 2011;21:358–369.
243. Karlas A, Machuy N, Shin Y, Pleissner KP, Artarini A, Heuer D, Becker D, Khalil H, Ogilvie LA, Hess S, Maurer AP, Muller E, Wolff T, Rudel T, Meyer TF. Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication. *Nature* 2010;463:818–822.
244. Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J Biol Chem* 1991;266:15771–15781.
245. Root CN, Wills EG, McNair LL, Whittaker GR. Entry of influenza viruses into cells is inhibited by a highly specific protein kinase C inhibitor. *J Gen Virol* 2000;81:2697–2705.
246. Siczarski SB, Brown HA, Whittaker GR. Role of protein kinase C betaII in influenza virus entry via late endosomes. *J Virol* 2003;77:460–469.
247. Ehrhardt C, Marjuki H, Wolff T, Nurnberg B, Planz O, Pleschka S, Ludwig S. Bivalent role of the phosphatidylinositol-3-kinase (PI3K) during influenza virus infection and host cell defence. *Cell Microbiol* 2006;8:1336–1348.
248. Ehrhardt C, Wolff T, Pleschka S, Planz O, Beermann W, Bode JG, Schmolke M, Ludwig S. Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses. *J Virol* 2007;81:3058–3067.
249. Hrinčius ER, Dierkes R, Anhlan D, Wixler V, Ludwig S, Ehrhardt C. Phosphatidylinositol-3-kinase (PI3K) is activated by influenza virus vRNA via the pathogen pattern receptor Rig-I to promote efficient type I interferon production. *Cell Microbiol* 2011;13:1907–1919.
250. Feeley EM, Sims JS, John SP, Chin CR, Pertel T, Chen LM, Gaiha GD, Ryan BJ, Donis RO, Elledge SJ, Brass AL. IFITM3 inhibits influenza A virus infection by preventing cytosolic entry. *PLoS Pathog* 2011;7:e1002337.
251. Hamilton BS, Whittaker GR, Daniel S. Influenza virus-mediated membrane fusion: determinants of hemagglutinin fusogenic activity and experimental approaches for assessing virus fusion. *Viruses* 2012;4:1144–1168.

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