Antiviral strategies against influenza virus: towards new therapeutic approaches

Arianna Loregian[‡]*, Beatrice Mercorelli[‡], Giulio Nannetti, Chiara Compagnin, Giorgio Palù

Department of Molecular Medicine, University of Padua, via Gabelli 63, 35121 Padua, Italy

[‡]A Loregian and B. Mercorelli contributed equally to this work.

*Corresponding author: Tel.: +39 049 8272363; Fax: +39 049 8272355; E-mail: arianna.loregian@unipd.it

Running Title: New anti-influenza strategies

Abstract (147 words)

Influenza viruses are major human pathogens responsible for respiratory diseases affecting millions of people worldwide and characterized by high morbidity and significant mortality. Influenza infections can be controlled by vaccination and antiviral drugs. However, vaccines need annual updating and give limited protection. Only two classes of drugs are currently approved for the treatment of influenza: M2 ion channel blockers and neuraminidase inhibitors. However, they are often associated with limited efficacy and adverse side effects. In addition, the currently available drugs suffer from rapid and extensive emergence of drug resistance. All this highlights the urgent need for developing new antiviral strategies with novel mechanisms of action and with reduced drug resistance potential. Several new classes of antiviral agents targeting viral replication mechanisms or cellular proteins/processes are under development. This review gives an overview of novel strategies targeting the virus and/or the host cell for counteracting influenza virus infection.

Keywords: Influenza virus, new antivirals, drug discovery, drug targets, virus-host interaction, signaling pathways.

Introduction

Influenza viruses (IV) represent one of the major threats to public health, as they are responsible for both epidemics and pandemics characterized by high morbidity and mortality. During the past century, the pandemics of Spanish flu (1918), Asian flu (1957), Hong Kong flu (1968), bird flu (2005), and recently, swine flu (2009) caused millions of deaths worldwide [1]. In addition, the seasonal influenza epidemic results in hundreds of thousands of deaths per year (http://www.who.int/). IV belong to the *Orthomyxoviridae* family and include A, B, and C types, which differ in host range and pathogenicity. In particular, influenza A viruses (IAV) infect a wide range of avian and mammalian hosts, while influenza B viruses (IBV) infect almost exclusively humans. IAV are further classified into subtypes based on the antigenic properties of two viral surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA): 17 HA (H1–H17) and 10 NA (N1–N10) antigenic subtypes have been identified so far [2]. Within a subtype, different strains can arise as a result of point mutations; indeed, IAV evolve constantly and new mutant strains replace the old ones in a process known as "genetic drift".

IAV possess a single-stranded, eight-segmented RNA genome of negative polarity, which encodes the surface glycoproteins HA and NA and the M2 ion channel, that are all inserted into the viral lipid envelope; the matrix protein 1 (M1), that lies beneath the membrane; the three subunits (PB1, PB2, and PA) of the RNA polymerase complex, that is associated with the encapsidated genome; the nucleoprotein (NP), that coats the viral genome; and the nonstructural proteins NS1 and NS2/NEP [2]. In addition, most IAV encode a nonstructural PB1-F2 protein of varying length, which has pro-apoptotic functions [3], and PA-x and N40, two newly identified proteins encoded by the *PA* and *PB1* genes, respectively [4,5]. The IV replication cycle initiates with the attachment of HA to sialic acid (SA)-containing glycoprotein and glycolipid receptors on cell surface. The virus particle then enters the cell via clathrin-dependent endocytosis and macropinocitosis. Following entry, the acidic environment of the late endosome triggers a conformational change of HA which drives fusion of the viral envelope with the endosomal membrane. Moreover, the M2 protein creates a proton flow from the endosome into the virion leading to the dissociation of M1 from the viral ribonucleoprotein complexes (vRNPs). The released vRNPs are then transported into the nucleus, wherein the viral RNA polymerase initiates genome transcription and replication. Newly synthesized viral genome segments and proteins (PB1, PB2, PA, and NP) are complexed with M1 and NEP and then exported from the nucleus to the cell membrane for the final assembly and budding phases. Finally, NA cleaves terminal SA residues from HA and the cellular receptors permitting the release of virions from the cell. In addition to the viral proteins, there are a number of

cellular proteins involved in each stage of IV replication, which could represent potential antiviral targets.

The current options for influenza therapy include vaccination and two classes of antiviral compounds, the M2 ion channel blockers (adamantanes) and the NA inhibitors. However, vaccines need to be reformulated each year due to the genetic instability of the virus and are not always protective; thus, vaccination is unlikely to be effective against a rapidly emerging influenza pandemic. Adamantanes inhibit IAV replication by blocking virus entry. However, they have no activity against IBV and are often associated with serious side effects. NA inhibitors block the release of virions after budding from the host cell. They exhibit activity against both IAV and IBV but can also cause side effects. In addition, a major problem with both classes of drugs is the rapid emergence of drug-resistant viral strains which have limited the use of the NA inhibitors and rendered the M2 blockers ineffective. Thus, there is a clear need to discover novel IV inhibitors.

The necessity of developing new drugs to overcome resistance and counteract threats of sporadic outbreaks of pandemic IAV has fueled the interest in gaining a deeper knowledge of the structures and functions of the viral components. The body of information coming out of new research initiatives may have the potential to be developed into useful therapeutic strategies. In addition, targeting of cellular factors involved in IAV replication represents a novel antiviral approach that could counteract viral drug resistance, as resistance against host-targeted antivirals would likely not emerge as rapidly as it is for virus-targeted inhibitors. In this review, we present an overview of recent progress in designing and developing new antivirals to block critical steps of the viral life cycle by inhibiting functions of viral proteins and/or host-virus interactions.

New virus-based anti-influenza virus strategies

In this section, we will discuss current and new anti-influenza approaches from the point of view of targeting the virus itself. A number of different novel virus-based anti-influenza strategies are being developed, which include improving currently available drugs in potency, spectrum of activity, or route of delivery; discovering new classes of compounds that target different viral proteins; and the application of combination therapy. An overview of the viral proteins/processes that are blocked by current drugs and by new inhibitors under development is depicted in Fig. 1.

Antiviral strategies targeting the M2 ion channel

Influenza M2 is a homotetrameric protein that acts as a proton channel [6]. After virus endocytosis into the host cell, M2 is activated in response to the low pH in the endosomal lumen and creates a proton flux from the endosome into the virion core [6]. M2 is essential for viral replication and its

short N-terminal extracellular domain is highly conserved in all human IAV [7]. For these reasons, M2 is considered an excellent target for antiviral agents. Indeed, the M2 protein of IAV (A/M2) is the target of two already licensed drugs for influenza treatment: amantadine and its methyl derivative rimantadine [8,9]. Both these adamantane derivatives bind the N-terminal channel lumen of the M2 pore and, upon binding, their charged amino group produces a positive electrostatic potential in the channel lumen, which involves an electrostatic repulsion of protons and prevents virus uncoating [8]. Unfortunately, these compounds are inactive against IBV, due to the fact that there are significant differences in the amino acid sequence between the A/M2 and the M2 of IBV, except for the H37xxxW41 sequence motif in the transmembrane domain required for the channel activity and proton selectivity [6]. Moreover, the rapid emergence of drug-resistant virus variants represents the main limit of these drugs. Indeed, almost all currently circulating IAV are resistant to amantadine and rimantadine, greatly limiting their utility in the clinical practice [10]. Thus, new M2 blockers active against amantadine-resistant viruses are urgently needed.

Drug-resistance to amantadine and rimantadine is associated to single or multiple amino acid substitutions at positions 26, 27, 30, 31, or 34 in the transmembrane region of A/M2 located outside of the H37xxxW41 motif [11]. More than 95% of the reported transmissible IAV carry the S31N mutation in the trans-membrane region of A/M2 [12,13]. For this reason, the possibility to target the predominant S31N mutant represents an attractive challenge. Recently, some small molecules were identified as potent inhibitors of the A/M2-S31N variant. Among these compounds, M2WJ332 exhibited an antiviral activity against the A/M2-S31N variant higher than that of amantadine against the wild-type A/M2 [14]. In addition to M2WJ332, some benzyl-substituted amantadine derivatives were recently found to inhibit the activity of both S31N and wild-type viruses [14]. Other frequent mutations in the A/M2 protein that confer resistance to amantadine and rimantadine are L26F and V27A [11,15]. Recently, novel small molecules with inhibitory activity against A/M2 bearing these mutations have been reported, including the spiroadamantane 9 [16], the spiran amine 8 [17], and some organosilane-based compounds [18]. In addition, a number of studies have reported other compounds (e.g., imines, adamantanaminoalcohols, adamantanamines, and spiro-piperidine) with inhibitory effects against the wild-type M2 protein more potent than amantadine; however, these compounds do not show any activity against amantadine-resistant variants [19-21]. Finally, a neutralizing antibody directed against the A/M2 ion channel, M2-7A, which is able to inhibit the replication of both amantadine-sensitive and amantadine-resistant viruses with similar IC_{50} values, has been identified [22]. Importantly, passive immunotherapy with M2-7A protected mice from a lethal IV challenge [22]. Although the exact mechanism of action of

M2-7A and its binding epitope have not yet been clarified, this inhibitor clearly deserves further investigation.

To date, there is no single M2 blocker capable of targeting both wild-type IV and all circulating amantadine-resistant strains. Nevertheless, the therapy with a combination of these inhibitors could provide an effective strategy to solve the problem of amantadine resistance.

Antiviral strategies targeting the neuraminidase

An attractive target for the development of new anti-influenza drugs is provided by the viral neuraminidase. NA is a homotetrameric glycoside hydrolase that binds and removes a terminal SA residue to the adjacent oligosaccharide moiety of the cellular receptors recognized by HA, playing a key role in promoting IV infectivity (for a review see [23]). Indeed, NA is responsible for virus penetration through mucosal secretions, helping the virus to access to the target cells by mucus degradation [24]. Moreover, NA allows the detachment of the virion from infected cells and avoids the self-aggregation of progeny virions at late stages of infection by disrupting HA-SA interactions, thus promoting the release and spread of IV [23].

The proof-of-principle for the "druggability" of this target is represented by zanamivir (Relenza) and oseltamivir (Tamiflu) that are the first two inhibitors of NA licensed for the treatment of IAV and IBV infection [25,26]; however, the development and rapid spread of IV resistant to these drugs have limited their efficacy [27]. This has prompted the search for new anti-NA drugs. On the basis of the mechanism of action and the chemical features of the molecules, NA inhibitors can be distinguished into two groups: (i) synthetic analogues of SA and (ii) natural molecules and plant extracts with anti-NA activity.

The mechanism of SA analogues is based on the competition with the natural substrate of NA, resulting in a block of the enzyme active site. These compounds are active against both IAV and IBV thanks to the fact that the NA active site is highly conserved among different NA subtypes of IAV and also among IBVs [28,29]. However, these inhibitors are effective against influenza infection only if administrated within 36-48 h of symptoms onset [29]. Zanamivir and oseltamivir, currently used worldwide as therapeutic and prophylactic agents against IAV and IBV, belong to this class. Zanamivir (GG167) is a 4-deoxy-4-guanidino analogue of SA and was the first approved NA inhibitor [26]. This compound is administrated in patients at least 7 years old via inhalation, due to its poor oral bioavailability (less than 20%) [30]. Although zanamivir is well tolerated and has few adverse reactions, the route of administration of this drug can represent a problem especially for children and elderly patients that could not be able to inhale zanamivir suitably [31]. To circumvent these issues, an intravenous formulation of zanamivir has been formulated and is

currently in Phase III clinical trial. Oseltamivir (GS4104) is an ethyl ester prodrug, which is orally administrated and is quickly converted into its active form oseltamivir carboxylate (GS4071) by hepatic esterases. Oseltamivir possesses higher bioavailability (around 80%) than zanamivir and can be administrated in patients ≥ 1 year old [32]. More recently, other two SA analogues, peramivir and laninamivir, have been licensed in some Asian countries and are currently under clinical evaluation in other countries. Peramivir (BCX-1812, RWJ-270201) is a cyclopentane compound, approved in Japan as Rapiacta and in South Korea as Peramiflu for use in adult and pediatric patients with IAV and IBV infection [33]; in addition, it is currently undergoing clinical trials in the USA and in other countries. This compound is administrated only as an intravenous formulation, due to its very low bioavailability [34]. Laninamivir (R-125489) is a SA analogue structurally similar to zanamivir. Laninamivir is administrated as octanoyl prodrug, laninamivir octanoate (LO; CS-8958, R-118958), and holds great promise for its long-acting inhibitory activity [35]. LO has been approved in Japan for clinical use (as Inavir) since September 2010, but is still undergoing clinical trials in the USA.

Although the NA active site is a highly conserved target, a number of mutations in the NA of viruses selected in vitro in the presence of NA inhibitors and also in patients have been identified, namely substitutions of residues E105, E119, I122, Q136, D151, R152, D198, R224, S246, H274, R292, N294, and R371 [27]. These mutations map in framework or catalytic residues of NA and can directly or indirectly alter the shape of the active site structure, thus leading to lower efficiency in inhibitor binding ability [27]. Some mutations were found to confer resistance to certain NA inhibitors but preserve the susceptibility to others [27]; for example, the main mutation conferring resistance to oseltamivir (H274Y) also confers resistance to peramivir, but not to zanamivir [36]. In addition, due to the similar binding properties and chemical structures of zanamivir and laninamivir [37], it is expected that mutations causing resistance to zanamivir confer cross-resistance to laninamivir. Currently, no mutation associated with resistance to laninamivir has been identified and, although this finding remains to be confirmed, at the moment laninamivir remains the only NA inhibitor not subjected to drug resistance [27]. Based on NA crystallographic structure and knowledge of the binding mode in which these NA inhibitors interact with the enzyme active site [38], many other analogues of SA have been synthesized and characterized, in order to further optimize the binding properties of NA inhibitors. Work on novel anti-NA compounds obtained by structure-based drug design strategies is well summarized in other recent reviews [38,39].

An alternative and interesting approach to increase the efficacy of the approved NA drugs is provided by the use of multivalent inhibitors, by conjugating the compounds to a biocompatible

polymer [40,41]. Indeed, several reports showed that such multivalent presentation of NA inhibitors results in a dramatically higher antiviral potency than that obtained with the monomeric drug [41-43]. As an example, zanamivir attached to the biodegradable polymer poly-L-glutamine exhibited an antiviral activity 1,000- to 10,000-fold higher than that of monomeric zanamivir [41]. In addition, a single dose of zanamivir dimers resulted in an in vivo longer-lasting activity against IV compared to monomeric zanamivir [43].

In recent years, natural products have acquired increasing relevance in drug discovery, including as anti-influenza agents. Among natural products, many classes of compounds with promising anti-NA activity have been identified (reviewed in [44]); however, flavonoids are the most studied as NA inhibitors [44]. Flavonoids are a group of secondary plant metabolites containing 15 carbon atoms. In addition to antioxidant effects [45], they possess antiviral activity against a wide array of viruses, including IV [44], showing anti-NA activity [46-49]. In addition, plant extracts have also been evaluated for their inhibitory potential on NA, especially extracts from plants of traditional Chinese medicine [44]; however, often the mechanism of action and/or the active principle(s) have not been yet clarified [44]. As an example, a screening of extracts from many medicinal plants led to the identification of five plant extracts endowed with anti-NA activity in vitro and among these, the extract of *Melia toosendan* was found to be also active against IV in a mouse model [50].

Antiviral strategies targeting the hemagglutinin

HA is a homotrimeric glycoprotein composed of a stem domain supporting a globular head. Each HA monomer consists of two disulfide-linked polypeptides, HA1 and HA2, derived from proteolytic cleavage of the single immature precursor HA0 by host proteases. The most part of HAs are activated by cleavage at a single arginine residue by extracellular proteases, whereas for other HAs, associated to highly pathogenic avian viruses, this cleavage is performed by furin-like intracellular proteases at sites characterized by multiple basic amino acids. HA is essential for the interaction of the virus to cells by binding to SA receptors on host cells and also it is involved in the low pH-induced membrane fusion between the viral envelope and the endosomal membrane [23].

A possible mechanism of inhibition of viral infection is to block the interaction between viral surface molecules and cellular receptors and the following fusion of the virion with the endosome membranes, thus preventing the entry of the virus into the host cell. Some drugs already approved for the treatment of human immunodeficiency virus (HIV) infection (enfuvirtide and maraviroc) and for the prevention of respiratory syncytial virus (RSV) infection (palivizumab) specifically act by interfering with this step of the viral life cycle [51-53]. In the case of IV, the

entry step can be blocked mainly by two strategies: i) by preventing the binding between the viral HA and the terminal SA of glycoproteins and glycolipids present on the cell membrane; ii) by blocking the process of fusion between the viral envelope and endosomal membrane, necessary for the release of the vRNP into the cytoplasm.

As for the first strategy, a variety of antiviral agents have been reported that can interfere with IV attachment to target cells. These inhibitors can be divided in different groups based on chemical properties and their mechanism of action:

1) Neutralizing monoclonal antibodies (mAbs) directed against the membrane-distal globular head domain of HA. The globular head of HA contains three receptor binding sites (RBS) and is responsible for the attachment to cellular receptors [23], thus representing the principal target of these inhibitors. The main limit of this antiviral strategy is the hyper-variability of the globular head of HA. In fact, several antibodies that target regions of globular head of HA, especially the exposed loops that surround the RBS, are typically able to neutralize a limited range of IVs, often showing strain-specific responses [54,55]. Despite the overall variability of the globular head of HA, the RBS is relatively conserved; thus, antibodies against this site possess a broader spectrum of neutralizing activity. An example is represented by CH65, which is able to neutralize several strains of IV in vitro, by inserting its heavy-chain CDR3 loop into the receptor-binding pocket [55]. Recent studies reported a few examples of RBS-directed antibodies with heterosubtypic anti-influenza activity. In particular, two mAbs, S139/1 and C05, showed neutralization activity against strains from multiple IV subtypes both in vitro and in mice [56,57]. Crystal structures of the antibody–HA complex confirmed that both antibodies are able to bind highly conserved residues in the RBS of HA [56,58].

2*) Decoy receptor or SA-containing inhibitors.* This class of agents that can subdivided in: (i) polyvalent synthetic SA-containing inhibitors; (ii) natural inhibitors containing SA. Both these types of inhibitors act as receptor mimics and compete with sialylated receptors on the target cells for binding to HA and thus neutralize the virus attachment. In addition, their binding to the virion surface can lead to virus particle aggregation, causing a reduction of virus infectivity. As for the first type of inhibitors, the design of polyvalent synthetic SA-containing compounds has been proposed to overcome the low affinity of HA binding to monovalent SA analogues, which are considered ineffective in competing with the highly multivalent interactions between the virus and the host cell [59]. In fact, polyvalent SA-containing inhibitors were more potent in vitro than corresponding monovalent molecules [59]. Some of these molecules also exhibited protective effects against IV infection in mice [60,61]. As an alternative to multivalent sialosides, several groups have proposed the use of liposomes with SA analogues on the surface in order to allow a

multivalent presentation of SA [62-64]. The second type of inhibitors are constituted by SA-containing natural molecules, such as glycoproteins or proteoglycans, which possess the ability to bind HA and at the same time to create a steric obstacle to the polyvalent interaction of the virus with cells, thus blocking the process of virus absorption to target cells. An example of these inhibitors is represented by the serum amyloid P component, which contains the α(2,6)-linked SA into the oligosaccharide side chains. This sialylated glycoprotein has been reported to limit IAV infection of airway epithelial cells and to have also therapeutic effects in mice [65].

3) *Peptides against HA.* Some peptides exhibiting potent and broad-spectrum anti-influenza activity recently emerged as inhibitors of viral attachment [66,67]. Similar to antibodies, these peptides specifically bind the HA protein and prevent IV absorption to the host cell. These peptides were found to be effective not only in vitro but also in vivo, even when administered post-infection.

4) *Carbohydrate-binding agents that recognize specific glycosylation sites on HA.* The mechanism of action of these inhibitors exemplified by cyanovirin-N (CV-N). This protein, which derives from the cyanobacterium *Nostoc ellipsosporum*, recognizes high-mannose oligosaccharide structures on HA (oligomannose-8 and -9) and its binding to HA prevents virus adsorption to the cell [68]. In fact, removal of these glycans from HA causes a decrease of viral sensitivity to CV-N [68]. Interestingly, CV-N showed antiviral activity not only against IAV and IBV, but also against a broad range of enveloped viruses, such as HIV [69], Ebola virus [70], human herpesvirus 6 [69,70], and hepatitis C virus (HCV) [71]. The same mechanism seems to be used by a lectin from Green Alga *Boodlea coacta* (BCA) [72]. Indeed, BCA exhibited a strong inhibition of HA activity by specifically interacting with 1–2-linked mannose at the nonreducing terminus of HA [72]. In addition, BCA showed activity against HIV [72].

5) *Natural molecules from plants.* Finally, some natural molecules which interfere with the binding of HA to the cell surface have also been identified [73-76]. However, the molecular details of the mechanism of these compounds have not yet been elucidated.

Another possible strategy of inhibition of viral entry consists in preventing the fusion of the viral envelope with the endosomal membrane, in order to avoid the release of virion components into the cytoplasm. There is a heterogeneous group of inhibitors that act at this step of the viral life cycle, with distinct mechanisms of action:

1) Small molecules that inhibit the low pH-induced conformational change of HA. After binding to the cellular receptor, IV is internalized into endosomes by clathrin-independent endocytosis [77]. The low pH inside the late endosomes triggers an irreversible conformational change of HA and enables the extrusion of the fusion peptide and its consequent insertion into the endosomal membrane [78]. Several small molecules that bind pockets in the stem region of the native form of

HA have been identified as specific fusion inhibitors of IV [79-86]. Most of them prevent the fusogenic activity of the virus by blocking the low pH-induced conformational change of HA [79,81-86]. The most recent example of compounds acting by this mechanism is RO5464466, which stabilizes the neutral pH conformation of HA in a pre-fusogenic state and prevents the fusogenic change of HA [86]. In contrast, others compounds, such as C22, inhibit membrane fusion by destabilizing the structure of HA, resulting in a premature and ineffective conformational change [80,87]. However, these inhibitors possess common drawbacks that would severely limit their potential utility in the clinical practice. In fact, these small molecules show a high propensity to the emergence of drug-resistant variants and exhibit limited protection against different HA subtypes. An exception is represented by arbidol (ARB, 1-metyl-2-phenyl-thiomethyl-3-carbotoxy-4-dimetylaminomethyl-5-hydroxy-6-bromoindolehydrochloridemonohydrate). In fact, ARB exhibits a broad spectrum of antiviral activity not only against IAV and IBV, but also against other viruses such as RSV, parainfluenza virus, coxsackie virus, rhinovirus, hepatitis B virus (HBV), and HCV [87,88]. This drug has been approved in Russia and in China for treatment and prophylaxis of IAV and IBV. Studies with viruses resistant to ARB bearing mutations which map in the HA2 subunit confirmed that ARB interacts with HA and acts by stabilizing its structure, thus preventing the low pH-induced fusogenic change of HA [89]. Recently, biochemical studies showed that ARB dually interacts both with cell membrane phospholipids and with aromatic residues of viral glycoproteins on the surface of enveloped viruses [90]. This mechanism of action of ARB can prevent the fusogenic change in viral glycoproteins required for membrane fusion and could explain the broad spectrum of antiviral activity of this compound.

2) *Neutralizing mAbs directed against the stem region of HA.* Another possible approach to interfere with the fusogenic activity of HA is to develop mAbs directed against its highly conserved stem region, mostly formed by HA2 monomers. Thanks to the high conservation of this region, such a strategy could allow to overcome the problem of the high variability associated to the HA globular head. Recently, a number of studies reported antibodies that recognize conserved epitopes of the stem region of HA and show a broad antiviral activity against IAV both in vitro and in animal models [91-97]. One of these antibodies, CR9114, exhibited antiviral effects also against IBV both in vitro and in mice [92]. These observations suggest that the epitope recognized by CR9114 is highly conserved not only among the different IAV subtypes, but also among IBV and this finding opens the possibility to develop an universal influenza vaccine against IAV and IBV based on this epitope [92].

3) *Broad-spectrum antivirals interfering with membrane fusion by a nonspecific mechanism.* An additional approach is to block the fusion between virus and endosomal membrane in a step

subsequent to the conformational rearrangement of HA. High-molecular weight molecules can prevent the fusion between the virus and endosomal membrane by acting as a steric barrier. For their nonspecific - still not entirely clear - mechanism of action, these compounds are active against a wide range of viruses, including IV. Dextran sulfate and retrocyclin 2 represent two examples of this type of inhibitors. Dextran sulfate is a sulfated polysaccharide which exhibits inhibitory effects not only against IAV, but also against HIV, cytomegalovirus, herpes simplex virus (HSV), vesicular stomatitis virus, and RSV [98-100]. Thanks to its negatively charged sulfated/carboxyl groups, dextran sulfate interacts with HA, that has a net positive charge at pH ≤7 [101]. Biochemical studies highlighted that there is an inhibitory effect on membrane fusion only when dextran sulfate is added at early steps of the fusion process [102]. However, this compound does not seem to inhibit the low pH-dependent conformational change of HA, rather suggesting an inhibitory activity due to steric hindrance in the following step [102]. Other sulfated polysaccharides, such as iota-carrageenan and pKG-03 derived from the marine microalga *Gyrodinium impudium*, have been reported to possess anti-IV activity, although with an unknown mechanism of action [103,104]. Another broad-spectrum antiviral that can be included in this class of inhibitors is retrocyclin 2. This molecule belongs to the family of theta-defensins and is able to inhibit not only IV, but also HIV and HSV [105-108]. Retrocyclin 2 was found to inhibit the process of membrane fusion even when HA is in a fusogenic conformation or when a state of membrane hemifusion is already induced [106]. Detailed studies on its mechanism of action demonstrated that retrocyclin 2 creates a network of crosslinked and immobilized surface glycoproteins both on the virus and on the host cell, thus blocking the successive membrane rearrangements necessary to complete the fusion process [106].

Antiviral strategies targeting the RNA polymerase

The viral RNA-dependent RNA polymerase (RdRP) is a heterotrimer composed of subunits PB1, PB2, and PA, which carry out both mRNA transcription and replication of the viral genome. During transcription, in a process known as “cap-snatching” PB2 binds to the 5′ methyl cap of host pre-mRNA molecules and PA, which has endonuclease activity, cleaves the pre-mRNA to produce a capped primer that is used to start transcription [109]. The PB1 protein possesses the RNA-dependent RNA polymerase activity and it is also responsible for the addition of a poly(A) tail to viral mRNA. PB1 also catalyzes the genome replication, which occurs via a positive sense cRNA intermediate that is an exact copy of the vRNA [109]. The three polymerase subunits interact each other, in particular the N-terminus of PB1 interacts with the C-terminus of PA [110-112], while the C-terminus of PB1 binds the N-terminus of PB2 [110,113]; in addition, a weak transient interaction

has been proposed for PA and PB2 [114]. Thanks to its multidomain structure and multiple enzymatic activities, the RdRP can be targeted at different sites.

While a number of nucleoside/nucleotide drugs have been developed against other viral polymerases and are commonly used for treating infections caused by HIV, HBV, and herpesviruses, very few compounds have been reported which target the polymerization activity of IAV RdRP. This is in part due to the fact that the RdRP active site of PB1 has not yet been structurally characterized and even the precise boundaries of this domain are not known. Almost twenty years ago, 2'-deoxy-2'-fluoroguanosine was described as an inhibitor of IV transcription, but it has not been further developed [115]. Ribavirin (1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide, RBV) is a nucleoside analogue that exhibits a broad antiviral activity against RNA and DNA viruses, including IV [116]. RBV is converted intracellularly into its monophosphate form, which inhibits the cellular enzyme inosine 5′-monophosphate (IMP) dehydrogenase, responsible for the conversion of IMP to xanthosine monophosphate during GTP synthesis, leading to inhibition of RNA synthesis [116]. The triphosphate form of RBV has been shown to interact with the IV RNA polymerase in a cell-free system [117]. However, despite evidence of in vitro antiviral activity of RBV against IAV RdRP, its clinical application for anti-influenza therapy has been rather limited, due to toxicity and poor in vivo efficacy. Indeed, variable results have been reported from clinical trials using oral or aerosolized [118] and intravenous [119] RBV for treating IAV- or IBV-infected patients. Thus, while RBV is currently approved for therapy against HCV and RSV, its clinical efficacy for the treatment of IV infection remains to be further investigated. In addition, an analogue of RBV - viramidine - was recently shown to have similar efficacy to RBV against IAV infections while exhibiting lower toxicity and thus may deserve further evaluation as a possible therapeutic agent [120]. Another anti-influenza nucleoside analogue is favipiravir (T-705; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide), which is a pyrazine derivative first identified in 2002 [121]. T-705 was shown to inhibit influenza A, B, and C viruses in vitro and to be more effective than oseltamivir in protecting mice infected with IAV [121]. By cellular kinases, T-705 is converted to the active form, ribofuranosyl triphosphate, which acts as a nucleoside inhibitor of IV RdRP [122]. Remarkably, fapiravir does not inhibit the synthesis of cellular RNA or DNA and in contrast to RBV, it is not an effective inhibitor of IMP dehydrogenase, thus showing less cytotoxity than RBV [123]. T-705 is active against a broad range of IAV and IBV strains, including 2009 pandemic strains, highly pathogenic avian H5N1 viruses, and the recently emerged H7N9 avian virus, and it also inhibits influenza strains resistant to current antiviral drugs [123,124]. Besides IV, T-705 inhibits a number of other RNA viruses, whereas it exhibits no inhibitory effect against DNA viruses. Importantly, very limited resistance to favipiravir has been reported [123]. A Phase III

clinical trial for evaluating favipiravir for influenza therapy began in Japan in October 2009 and has been completed, and two Phase II studies have been conducted in the United States since February 2010 and the results are being reviewed [124]. In the near future, we may therefore see approval of clinical use of favipiravir for the treatment of influenza.

Another possible strategy for selectively inhibiting IV replication is to target the endonuclease cap-snatching activity of the RdRP complex, which resides in the N-terminal region of PA [125,126]. In past years, a number of inhibitors (e.g., flutimide and L-735882) of PA endonuclease activity have been discovered by Roche, Merck, and other pharmaceutical companies using a structure-activity relationships (SAR) approach [127-129], but most of them have not been developed further. More recently, crystallographic studies revealed that the endonuclease active site of PA contains a conserved deep cleft which could be an excellent target for structure-based design of novel anti- IV drugs [125,126]. On this line, two groups reported co-crystal structures of the PA endonuclease domain with known or predicted inhibitors [130,131], providing insights that could be useful for the structure-based design of new PA inhibitors. In addition, a fragment screening using a high-resolution crystal structure of the N-terminal endonuclease domain of pandemic 2009 H1N1 IV and structure-based optimization led to the identification of a hydroxypyridinone series of compounds exhibiting promising enzymatic inhibition; a compound from this series was also found to have a significant antiviral activity in cells [132]. Using a different approach, Iwai et al. screened 33 different types of phytochemicals using a PA endonuclease inhibition assay in vitro and identified marchantins as PA inhibitors [133]. In particular, marchantin E docked well into the endonuclease active site and inhibited the growth of both AV and IBV.

The cap-binding activity of PB2 might also be targeted by anti- IV agents. The crystal structure of the PB2 cap-binding domain (residues 318–483) bound to a 5′-cap analogue (m_7GTP) [134] revealed a pocket in this domain of PB2 which could be a possible drug target. An m7GTP-mimic, if developed, would inhibit the transcription of IV mRNAs, but most likely would also be recognized by cellular cap-binding proteins, thus posing significant selectivity and cytotoxicity issues. Another target for the development of anti-PB2 compounds may be the packaging signal at the 5’ end of the PB2 RNA. In fact, a recent study reported that a 15-mer phosphorothioate oligonucleotide derived from the 5’ end of the viral *PB2* RNA, complementary to the 3’ end of its coding region (nucleotides 2279–2293), markedly inhibited IV replication [135]. However, the suitability of this type of inhibitors for in vivo anti-influenza therapy remains to be investigated.

The interactions between the PA and PB1 as well as the PB1 and PB2 subunits have been shown to be essential for polymerase function [136,137]. In addition, the subunits binding interfaces are highly conserved between different viral strains [2]. Thus, inhibition of these interactions

represents an attractive strategy for the development of drugs with broad efficacy against all IV strains [138,139]. The feasibility of this approach was first proved by studies showing that short N-terminal PB1 peptides, corresponding to the PA-binding domain of PB1, were able to block the activity of IAV polymerase and also inhibit viral replication [140,141]. Recently, two crystal structures of a truncated form of PA bound to a PB1-derived peptide have been published [142,143]. Importantly, these structures showed that relatively few residues drive binding of PB1 to PA, suggesting the potential for small molecule-mediated inhibition. On this line, an in silico screening of 3 million small-molecule structures using one of these crystal structures [142] led to the identification of two compounds (compounds 1 and 5) able to interfere with the interaction between PB1 and PA both in vitro and in cells, as well as transcription by the RdRP [144]. One of these molecules (compound 1) also inhibited the replication of a panel of IAV strains, including 2009 pandemic strains and an oseltamivir-resistant isolate, as well as several IBV strains, with EC_{50} values in the low micromolar range [144]. Interestingly, a compound, AL18, previously shown to inhibit subunit interactions of human cytomegalovirus DNA polymerase [145] was found to also block the PA/PB1 interaction as well as the replication of IAV and IBV [146]. In a similar, but more restricted screening, Fukuoka and colleagues performed a docking simulation using a drug database of ~4000 compounds and selected candidate compounds targeting the PA/PB1 interface [147]. Among these, benzbromarone, diclazuril, and trenbolone acetate exhibited anti-IAV activity. In addition, benzbromarone and diclazuril were shown to bind the PA subunit and to decrease the transcriptional activity of the viral RdRP. In a different approach, a total of 15,000 molecules were tested in an ELISA-based screening, which led to the identification of a benzofurazan compound that also showed inhibition of viral replication at micromolar concentrations [148]. However, both this compound and its derivatives exhibited significant cytotoxicity, thus likely excluding an in vivo use. Overall, the compounds targeting the PA/PB1 binding interface could provide the basis for the development of a new generation of therapeutic agents against IAV and IBV.

The possibility of targeting other interaction sites in the polymerase complex, e.g., those between PB1 and PB2 subunit, recently emerged with the publication of the crystal structure of the PB1/PB2 binding interface [137]. The structure showed that only small regions of PB1 (residues 678–757) and of PB2 (residues 1–37) are required for tight binding. Since the PB1/PB2 interface has a crucial function in regulating the polymerase complex and it is highly conserved among IV, it appears as a promising target for novel broad-spectrum anti-influenza drugs. As a proof-of-principle, a synthetic peptide corresponding to residues 1–37 of PB2 was shown to inhibit the PB1/PB2 interaction in vitro [149]. However, to date no small molecule targeting this protein-protein interaction has been yet reported, and indeed the flat PB1/PB2 interface may pose

significant challenges in developing a nonpeptide small-molecular-weight inhibitor. Recently, Li and colleagues reported that a peptide derived from amino acids 731–757 of PB1 can disrupt the interaction between the C-terminal part of PB1 (aa 676–757) and the N-terminal part of PB2 (aa 1-40) and also inhibit viral RdRP activity and IV replication [150]. Surprisingly, the authors showed that this peptide interacts with PB1 rather than PB2. Furthermore, mutational analyses and computational modelling suggested that $PB1_{731–757}$ peptide acts as a competitor of PB2 with respect to binding to PB1. Thus, the inhibitory mechanism of the $PB1_{731–757}$ peptide is likely different from that of the interfacial peptides $PB1_{1–25}$ and $PB2_{1–37}$, which inhibit complex assembly by binding to its interaction partner PA or PB1, respectively, and could suggest new avenues for antiviral discovery.

Antiviral strategies targeting the nucleoprotein

Given the pivotal role of NP during the IV life cycle, it represents an emerging target for new antiviral approaches [100]. IAV NP orchestrates the vRNP assembly by covering the genomic RNA segments, facilitating the correct folding, and by directing the proper constitution of vRNP via protein-protein interactions with RdRP subunits. Oligomerization of NP into a trimer coating viral RNA is essential to maintain vRNP structure; however, IAV NP assembles into a trimer through monomer interactions independently from the presence of RNA.

NP is a multifunctional, essential protein, which plays an active role not only in vRNP architecture, but also in transcription and replication of the viral genome and in vRNPs nuclear shuttling and cytoplasmic trafficking [7]. In the recent years, the interactions of NP with itself [151-155], with viral RNA [156], with viral RdRP, or with cellular factors were exploited for the development of new anti-IV strategies, demonstrating the significant druggable potential of NP. As an example, short interfering RNA designed for *NP* gene silencing and possessing a 5'-triphosphate moiety to induce a RIG-I-mediated interferon (IFN) response exhibited potent inhibitory effects both in infected cells and in mice, demonstrating that NP knockdown is a successful strategy to inhibit IAV propagation [157,158]. Perhaps, the most promising feature of NP from a pharmaceutical point of view is the ability of self-interaction mediated by a flexible tail loop present in each monomer that inserts in the neighboring monomer. This interaction is stabilized by electrostatic interactions between E339 and R416 residues [154]. In addition, the phosphorylation at Ser-165 seems to regulate the polymerization status and RNA binding activity of NP in infected cell [159]. Small molecules able to interfere with correct protein-protein interactions between NP monomers were independently identified by different groups [151,153-155]; among these, nucleozin (NCZ) is the most studied. It is widely accepted that such compounds bind at least two

different sites on NP and act by either stabilizing monomeric NP or by inducing the formation of NP aggregates. The improper interactions of NP monomers occurring in the presence of these antiviral compounds interfere with the different functions of NP in the virus cycle. In fact, NCZ and its derivatives were reported to exert antiviral activity at different times of IAV cycle [160]: there is an early inhibitory effect of NCZ on viral RNA transcription and replication as well as a recently identified antiviral effect exerted on the cytoplasmic trafficking of newly synthesized vRNPs. NCZ is supposed to affect both the transport of vRNPs into the nucleus and, after nuclear export, the transport of newly synthesized vRNP through the cytoplasm that involves the cellular protein Rab11 [160]. Interestingly, also cellular proteins have been recently identified as restriction factors for IAV replication by interfering with NP oligomerization, such as cyclophylin E [161], or with the NP-PB2 interaction, such as the IFN-inducible Mx1 protein [162], thus highlighting NP interactions with both cellular and viral partners as potential targets of antiviral strategies. Finally, very recently naproxen, a clinically-approved inhibitor of inducible COX-2, was identified through a structure-based in silico screening as the first inhibitor of the interaction between NP and RNA [156]. It was demonstrated that naproxen targets the RNA-binding groove of NP and blocks it in a monomeric form. Naproxen is effective against IAV H1N1 and H5N1 replication in infected cells and in mice. As prospected by the authors, the dual antiviral effect of this drug against IAV, i.e., inhibition of NP functions and of COX-2-induced pro-inflammatory response, could be particularly useful for the treatment of emerging pandemic IAV infections.

Antiviral strategies targeting the Nonstructural protein 1

NS1 is a multifunctional viral protein, whose major role is to antagonize the cellular antiviral response and in particular the IFN-mediated response [163,164]. Actually, NS1 IFN-antagonistic properties seem to be strain-specific [164]. NS1 subverts the cellular IFN response by different strategies: (i) it cooperatively binds to viral dsRNA [165], thus protecting it from the recognition of two cytoplasmic “sentinels” of viral infection, i.e., protein-kinase RNA-activated (PKR) [166] and 2’-5’-oligo(A) synthetase (OAS)/RNaseL [167], which are activated by dsRNA and serve to shut off host protein synthesis (and also IFN pathway effectors) and to induce viral RNA degradation, respectively; (ii) it binds to cellular mRNAs processing factors, such as cellular polyadenylation specifity factor 30 (CPSF30) and polyA-binding protein II (PABP2) [168,169], thus inhibiting 3’-mRNAs (such as IFN mRNAs) processing and nuclear/cytoplasmic trafficking; (iii) it interacts with the ubiquitin-ligase TRIM25 and blocks RIG-I activation, thus preventing subsequent IFN cascade activation [169]; (iv) it interacts with cellular IFN-inducible hGBP1 to antagonize its antiviral activity [170]; and finally (v) it targets IKK to block NF-kB activation [171]. NS1 post-translational

modifications such as phosphorylation and SUMOylation may modulate its activity and also the abundance of NS1 dimers and trimers in infected cells [172]. In fact, NS1 dimerization is essential for RNA binding, since mutations that block dimers formation also affect the RNA binding ability of NS1 [173].

Taken together, NS1 represents an attractive target for new chemotherapeutic strategies, but to date no anti-influenza drug targeting this viral protein is under clinical development. However, some molecules were recently identified by a screen aimed at searching compounds that phenotypically suppress NS1 functions [174]. By this approach, 2,000 compounds were tested for their ability to suppress the slow-growth phenotype in yeasts expressing IAV NS1; four molecules able to restore normal growth in yeasts and also to inhibit IAV replication in cells were identified. These compounds and some derivatives showed anti-influenza activity only in IFN-competent cells, and the ability to reverse NS1-mediated block of IFN response [175]. One derivative, JJ3297, resulted to be dependent on cellular RNAseL functions for antiviral activity [176]. These compounds provide the proof-of-principle that NS1 activity can be blocked by small molecules that could be also used in combination with IFN agonists to enhance their antiviral therapeutic potential. Another target of NS1-based antiviral strategies recently reported is the interaction of NS1 with viral RNA. Indeed, compounds able to interfere with NS1/viral RNA binding have been identified both by in silico screening [177] and by high-throughput screening (HTS) developed to search for inhibitors of the binding of recombinant NS1 to a viral RNA construct in vitro [178]. By this combinatorial approach, three compounds able to effectively inhibit NS1 binding to RNA in vitro and to reduce the cytopathic effect of IAV in infected cells were identified. By another approach, i.e., a fluorescence polarization-based assay other inhibitors of the binding between IAV NS1 and dsRNA, a library of quinoxoline derivatives, and a large small molecule library were tested leading to the identification of a compound, epigallocatechine gallate, able to inhibit virus growth [179,180]. These compounds provide the proof that also the interference with NS1 binding to viral RNA could be a feasible anti-influenza strategy. On this line, also the dimerization of NS1 could represent an interesting target of new anti-influenza interventions; however, to date no compound that acts by this mechanism has been reported yet.

Antiviral strategies using drug combinations

Combination therapies that target multiple viral protein functions have been proposed to achieve greater antiviral effects than each compound given individually, reduce the development of drug-resistance and administer lower drug doses, thereby decreasing adverse effects. Analogous to the treatments used against HIV and HCV, a combination of anti- IV drugs would be expected to be

more effective than single-agent chemotherapy in treating serious influenza cases. The combined use of amantadine + oseltamivir [181] and oseltamivir + T-705 [182] has shown therapeutic synergism in infected mice. In addition, association of oseltamivir and amantadine has been demonstrated to reduce the emergence of drug-resistant IAVs [183]. A preliminary, controlled clinical study comparing the therapy with rimantadine plus inhaled zanamivir versus rimantadine alone in hospitalized adult patients with serious influenza showed a higher efficacy for the combination of zanamivir with rimantadine [184]. Several other double-, triple-, even quadruple-drug combinations could be envisaged for achieving additive or synergistic antiviral effects, based not only on the currently available drugs but also on new compounds under development.

New host-based anti-influenza virus strategies

IV replication is strictly host-dependent. In fact, a plethora of cellular proteins are engaged in each step of the virus life cycle have been identified as restriction factors, since their activities exert a dramatic effect on IV replication [185-187]. In recent years, several studies reported different experimental approaches aimed at identifying the cellular proteins that are essential for IV replication and could be potential targets of new antiviral strategies. Among these, there are for example genome-wide RNA interference screenings (reviewed in [188]), proteomic approaches [189], and yeast two-hybrid screenings [190,191]. Antiviral drugs targeting cellular functions are expected to have some advantages over inhibitors exclusively directed against viral targets, in particular for highly genetically unstable viruses such as IAV. In fact, inhibitors of cellular proteins and/or pathways should be less prone to induce the emergence of resistant strains; on the other hand, perturbing the cellular environment to disrupt viral functions could have adverse side-effects that should be carefully considered.

Cellular proteins are found to be actively involved in virus attachment and entry, in endocytosis of the virus particle and subsequent uncoating, in primary viral RNA transcription and protein synthesis as well as in genome replication, and finally in assembly, budding, and release of new IV particles. From a pharmaceutical point of view, the host-virus interplay is highly challenging, since each interaction between viral and cellular partners virtually represents a potential antiviral target for the development of new drugs and therapeutic strategies.

Here, we will discuss a series of new antiviral approaches based on the inhibition of cellular functions that have emerged as essential for IV replication. We will describe cellular proteins and/or pathways endowed with pharmaceutical potential due to their active engagement in IV cycle and some already identified small-molecular weight inhibitors that are currently under preclinical or

clinical investigation as anti-IV agents. An overview of the cellular proteins/processes that could be targeted to block IV replication is illustrated in Figures 2 and 3.

Antiviral strategies involving host factors that are engaged in virus attachment, entry, and release of virus particles

As already mentioned above, the first step in the initiation of IV infection is the attachment of HA to SA-containing glycoprotein or glycolipid receptors on the host cell surface. An option for influenza therapy is the development of drugs targeting cellular components involved in this step rather than targeting the viral HA protein. The proteolytic activation of HA by cellular proteases is essential for IV propagation and may also represent an attractive antiviral target. Human airway trypsin-like protease (HAT), and transmembrane protease serine S1 members (TMPRSS) belong to the type II transmembrane serine proteases family expressed in human lungs and involved in HA activation. These proteases cleave some HAs of human IV strains with a monobasic cleavage site, in particular of H1, H2, and H3 strains [192]. TMPRSS2 cleaves HA intracellularly, while HAT cleaves HA at the plasma membrane; indeed, the cleavage by TMPRSS2, but not HAT, is resistant to certain protease inhibitors [193,194]. In addition, TMPRSS4 has been shown to be responsible of the activation of HA of 1918 H1N1 IAV, which caused the Spanish flu [195]. Protease inhibitors are thus potential anti-influenza agents [196]; in fact, some have been shown to suppress IV spread in cell culture [193], in animal models[197], and in humans [198]. IV propagation in HAT- or TMPRSS2-expressing cells the inhibition of HA cleavage was efficiently suppressed using specific low-molecular-weight peptide mimetic inhibitors [193,194]. The selectivity and potency of some of these peptide mimetic protease inhibitors were improved by incorporation of a synthetic amino acid residue, norvaline [199]. In addition, very recently a screening with the catalytic domain of TMPRSS2 and known trypsin-like serine proteases inhibitors identified a sulfonylated 3-amindinophenylalanylamide derivative able to block IV propagation in human airway epithelial cells [200]. The soluble proteases secreted by lung epithelial cells, such as tryptase Clara [201], mini-plasmin [202], and ectopic anionic trypsin I [203] are involved in low pathogenic avian IV activation. Indeed, Kido et al. have shown that viral replication is inhibited in vitro and in infected rats by endogenous inhibitors of these enzymes, such as secretory leuko-protease inhibitor and pulmonary surfactant [196]. An inhibitor of serine proteases is aprotinin, a 58- amino-acid single-chain globular polypeptide purified from bovine lung tissue. Aprotinin has a wide anti-protease spectrum and good physiologic tolerance in animals and humans [204]. There are various licensed formulations of aprotinin, including Trasylol, Gordox, Antagosan, and Contrycal [198]. Zhirnov et al. demonstrated that in the presence of aprotinin, IV prevalently contains uncleaved HA0 and the

resulting viral progeny is less infectious [205]. Aprotinin blocks IV replication in chicken embryonated eggs [206], cultured cells [194,195], mouse lung and heart [207], and airway epithelial cell cultures [208]. Other drugs similar to aprotinin, such as leupeptin and camostat and their nucleoside and nonnucleoside analogues, proved anti-IV activity [209]. Another circulating serine proteases inhibitor is α_1-antitrypsin (AAT), a 52 kDa glycoprotein produced by the liver, also known as SERPINA 1 [210]. Independently of protease inhibition, AAT appears to possess anti-inflammatory and tissue-protective effects, as well as antiviral activity especially against IV and HIV [210]. Finally, as mentioned above, the HA of some highly pathogenic IV strains, such as H5 and H7 subtypes of avian IV [211], contains a multibasic cleavage site and are activated during the transport from ER to the plasma membrane by furin and other cellular proteases located in the trans-Golgi network. Thus, the inhibition of furin or furin-like proprotein convertases may also represent an anti-IV strategy [212,213].

Another possible approach to block virus-host interactions is the removal of SA from the cellular membrane. Sialidases, that catalyze the removal of terminal SA residues from glycoproteins and glycolipids, have been demonstrated to be effective inhibitors of IV infection [214]. A novel drug currently in clinical development as a candidate inhibitor of influenza infections is DAS181 (also known as Fludase), a recombinant fusion protein that prevents IV attachment by enzimatically removing SA receptors from the epithelial surface of the human airway [214]. DAS181 is composed of the catalytic domain of *Actinomyces viscosus* sialidase fused with the respiratory epithelium anchoring domain of human protein amphiregulin [215,216]. The advantages of *A. viscosus* sialidase with respect to other bacterial sialidases are broad substrate specificity, higher specific activity, and good tolerance by the human immune system [214]. Preclinical in vitro and in vivo studies demonstrated the inhibitory activity of DAS181 against various seasonal strains of IAV and IBV and prophylactic and therapeutic effects against H5N1 virus infection in mice [217,216]. Importantly, this compound is also effective against strains resistant to the existing antiviral drugs, for example against oseltamivir-resistant H1N1 clinical isolates [216]. Moreover, a recent phase II clinical study of inhaled DAS181 showed a significant decrease of viral load in influenza-infected patients [218].

The attachment of IV to the host cell membrane is followed by the internalization of viral particles via pH-dependent receptor-mediated endocytosis [219]. IV infection is in fact inhibited by lysosomotropic agents, such as ammonium chloride [220] and chloroquine [221]. IAV endocytic uptake is promoted by the activation of cellular receptors triggering signaling cascades, in particular the members of receptor tyrosine kinases (RTKs) family. A possible anti-influenza strategy is thus the inhibition of these receptors. Recently, Eirhoff et al. showed that the modulation of the

expression or the activity of epidermal growth factor receptor (EGFR) and c-Met receptor causes the alteration of IAV entry, suggesting the involvement of these receptors in the transmission of entry signals upon virus attachment [222]. In fact, a virus uptake reduction and consequently a decrease of progeny virus titer were observed upon treatment with small molecule inhibitors of tyrosine kinases (i.e., genistein, SU4312, picropodophyllin, and gefitinib), as well as by a specific EGFR and c-Met inhibition via siRNA [222].

The internalized influenza virions are trafficked along the endocytic pathway to acidic late endosomes [77]. Cellular ATP-dependent proton pumps, named vacuolar-type H^+-ATPases (vATPases), mantain the acidification of endosomes, lisosomes, and Golgi-derived secretory vesicles in eukaryotic cells [223]. Selective inhibition of acidification of virus-containing endosomes could provide effective protection from viral infection [224]. On this line, Müller et al. reported the inhibition of IAV entry by the new vATPase inhibitor saliphenylhalamide (SaliPhe), a phenyl derivative of salicylihalamide A (SaliA), which exhibited a better antiviral effect/toxicity profile in comparison with various old generation vATPase inhibitors, such as concanamycin (ConmyA), bafilomycin A1 (BafA1), and archazolid (ArchB) [225]. Also in previous anti-influenza studies ConmyA and BafA1 were used as specific potent blockers of vATPase activity, but their pharmacological use in vivo as antivirals resulted incompatible because of their toxicity in animals [226]. The target of BafA1, ArchB, and ConmyA is the subunit c of the V_0 domain of vATPase [227,228]. The new generation vATPase inhibitors with reduced toxicity probably target a different binding site of the proton translocation domain with respect to older inhibitors [225]. Recently, other small molecules, e.g. the natural compound diphyllin, have also been shown to prevent IV entry by inhibiting vATPases [229].

Many essential cellular processes are regulated by the ubiquitin-proteasome system (UPS), an important intracellular protein degradation pathway. Different studies reported that the UPS is involved in IAV replication cycle and UPS inhibitors impair viral growth. A study by Khor et al. showed that treatment of infected cells in vitro with the proteasome inhibitor MG132 drastically reduced IV infectivity by blocking the virus entry process but not virus replication and budding [230]. In the presence of MG132, virus particles resulted trapped into endocytic compartments distinct from the classical early and late endosomes [230]. Widjaja and coworkers investigated the role of the UPS in IAV entry and replication using MG132 in combination with BafA1 and the E36ts20 hamster cell line [231], which expresses a temperature-sensitive E1 [232]. The results showed that IAV RNA synthesis depends on UPS; indeed, the inhibition of the proteasome affected IAV RNA synthesis and consequently viral protein expression. Actually, the mechanism of inhibition of IAV RNA synthesis by proteasome inhibitors is still unknown. The experiments

performed with MG132 combined with BafA1 showed that these proteasome inhibitors affect IAV replication at a post-fusion step. A possible mechanism of antiviral activity of proteasome inhibitors might be interference with the disassembly of viral particles and subsequent uncoating, resulting in retention of the vRNPs in the cytoplasm [231]. Also studies with the FDA-approved proteasome inhibitor PS-341, a dipeptidyl boronic acid also known as bortezomib or velcade, revealed anti-IV activity [233]. The inhibition of the activation of NF-κB pathway as a consequence of the lack of IκB degradation was suggested as another possible explanation of the antiviral effect of proteasome inhibition. In addition, a new proteasome inhibitor, VL-01, was shown to inhibit IAV replication in vitro in A549 cells infected with H1N1 and H5N1 IAV strains and in infected mice when administered by aerosolic route, as well to reduce the systemic pro-inflammatory cytokine and chemokine release [234]. For these reasons, although the mechanism of action of protesome inhibitors against IV is not yet completely clear, this class of compounds deserves further investigation as possible new anti-influenza drugs.

Antiviral strategies involving host factors that are engaged in vRNP nuclear trafficking, viral RNA transcription/replication, and processing

Viral RNA synthesis, which entails both primary mRNA transcription and vRNA replication processes, is directed by the virus-encoded RdRP, either transported into the nucleus directly after viral entry or *de novo* synthesized in the infected cell. The initiation of primary mRNA transcription also depends on the activity of host RNA polymerase II (RNAP-II) and other accessory factors such as the positive transcription elongation factor 1b (pTEF1b), which consists of the cyclin-dependent kinase 9 (Cdk9) and Cyclin T1 complex [235]. In uninfected cells, pTEF1b promotes the switch from transcription initiation to elongation step by phosphorylating the C-terminal domain (CTD) of the largest subunit of RNAP-II at Ser-5. Active pTEF1b is recruited for the replication of different viruses, including IV, for which it was demonstrated that pTEF1b interacts with viral RdRP complex, thus facilitating its association with host RNAP-II and the cap-snatching process [236]. The cellular RNAP-II in the "initiating status", when associated with a nascent pre-mRNA, is essential for viral mRNA transcription. In fact, there is a physical interaction between viral RdRP and hyperphosphorylated CTD of RNAP-II that results in cap-snatching by PB2 and cleavage of the 5'-end of cellular pre-mRNA by PA. Since siRNA-mediated knock-down of cyclin T1 resulted in strong inhibition of viral transcription and replication, while the enzymatic activity of Cdk9 was not required, Zhang and colleagues hypothesized that, to enhance viral transcription, pTEF1b might function as an adaptor to facilitate the association between viral RdRP and stalled cellular RNAP-II [236]. On this line, inhibitors of RNAP-II, such as α-amanitin and actinomycin D, also inhibited IV

replication [237]. Furthermore, 5,6-dichloro-1-beta-D-ribofuranosyl-benzimidazole, an inhibitor of Cdk9 that prevents elongation catalyzed by RNAP-II, was reported to block IV replication [238]. However, the potential of CyclinT1/Cdk9 as an anti-influenza target should be more deeply investigated, given the raising interest on the role of pTEF1b in many pathogenic processes, including HIV replication, and the number of inhibitors that are under clinical investigation as potential antiretroviral drugs [239,240]. The interacting portions of viral RdRP and pTEF1b remain to be yet identified. The identification of the residues essential for their interaction could prompt the design of protein-protein interactions (PPI) inhibitors, similar to the cyclin T1-derived peptides with anti-HIV activity [241].

Not only the cellular transcription machinery but also polyadenylation and splicing factors are involved in viral mRNA processing. As already mentioned, NS1 specifically interacts with cleavage and CPSF30 and PABII to achieve optimal viral mRNA processing [242,243]. In particular, given the essential nature of the NS1/CPSF30 interaction for IV replication, it has been proposed as a potential target of antiviral strategies based on PPI disruption [243]. The same strategy could be applied once the interactome of viral RdRP and other viral and cellular proteins will be fully dissected and PPI essential for IV productive replication will be identified. For example, six cellular proteins that functionally interact with RdRP have been recently identified as host factors essential for RdRP activity [191]. RNAP-II is also required for nuclear export of certain viral mRNAs [244], thus also this additional activity of cellular RNAP-II in IV life cycle might represent a potential target for novel host-based antiviral strategies.

The nuclear translocation of vRNP components to promote new vRNP assembly and export depends on a number of host factors such as importins and molecular chaperones that may represent antiviral targets less prone to raise resistant viruses. In particular, the Hsp90 protein plays a key role in the nuclear translocation of vRNP components PB1 and PB2, by forming a PB1-PB2-Hsp90 complex prior to the assembly of the RdRP complex [245]. Inhibitors of Hsp90, such as geldanamycin and 17-AAG, affect IV replication in cell culture possibly by blocking the nuclear import of PB1 and PB2 and by inducing their degradation [246]. Other host factors representing possible antiviral targets are proteins involved in the nuclear export of vRNP. For instance, the interaction between nucleoporin Nup98 and viral NS2, also called viral nuclear export protein (NEP), has been reported [247]. The GLFG repeat domain of Nup98 has a transdominant effect on Nup98/NS2 and blocks virus propagation [247], suggesting that this interaction could be a target of an antiviral strategy based on PPI disruption. Also leptomycin B, which is a specific inhibitor of the binding of chromosome region maintenance 1 protein (CRM1)/Exportin complex to nuclear export signals, blocks IAV vRNPs nuclear export in infected cells [248]. Thus, vRNPs nuclear export is

CRM1 pathway-dependent and is potentially susceptible to inhibition by small molecules. Very recently, other specific nuclear export inhibitors of natural origin, valtrate and 1-acetoxychlavicol acetate, that target cellular CRM1 have been reported to be potent inhibitors of IAV replication in infected cells [249]. The cellular protein Hsc70 is also involved in the regulation of the nuclear export (but not import) of vRNPs, via an interaction with viral M1 protein. The nuclear export of Hsc70 is also blocked by leptomycin B; thus also this host factor could represent a possible antiviral target [250]. Very recently, the serum- and glucocorticoid-regulated kinase 1 (SGK1) has been identified as essential for IAV replication in A549 cells [251]. By using siRNA-mediated knockdown and pharmacological inhibition, it was demonstrated that SGK1 is involved in vRNPs nuclear export and that a specific SGK1 inhibitor, GSK 650394, is able to block IAV replication in A549 cells. Thus, inhibition of SGK1 may represent another anti-IV strategy.

Antiviral strategies involving host signaling pathways

Many host cell signaling pathways are affected during IV infection; among these there are the mitogen-activated protein kinase Raf/MEK/ERK pathway, the phophatidylinositol-3-kinase/Akt/mTOR pathway, the protein kinase C (PKC) pathway, the NF-κB pathway, and a more recently identified inositol-requiring enzyme (IRE) stress pathway. The activation of host signaling pathways is essential for productive IV infection, since small molecules that inhibit any step of signal transduction are also able to block virus propagation by affecting different events in IV life cycle as it will be described below.

The mitogen-activated protein kinase (MAPK) signaling pathway is activated in response to a variety of stimuli, such as mitogenic factors, oxidative stress, inflammatory cytokines, and morphogenic stimuli. This pathway is mainly constituted by a tripartite module effectors consisting of a series of protein kinases that activate the downstream effectors by phosphorylation [252]. There are three principal MAPKs pathways depending on the stimuli, effectors, and response. It is mainly accepted that the extracellular-regulated kinase (ERK) module is activated by mitogenic stimuli and leads to cell proliferation; the JNK/p38 module is activated by oxidative stress, inflammatory cytokines, and other stimuli and leads to apoptosis and inflammation; and the ERK5 module is activated by morphogenic stimuli [252]. IV is known to activate all the MAPKs pathways and the inhibition of this activation has a detrimental effect on virus replication [253]. The phosphorylating activity of MAPKs pathways effectors plays a major role in vRNP trafficking, particularly in NEP-mediated vRNP nuclear export, and in viral infectious particle production [254-256]. Of particular interest, MAPKs inhibition can be obtained both pharmacologically (by using inhibitors) and by restoring the physiological reduced state of intracellular environment of the infected cells after the

oxidative stress caused by IV infection [257]. In fact, MAPKs pathway, like other cellular signaling pathways activated by IV, such as PI3K and NF-κB-mediated signaling, is strictly dependent on host cell redox state [258-260]. On this line, MAPKs inhibitors that can block IV replication and the pro-inflammatory cascade are represented by kinase inhibitors, such as the MEK-specific inhibitor UO126 [261,262,253], and antioxidant compounds, such as p38 inhibitor SB203580 and N-acetylcysteine [263,264], glycyrrizin [265], glutathione and its derivatives [266,267], and curcumin and resveratol derivatives [268]. All these compounds are potential new host-based anti-influenza drugs that could be used in combination with "old" anti-influenza drugs such as oseltamivir, as prospected in [269].

PKC plays a role at different stages of IAV replication, in particular in virus entry. In fact, the binding of viral HA to the host cell activates PKC signaling [270] and, according to this, the PKC signaling inhibitors bisindolylmaleimides block viral entry possibly at the stage of post-fusion endocytosis [271,272]. Furthermore, also the viral M1, PB1-F2, and NS1 proteins are substrates of PKC [273-275]. In keeping with the importance of PKC signaling during IV infection, the block of its activation or the treatment with specific inhibitors affect not only virus entry [272] but also vRNP trafficking [273] and viral propagation [276].

The redox state of the infected cell also influences other two important processes in the IV life cycle, i.e., HA maturation and NF-kB activation. HA is a disulfide-rich protein and is initially found as a glycosylated monomer in the endoplasmic reticulum (ER). Then it undergoes trimerization, oxidation catalyzed by the cellular protein disulfide isomerase (PDI), cleavage, and finally is inserted into the plasma membrane. The activity of PDI is enhanced by the oxidative environment produced by IV infection. Thus, compounds that are able to restore the cellular redox state, such as glutathione, are also able to exert antiviral activity by two distinct mechanisms: (i) by interference with the activation of redox-sensitive signaling pathways exploited by the virus, such as MAPKs and PI3K pathways and (ii) by blocking the maturation of viral HA protein [257,256]. Also thiazolides are potential broad-spectrum antivirals that exert anti-influenza activity by blocking HA terminal glycosylation and impairing the trafficking of HA precursors from ER to Golgi [277]. However, the exact mechanism of action is still to be elucidated, although a cell-mediated effect of thiazolides has been postulated due to the observed inhibition of different viruses by this class of molecules.

The redox-sensitive signaling pathway of phosphoinositide 3-kinase (PI3K)/Akt/mTOR is also activated by different extracellular stimuli and by IV infection. Its activation transduces the transcriptional signal through a phosphorylation cascade of other downstream kinases such as Akt/PKB, cAMP-dependent kinase (PKA), and ribosomal S6 kinases and mTOR. The role of PI3K

pathway activation during IV infection is still controversial. There is increasing evidence that either pro-viral or anti-viral effects following PI3K pathway activation might be dependent on the stage of virus cycle at which activation occurs, as well as on the viral strain and the spatial localization of kinase effectors [164,278,279]. The IV NS1 protein is known to bind PI3K [280] and this interaction could play a role in the activation of NF-κB activation and pro-inflammatory cascade and anti-apoptotic signaling; however, other viral factors could contribute to PI3K pathway activation. Thus, there are still a number of unresolved questions about IV and PI3K pathway interplay [281]. Recently, a role for PI3K and ERK activation in virus entry was reported, since the early activation of both kinases stimulates acidification of the intracellular environment induced by the vATPase and the internalization process [255]. The key role of the activation of PI3K pathway, although still a little fuzzy, is mirrored by the evidence that compounds targeting PI3K or downstream effectors, such as PI3K inhibitors wortmannin [282] and LY294002 [283], or mTOR inhibitors [284] block IAV replication in vitro. For this reason, considering the increasing availability of PI3K/Akt/mTOR inhibitors already approved or under clinical investigation as anticancer drugs [285], the inhibition of this host cell pathway as a new anti-influenza approach clearly deserves further investigation. Finally, very recently the small molecule multi-kinase inhibitor ON108110 was reported to reduce the IAV replication by restricting viral RNA synthesis [286].

Another pathway targeted by IV infection is that of NF-κB, a family of transcription factors that play a role in induction of inflammation, activation of immune response, proliferation, and apoptosis [287]. The activation of NF-κB pathway is critical for IV productive replication [288-290] and is the result of the ability of viral HA, NP, and M1 proteins to produce oxidative radicals. The oxidative stress activates redox-sensitive signaling pathways and the NF-κB inhibitor IκB kinase (IKK), which is responsible of its derepression and transcriptional activation [291]. The essential nature of active NF-κB for IV replication makes it a promising target of antiviral strategies. On this line, a number of inhibitors of NF-κB activation, such as acetylsalicylic acid [288], SC75741 [292], BAY 11-7082 [293], pyrrolidine dithiocarbamate [294], as well as antioxidant agents (see above and [295]), have been reported to block IV replication and propagation.

Recently, the unfolded protein response (UPR) pathway, which is activated upon ER stress [296], has also been proposed for new host-based anti- IV strategies. The UPR pathway is activated in the case of increasing protein misfolding induced by ER stress. The high presence of misfolded proteins in the ER induces UPR activation, upregulation of chaperones transcription, the decrease of cellular mRNA transcription, and the increase of ER-associated proteasomal degradation of

unfolded proteins. Inositol-requiring enzyme 1 (IRE1) is involved in the latter process and is also the only UPR pathway branch activated by IAV infection [296]. IRE1 possesses both kinase and endoribonuclease activity and is able to activate the MAPKs signalling and to activate, by favouring its unusual splicing, the transcription factor XBP1 involved in ER-associated protein degradation. Inhibition of IRE1 activation by chemical chaperones that alleviate ER stress or its enzymatic inhibition by small molecule compounds also block IAV replication, thus opening a new therapeutic intervention option by targeting a host cellular mechanism; however, possible off-target effects related to the inhibition of the cellular UPR pathway should also be carefully considered [296].

The cellular cyclooxigenases (COX) pathway is also engaged during IV infection, in particular in the case of human infection with highly pathogenic avian strains such as H5N1 [297]. In fact, the H5N1 strain strongly upregulates COX-2 during the infection of human macrophages, and COX-2 mediated pro-inflammatory signaling could be responsible of the hypercytokinemia that distinguishes the pathogenic mechanism of H5N1 strain in humans. Recently, it was demonstrated that the non-steroidal COX-2 inhibitor nimesulide is able to inhibit H5N1 strain replication in human macrophages; thus, also COX-2 could represent a promising cellular target for new anti- IV strategies [298].

In conclusion, there is increasing evidence that understanding the molecular mechanisms underlying how IV modulates the host cell signaling pathways could be the key that opens the door to innovative therapeutic antiviral strategies.

Antiviral strategies involving host cell metabolism

IV uses cellular constituents to produce its own viral RNAs, proteins, and lipid envelope. However, the virus needs to purchase dNTPs, amino acids, and membrane components from the host cell and the best way is to induce the degradation of host nucleic acids, proteins, and organelles to obtain new building blocks to be recycled in the synthesis of viral components. Autophagy is a physiological process aimed at self-renewal and recycling of cellular constituents and consists of degradation of organelles and proteins that occurs into lysosomes; however, it can be induced by various stress conditions and by infection with certain viruses, including IV, through the inhibition of the mTORC1 pathway [299]. The mTORC1 inhibition, obtained either pharmacologically or under stress conditions, results in the activation of the so-called autophagy-related genes and in autophagosome biogenesis. However, it is still matter of debate whether IV really induces autophagy or if autophagy is just a consequence of the perturbation of cellular pathways and homeostasis induced by IV infection [299]. Waiting for answers to the high number of open questions, the presumed IV-induced autophagy is an emerging target of new antiviral strategies.

Indeed, two molecules recently identified by cell-based screenings, i.e., procyanidin and evodiamine, were able to block autophagy and also IAV replication in infected cells [300,301]. Other cellular proteins recently have emerged as host restriction factors and highlight the strict interplay with viral proteins, particularly NS1. This is the case of REDD1 protein, a major negative regulator of the mTORC1 pathway, whose expression is increased by the treatment with naphtalimides, antiviral compounds that are able to antagonize NS1 and thus to reduce IV propagation [302].

The degradation of cellular nucleic acids is not the only strategy whereby IV enriches the dNTPs pool for viral RNA synthesis [303]; in fact, also *de novo* synthesis of pyrimidine and uracil salvage pathways are engaged during IV infection, depending on the cell type [304]. Pyrimidines are important precursors used for RNA (uracil and cytosine), glycoproteins, and phospholipids biosynthesis. On this line, compounds able to deplete the cellular pyrimidine pool show anti-influenza activity. An example is the small molecule A3, identified by HTS, which possesses broad-spectrum antiviral activity by targeting pyrimidine metabolism and in particular the mitochondrial enzyme dihydroorotate dehydrogenase (DHODH) [304]. This enzyme is an antiviral target of other two antiviral drugs, i.e., leflunimide and brequinar [305]; however, the authors hypothesized a different mechanism of action for A3. DHODH has emerged as a possible anti-IV target of another class of inhibitors, the quinolone carboxylic acid derivatives, which were reported to inhibit IAV replication in infected cells [306]. Furthermore, by studying their effects on DHODH inhibition, it was demonstrated that DHODH block leads to the up-regulation of cellular antiviral factors, such as the NXF1 protein, which is able to reverse the cellular mRNA export block mediated by viral NS1 protein [306]. However, de novo pyrimidine synthesis requirement for efficient viral replication may be cell- and species-specific, since it was reported that the nonnucleoside DHODH inhibitor D282 showed anti-IAV and IBV activity in vitro but not in infected mice [307].

Lipid mediators and lipid metabolic pathways are also involved in IV infection. Very recently, it was reported the identification of a potent IV replication inhibitor identified by a screening using derivatives of omega-3 polyunsaturated fatty acid (PUFA) [308], which are biosynthetic precursors of lipid mediators with anti-inflammatory and pro-resolving properties [309]. The omega-3 PUFA-derived lipid mediator protectin D1 (PD1) is able to potently inhibit IAV replication in vitro and in infected mice also at late stages of infection [308]. Furthermore, it was demonstrated that its mechanism of action specifically involves the impairment of viral transcripts nuclear export mediated by the cellular protein NXF1, without significant effects on cellular mRNA export or antiviral response. Thus, the further clinical development of PD1, given its potency and late-stage efficacy, is strongly encouraged.

Not only lipid soluble mediators, but also lipid metabolism has been involved in IV infection, since it was demonstrated that budding and release of new virus particles occur at specific domains of the plasma membrane, called lipid rafts [310]. Lipid rafts are dynamic microdomains of the cell membrane characterized by a higher percentage of cholesterol, sphingolipids, and phospholipids containing saturated fatty acids; this particular composition makes lipid rafts extremely flexible. IV HA and NA proteins are recruited at these specific domains, together with other cellular proteins responsible of vesicle formation, thus they are considered to be the sites of budding initiation [310]. It was reported that cholesterol depletion [311] or the expression of an IFN-inducible cellular protein, viperin [312], disrupt plasma membrane by perturbing lipid raft formation. Viperin interacts with and inhibits farnesyl diphosphate synthase (FPPS), a cellular enzyme involved in the synthesis of various isoprenoid-derived precursors of cholesterol and other essential cellular components [312] Furthermore, inhibition of FPPS, either by siRNA or by viperin expression, also inhibits IAV release and replication by perturbing lipid raft correct formation [312]. These findings suggest that perturbing membrane fluidity and/or composition could be a new host-based anti-influenza strategy. The recent identification by in silico screenings of new FPPS inhibitors, beside traditional bisphosphonate FPPS inhibitors, opens new avenues for further investigation of the potential anti-influenza activity of FPPS inhibitors, which are already under clinical investigation as anti-cancer and anti-infective agents [313,314]. In keeping with the importance of membrane composition for efficient IV replication, the inhibition of cellular sphingolipids biosynthesis has also been reported to have an abortive effect on the replication of IAV and IBV and to perturb the intracellular distribution of viral HA protein [315]. Very recently, also the biosynthetic pathway of sphingomyelin was reported to be essential for the intracellular transport of viral glycoproteins; thus, its modulation could also represent a possible antiviral strategy [316].

Antiviral strategies involving host cell antiviral response

Infection of ssRNA viruses, including IV, is known to be recognized by certain cellular pattern recognition receptors (PRRs), such as transmembrane Toll-like receptors (TLR) 3, 7, and 9, and cytoplasmic RIG-I like receptors (RLRs), which sense the invasion of a pathogen and counteract it both by stimulating an intracellular antiviral state and by sensitizing neighboring cells. One of the most important cellular defense strategy against IV infection is the activation of type I and III IFN pathway [317]. Type I IFN expression is the result of the PRRs activation and downstream signaling and leads in turn to the activation of more than 300 IFN-stimulated genes , whose products cooperate to inhibit IV replication at different stages, to trigger the adaptive immune

response, and ultimately to induce apoptosis of the infected cell. IVs have evolved various strategies to evade IFN response (reviewed in [317]) with NS1 representing the major player (see above).

Given the pivotal role of IFN pathway in the early anti-influenza cellular response, its activation or enhancement represent an important option for antiviral intervention. Small molecules that act as agonists of TLRs and RLRs are able to induce IFN and other pro-inflammatory cytokines and chemokines, as well as to function as vaccine adjuvants. Indeed, treatment with poly(I·C), CpG oligodeoxynucleotides (ODNs), and other TLRs ligands proved to be effective in protecting aged mice against lethal IAV infection. Recently, a cell-based HTS aimed at identifying small molecules able to induce IFN was reported [318]. The lead compound 3 identified by this HTS is able to induce an IFN-dependent antiviral state that cannot be counteracted by IAV NS1 protein [318]. Furthermore, a novel small molecule, ASN2, which inhibits viral RdRP subunit PB1 was reported to have additional IFN-inducing properties [319]. ASN2 was identified by a cell-based HTS aimed at identifying molecules able to activate luciferase expression under control of INF-β promoter and is an effective inhibitor of IAV replication both in infected cells and in mice. The IFN-inducing properties are most likely due to the ability of ASN2 to inactivate PB1 and therefore to the loss of NS1 expression [319]. Also the treatment with high-molecular-weight poly-γ-glutamate (HM-γ-PGA) produced by a bacterium from *Bacillus* sp. was reported to induce IFN response and to exert potent inhibition of highly pathogenic IAV infection in humanized mice [320]. Short synthetic RNA molecules with an exposed 5'-triphosphate moiety are substrate for RIG-I and were reported to be able to induce a potent antiviral response that results in inhibition of IAV replication in A549 cells and also to protect mice from lethal IV infection [321].

In conclusion, the induction of IFN antiviral response by small molecules or polymers is another promising strategy for the development of new anti-influenza drugs that could be used in combination with other already available drugs to enhance their efficacy and to counteract synergistically the viral infection.

Concluding remarks

Infection with IV is a life-threatening event for high-risk patients, such as the elderly and patients with cardiovascular, pulmonary, or renal diseases, diabetes, and immunodeficiency, but also for the whole human population. The IV disseminate rapidly around the world, and how and when new IAV emerge as pandemic strains and their mechanism of pathogenesis are still poorly understood. The virus changes very quickly, humans have no natural immunity to it and infected birds may be able to transmit it before they show symptoms. Any virus with these characteristics could devastate the human population while causing massive economic and social chaos. The last - but not least -

emerged avian highly pathogenic IAV to be reported is the H7N9 virus, which is a new reassortant of avian origin isolated in China and associated with severe respiratory disease with 40% of mortality [322]. To date, this virus represents a major concern, since it might be able to become pandemic [323]. Besides vaccination, treatment with M2 ion channel blockers and NA inhibitors is currently the only option for influenza management. However, these weapons are clearly insufficient and the rapid and extensive development of resistance to the existing drugs has raised great public health concern. There is therefore an urgent need to develop new antiviral strategies targeting other processes in the IV life cycle. Current research efforts include (i) the improvement of existing drugs; (ii) the development of inhibitors against other antiviral targets, in particular RdRP, NP, and NS1; (iii) strategies to block virus-cell interactions occurring at different stages of IV replication, such as attachment, entry, viral genome transcription and replication, nuclear export of viral products, and viral particles release; and (iv) modulation of cell metabolism and host antiviral response. In addition, like for other fast mutating viruses such as HIV and HCV, the application of antiviral drugs in combination, with different mechanisms of action, is being actively pursued, as it could be more effective in treating virulent and pandemic IV strains. Given the broad array of different anti-IV strategies under development, it is our hope that the discovery of new drugs will very soon provide wider options for improved prophylactic and therapeutic approaches against influenza infection.

Acknowledgments

Work in the authors' laboratory was supported by MURST EX60%, Progetto di Ricerca di Ateneo 2007 (grant n. CPDA074945), and PRIN 2008 (grant n. 20085FF4J4) to A.L, by Regione Veneto and Progetto Strategico di Ateneo 2008 to G.P., by Italian Ministry of Health and Istituto Superiore Sanità, Progetto finalizzato 2009 “Studio e sviluppo di nuovi farmaci antivirali contro infezioni da virus influenzale A-H1N1” to A.L. and G.P., and by ESCMID Research Grant 2013 to B.M and A.L.

References

1. Cox NJ, Subbarao K (2000) Global epidemiology of influenza: past and present. Annu Rev Med 51:407-421

2. Palese P, Shaw ML. (2007) Orthomyxoviridae: the viruses and their replication. In Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (eds), Fields Virology, 5th edn. Lippincott Williams & Wilkins, Philadelphia

3. Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, Basta S, O'Neill R, Schickli J, Palese P, Henklein P, Bennink JR, Yewdell JW (2001) A novel influenza A virus mitochondrial protein that induces cell death. Nat Med 7 (12):1306-1312

4. Jagger BW, Wise HM, Kash JC, Walters KA, Wills NM, Xiao YL, Dunfee RL, Schwartzman LM, Ozinsky A, Bell GL, Dalton RM, Lo A, Efstathiou S, Atkins JF, Firth AE, Taubenberger JK, Digard P (2012) An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. Science 337 (6091):199-204

5. Wise HM, Foeglein A, Sun J, Dalton RM, Patel S, Howard W, Anderson EC, Barclay WS, Digard P (2009) A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. J Virol 83 (16):8021-8031

6. Pinto LH, Lamb RA (2006) The M2 proton channels of influenza A and B viruses. J Biol Chem 281 (14):8997-9000

7. Ito T, Gorman OT, Kawaoka Y, Bean WJ, Webster RG (1991) Evolutionary analysis of the influenza A virus M gene with comparison of the M1 and M2 proteins. J Virol 65 (10):5491-5498

8. Leonov H, Astrahan P, Krugliak M, Arkin IT (2011) How do aminoadamantanes block the influenza M2 channel, and how does resistance develop? J Am Chem Soc 133 (25):9903-9911

9. Wang C, Takeuchi K, Pinto LH, Lamb RA (1993) Ion channel activity of influenza A virus M2 protein: characterization of the amantadine block. J Virol 67 (9):5585-5594

10. Moss RB, Davey RT, Steigbigel RT, Fang F (2010) Targeting pandemic influenza: a primer on influenza antivirals and drug resistance. J Antimicrob Chemother 65 (6):1086-1093

11. Bright RA, Medina MJ, Xu X, Perez-Oronoz G, Wallis TR, Davis XM, Povinelli L, Cox NJ, Klimov AI (2005) Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. Lancet 366 (9492):1175-1181

12. Krumbholz A, Schmidtke M, Bergmann S, Motzke S, Bauer K, Stech J, Durrwald R, Wutzler P, Zell R (2009) High prevalence of amantadine resistance among circulating European porcine influenza A viruses. J Gen Virol 90 (Pt 4):900-908

13. Wang J, Wu Y, Ma C, Fiorin G, Pinto LH, Lamb RA, Klein ML, Degrado WF (2013) Structure and inhibition of the drug-resistant S31N mutant of the M2 ion channel of influenza A virus. Proc Natl Acad Sci U S A 110 (4):1315-1320

14. Wang J, Ma C, Jo H, Canturk B, Fiorin G, Pinto LH, Lamb RA, Klein ML, DeGrado WF (2013) Discovery of novel dual inhibitors of the wild-type and the most prevalent drug-resistant mutant, S31N, of the M2 proton channel from influenza A virus. J Med Chem 56 (7):2804-2812

15. Furuse Y, Suzuki A, Oshitani H (2009) Large-scale sequence analysis of M gene of influenza A viruses from different species: mechanisms for emergence and spread of amantadine resistance. Antimicrob Agents Chemother 53 (10):4457-4463

16. Wang J, Ma C, Fiorin G, Carnevale V, Wang T, Hu F, Lamb RA, Pinto LH, Hong M, Klein ML, DeGrado WF (2011) Molecular dynamics simulation directed rational design of inhibitors targeting drug-resistant mutants of influenza A virus M2. J Am Chem Soc 133 (32):12834-12841

17. Balannik V, Wang J, Ohigashi Y, Jing X, Magavern E, Lamb RA, Degrado WF, Pinto LH (2009) Design and pharmacological characterization of inhibitors of amantadine-resistant mutants of the M2 ion channel of influenza A virus. Biochemistry 48 (50):11872-11882

18. Wang J, Ma C, Wu Y, Lamb RA, Pinto LH, DeGrado WF (2011) Exploring organosilane amines as potent inhibitors and structural probes of influenza a virus M2 proton channel. J Am Chem Soc 133 (35):13844-13847

19. Wang J, Cady SD, Balannik V, Pinto LH, DeGrado WF, Hong M (2009) Discovery of spiro-piperidine inhibitors and their modulation of the dynamics of the M2 proton channel from influenza A virus. J Am Chem Soc 131 (23):8066-8076

20. Zhao X, Li C, Zeng S, Hu W (2011) Discovery of highly potent agents against influenza A virus. Eur J Med Chem 46 (1):52-57

21. Zoidis G, Kolocouris N, Kelly JM, Prathalingam SR, Naesens L, De Clercq E (2010) Design and synthesis of bioactive adamantanaminoalcohols and adamantanamines. Eur J Med Chem 45 (11):5022-5030

22. Wei G, Meng W, Guo H, Pan W, Liu J, Peng T, Chen L, Chen CY (2011) Potent neutralization of influenza A virus by a single-domain antibody blocking M2 ion channel protein. PLoS One 6 (12):e28309

23. Gamblin SJ, Skehel JJ (2010) Influenza hemagglutinin and neuraminidase membrane glycoproteins. J Biol Chem 285 (37):28403-28409

24. Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD (2004) Human and avian influenza viruses target different cell types in cultures of human airway epithelium. Proc Natl Acad Sci U S A 101 (13):4620-4624

25. Kim CU, Lew W, Williams MA, Liu H, Zhang L, Swaminathan S, Bischofberger N, Chen MS, Mendel DB, Tai CY, Laver WG, Stevens RC (1997) Influenza neuraminidase inhibitors possessing a novel hydrophobic interaction in the enzyme active site: design, synthesis, and structural analysis of carbocyclic sialic acid analogues with potent anti-influenza activity. J Am Chem Soc 119 (4):681-690

26. von Itzstein M, Wu WY, Kok GB, Pegg MS, Dyason JC, Jin B, Van Phan T, Smythe ML, White HF, Oliver SW, et al. (1993) Rational design of potent sialidase-based inhibitors of influenza virus replication. Nature 363 (6428):418-423

27. Samson M, Pizzorno A, Abed Y, Boivin G (2013) Influenza virus resistance to neuraminidase inhibitors. Antiviral Res 98 (2):174-185

28. Russell RJ, Haire LF, Stevens DJ, Collins PJ, Lin YP, Blackburn GM, Hay AJ, Gamblin SJ, Skehel JJ (2006) The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. Nature 443 (7107):45-49

29. Gubareva LV, Kaiser L, Hayden FG (2000) Influenza virus neuraminidase inhibitors. Lancet 355 (9206):827-835

30. Cass LM, Efthymiopoulos C, Bye A (1999) Pharmacokinetics of zanamivir after intravenous, oral, inhaled or intranasal administration to healthy volunteers. Clin Pharmacokinet 36 Suppl 1:1-11

31. Tsang KW, Eng P, Liam CK, Shim YS, Lam WK (2005) H5N1 influenza pandemic: contingency plans. Lancet 366 (9485):533-534

32. Davies BE (2010) Pharmacokinetics of oseltamivir: an oral antiviral for the treatment and prophylaxis of influenza in diverse populations. J Antimicrob Chemother 65 Suppl 2:ii5-ii10

33. Kohno S, Kida H, Mizuguchi M, Hirotsu N, Ishida T, Kadota J, Shimada J (2011) Intravenous peramivir for treatment of influenza A and B virus infection in high-risk patients. Antimicrob Agents Chemother 55 (6):2803-2812

34. Barroso L, Treanor J, Gubareva L, Hayden FG (2005) Efficacy and tolerability of the oral neuraminidase inhibitor peramivir in experimental human influenza: randomized, controlled trials for prophylaxis and treatment. Antivir Ther 10 (8):901-910

35. Koyama K, Takahashi M, Oitate M, Nakai N, Takakusa H, Miura S, Okazaki O (2009) CS-8958, a prodrug of the novel neuraminidase inhibitor R-125489, demonstrates a favorable long-retention profile in the mouse respiratory tract. Antimicrob Agents Chemother 53 (11):4845-4851

36. Pizzorno A, Bouhy X, Abed Y, Boivin G (2011) Generation and characterization of recombinant pandemic influenza A(H1N1) viruses resistant to neuraminidase inhibitors. J Infect Dis 203 (1):25-31

37. 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 (2011) Structural and functional analysis of laninamivir and its octanoate prodrug reveals group specific mechanisms for influenza NA inhibition. PLoS Pathog 7 (10):e1002249

38. Feng E, Ye D, Li J, Zhang D, Wang J, Zhao F, Hilgenfeld R, Zheng M, Jiang H, Liu H (2012) Recent advances in neuraminidase inhibitor development as anti-influenza drugs. ChemMedChem 7 (9):1527-1536

39. Mitrasinovic PM (2010) Advances in the structure-based design of the influenza A neuraminidase inhibitors. Curr Drug Targets 11 (3):315-326

40. Lee CM, Weight AK, Haldar J, Wang L, Klibanov AM, Chen J (2012) Polymer-attached zanamivir inhibits synergistically both early and late stages of influenza virus infection. Proc Natl Acad Sci U S A 109 (50):20385-20390

41. Weight AK, Haldar J, Alvarez de Cienfuegos L, Gubareva LV, Tumpey TM, Chen J, Klibanov AM (2011) Attaching zanamivir to a polymer markedly enhances its activity against drug-resistant strains of influenza a virus. J Pharm Sci 100 (3):831-835

42. Honda T, Yoshida S, Arai M, Masuda T, Yamashita M (2002) Synthesis and anti-influenza evaluation of polyvalent sialidase inhibitors bearing 4-guanidino-Neu5Ac2en derivatives. Bioorg Med Chem Lett 12 (15):1929-1932

43. Macdonald SJ, Cameron R, Demaine DA, Fenton RJ, Foster G, Gower D, Hamblin JN, Hamilton S, Hart GJ, Hill AP, Inglis GG, Jin B, Jones HT, McConnell DB, McKimm-Breschkin J, Mills G, Nguyen V, Owens IJ, Parry N, Shanahan SE, Smith D, Watson KG, Wu WY, Tucker SP (2005) Dimeric zanamivir conjugates with various linking groups are potent, long-lasting inhibitors of influenza neuraminidase including H5N1 avian influenza. J Med Chem 48 (8):2964-2971

44. Grienke U, Schmidtke M, von Grafenstein S, Kirchmair J, Liedl KR, Rollinger JM (2012) Influenza neuraminidase: a druggable target for natural products. Nat Prod Rep 29 (1):11-36

45. Heim KE, Tagliaferro AR, Bobilya DJ (2002) Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. J Nutr Biochem 13 (10):572-584

46. Jeong HJ, Ryu YB, Park SJ, Kim JH, Kwon HJ, Park KH, Rho MC, Lee WS (2009) Neuraminidase inhibitory activities of flavonols isolated from Rhodiola rosea roots and their in vitro anti-influenza viral activities. Bioorg Med Chem 17 (19):6816-6823

47. Miki K, Nagai T, Suzuki K, Tsujimura R, Koyama K, Kinoshita K, Furuhata K, Yamada H, Takahashi K (2007) Anti-influenza virus activity of biflavonoids. Bioorg Med Chem Lett 17 (3):772-775

48. Ryu YB, Curtis-Long MJ, Kim JH, Jeong SH, Yang MS, Lee KW, Lee WS, Park KH (2008) Pterocarpans and flavanones from Sophora flavescens displaying potent neuraminidase inhibition. Bioorg Med Chem Lett 18 (23):6046-6049

49. Ryu YB, Kim JH, Park SJ, Chang JS, Rho MC, Bae KH, Park KH, Lee WS (2010) Inhibition of neuraminidase activity by polyphenol compounds isolated from the roots of Glycyrrhiza uralensis. Bioorg Med Chem Lett 20 (3):971-974

50. Tian L, Wang Z, Wu H, Wang S, Wang Y, Xu J, Wang L, Qi F, Fang M, Yu D, Fang X (2011) Evaluation of the anti-neuraminidase activity of the traditional Chinese medicines and determination of the anti-influenza A virus effects of the neuraminidase inhibitory TCMs in vitro and in vivo. J Ethnopharmacol 137 (1):534-542

51. Este JA, Telenti A (2007) HIV entry inhibitors. Lancet 370 (9581):81-88

52. Hu J, Robinson JL (2010) Treatment of respiratory syncytial virus with palivizumab: a systematic review. World J Pediatr 6 (4):296-300

53. Matthews T, Salgo M, Greenberg M, Chung J, DeMasi R, Bolognesi D (2004) Enfuvirtide: the first therapy to inhibit the entry of HIV-1 into host CD4 lymphocytes. Nat Rev Drug Discov 3 (3):215-225

54. Fleury D, Barrere B, Bizebard T, Daniels RS, Skehel JJ, Knossow M (1999) A complex of influenza hemagglutinin with a neutralizing antibody that binds outside the virus receptor binding site. Nat Struct Biol 6 (6):530-534

55. 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 (2011) Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin. Proc Natl Acad Sci U S A 108 (34):14216-14221

56. 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 (2012) Cross-neutralization of influenza A viruses mediated by a single antibody loop. Nature 489 (7417):526-532

57. Yoshida R, Igarashi M, Ozaki H, Kishida N, Tomabechi D, Kida H, Ito K, Takada A (2009) Cross-protective potential of a novel monoclonal antibody directed against antigenic site B of the hemagglutinin of influenza A viruses. PLoS Pathog 5 (3):e1000350

58. Lee PS, Yoshida R, Ekiert DC, Sakai N, Suzuki Y, Takada A, Wilson IA (2012) Heterosubtypic antibody recognition of the influenza virus hemagglutinin receptor binding site enhanced by avidity. Proc Natl Acad Sci U S A 109 (42):17040-17045

59. Sun XL (2007) Recent anti-influenza strategies in multivalent sialyloligosaccharides and sialylmimetics approaches. Curr Med Chem 14 (21):2304-2313

60. Gambaryan AS, Tuzikov AB, Chinarev AA, Juneja LR, Bovin NV, Matrosovich MN (2002) Polymeric inhibitor of influenza virus attachment protects mice from experimental influenza infection. Antiviral Res 55 (1):201-205

61. Gambaryan AS, Boravleva EY, Matrosovich TY, Matrosovich MN, Klenk HD, Moiseeva EV, Tuzikov AB, Chinarev AA, Pazynina GV, Bovin NV (2005) Polymer-bound 6' sialyl-N-acetyllactosamine protects mice infected by influenza virus. Antiviral Res 68 (3):116-123

62. Guo CT, Sun XL, Kanie O, Shortridge KF, Suzuki T, Miyamoto D, Hidari KI, Wong CH, Suzuki Y (2002) An O-glycoside of sialic acid derivative that inhibits both hemagglutinin and sialidase activities of influenza viruses. Glycobiology 12 (3):183-190

63. Hendricks GL, Weirich KL, Viswanathan K, Li J, Shriver ZH, Ashour J, Ploegh HL, Kurt-Jones EA, Fygenson DK, Finberg RW, Comolli JC, Wang JP (2013) Sialylneolacto-N-tetraose c (LSTc)-bearing liposomal decoys capture influenza A virus. J Biol Chem 288 (12):8061-8073

64. Spevak W, Nagy JO, Charych DH, Schaefer ME, Gilbert JH, Bednarski MD (1993) Polymerized liposomes containing C-glycosides of sialic acid: potent inhibitors of influenza virus in vitro infectivity. J Am Chem Soc 115 (3):1146–1147

65. Job ER, Bottazzi B, Gilbertson B, Edenborough KM, Brown LE, Mantovani A, Brooks AG, Reading PC (2013) Serum amyloid P is a sialylated glycoprotein inhibitor of influenza A viruses. PLoS One 8 (3):e59623

66. Jones JC, Turpin EA, Bultmann H, Brandt CR, Schultz-Cherry S (2006) Inhibition of influenza virus infection by a novel antiviral peptide that targets viral attachment to cells. J Virol 80 (24):11960-11967

67. Nicol MQ, Ligertwood Y, Bacon MN, Dutia BM, Nash AA (2012) A novel family of peptides with potent activity against influenza A viruses. J Gen Virol 93 (Pt 5):980-986

68. Smee DF, Bailey KW, Wong MH, O'Keefe BR, Gustafson KR, Mishin VP, Gubareva LV (2008) Treatment of influenza A (H1N1) virus infections in mice and ferrets with cyanovirin-N. Antiviral Res 80 (3):266-271

69. Dey B, Lerner DL, Lusso P, Boyd MR, Elder JH, Berger EA (2000) Multiple antiviral activities of cyanovirin-N: blocking of human immunodeficiency virus type 1 gp120 interaction with CD4 and coreceptor and inhibition of diverse enveloped viruses. J Virol 74 (10):4562-4569

70. Barrientos LG, O'Keefe BR, Bray M, Sanchez A, Gronenborn AM, Boyd MR (2003) Cyanovirin-N binds to the viral surface glycoprotein, GP1,2 and inhibits infectivity of Ebola virus. Antiviral Res 58 (1):47-56

71. Helle F, Wychowski C, Vu-Dac N, Gustafson KR, Voisset C, Dubuisson J (2006) Cyanovirin-N inhibits hepatitis C virus entry by binding to envelope protein glycans. J Biol Chem 281 (35):25177-25183

72. Sato Y, Hirayama M, Morimoto K, Yamamoto N, Okuyama S, Hori K (2011) High mannose-binding lectin with preference for the cluster of alpha1-2-mannose from the green alga Boodlea coacta is a potent entry inhibitor of HIV-1 and influenza viruses. J Biol Chem 286 (22):19446-19458

73. Chen JX, Xue HJ, Ye WC, Fang BH, Liu YH, Yuan SH, Yu P, Wang YQ (2009) Activity of andrographolide and its derivatives against influenza virus in vivo and in vitro. Biol Pharm Bull 32 (8):1385-1391

74. Droebner K, Ehrhardt C, Poetter A, Ludwig S, Planz O (2007) CYSTUS052, a polyphenol-rich plant extract, exerts anti-influenza virus activity in mice. Antiviral Res 76 (1):1-10

75. Haruyama T, Nagata K (2013) Anti-influenza virus activity of Ginkgo biloba leaf extracts. J Nat Med 67 (3):636-642

76. Kwon HJ, Kim HH, Yoon SY, Ryu YB, Chang JS, Cho KO, Rho MC, Park SJ, Lee WS (2010) In vitro inhibitory activity of Alpinia katsumadai extracts against influenza virus infection and hemagglutination. Virol J 7:307

77. Lakadamyali M, Rust MJ, Zhuang X (2004) Endocytosis of influenza viruses. Microbes Infect 6 (10):929-936

78. Harrison SC (2008) Viral membrane fusion. Nat Struct Mol Biol 15 (7):690-698

79. Bodian DL, Yamasaki RB, Buswell RL, Stearns JF, White JM, Kuntz ID (1993) Inhibition of the fusion-inducing conformational change of influenza hemagglutinin by benzoquinones and hydroquinones. Biochemistry 32 (12):2967-2978

80. Hoffman LR, Kuntz ID, White JM (1997) Structure-based identification of an inducer of the low-pH conformational change in the influenza virus hemagglutinin: irreversible inhibition of infectivity. J Virol 71 (11):8808-8820

81. Luo G, Torri A, Harte WE, Danetz S, Cianci C, Tiley L, Day S, Mullaney D, Yu KL, Ouellet C, Dextraze P, Meanwell N, Colonno R, Krystal M (1997) Molecular mechanism underlying the action of a novel fusion inhibitor of influenza A virus. J Virol 71 (5):4062-4070

82. Plotch SJ, Bouloy M, Krug RM (1979) Transfer of 5'-terminal cap of globin mRNA to influenza viral complementary RNA during transcription in vitro. Proc Natl Acad Sci U S A 76 (4):1618-1622

83. Staschke KA, Hatch SD, Tang JC, Hornback WJ, Munroe JE, Colacino JM, Muesing MA (1998) Inhibition of influenza virus hemagglutinin-mediated membrane fusion by a compound related to podocarpic acid. Virology 248 (2):264-274

84. Yoshimoto J, Kakui M, Iwasaki H, Fujiwara T, Sugimoto H, Hattori N (1999) Identification of a novel HA conformational change inhibitor of human influenza virus. Arch Virol 144 (5):865-878

85. Yu KL, Torri AF, Luo G, Cianci C, Grant-Young K, Danetz S, Tiley L, Krystal M, Meanwell NA (2002) Structure-activity relationships for a series of thiobenzamide influenza fusion inhibitors derived from 1,3,3-trimethyl-5-hydroxy-cyclohexylmethylamine. Bioorg Med Chem Lett 12 (23):3379-3382

86. 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 (2011) Inhibition of influenza A virus (H1N1) fusion by benzenesulfonamide derivatives targeting viral hemagglutinin. PLoS One 6 (12):e29120

87. Boriskin YS, Leneva IA, Pecheur EI, Polyak SJ (2008) Arbidol: a broad-spectrum antiviral compound that blocks viral fusion. Curr Med Chem 15 (10):997-1005

88. Pecheur EI, Lavillette D, Alcaras F, Molle J, Boriskin YS, Roberts M, Cosset FL, Polyak SJ (2007) Biochemical mechanism of hepatitis C virus inhibition by the broad-spectrum antiviral arbidol. Biochemistry 46 (20):6050-6059

89. Leneva IA, Russell RJ, Boriskin YS, Hay AJ (2009) Characteristics of arbidol-resistant mutants of influenza virus: implications for the mechanism of anti-influenza action of arbidol. Antiviral Res 81 (2):132-140

90. Teissier E, Zandomeneghi G, Loquet A, Lavillette D, Lavergne JP, Montserret R, Cosset FL, Bockmann A, Meier BH, Penin F, Pecheur EI (2011) Mechanism of inhibition of enveloped virus membrane fusion by the antiviral drug arbidol. PLoS One 6 (1):e15874

91. 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 (2011) A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. Science 333 (6044):850-856

92. Dreyfus C, Laursen NS, Kwaks T, Zuijdgeest 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 (2012) Highly conserved protective epitopes on influenza B viruses. Science 337 (6100):1343-1348

93. Ekiert DC, Bhabha G, Elsliger MA, Friesen RH, Jongeneelen M, Throsby M, Goudsmit J, Wilson IA (2009) Antibody recognition of a highly conserved influenza virus epitope. Science 324 (5924):246-251

94. 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 (2011) A highly conserved neutralizing epitope on group 2 influenza A viruses. Science 333 (6044):843-850

95. Kashyap AK, Steel J, Rubrum A, Estelles A, Briante R, Ilyushina NA, Xu L, Swale RE, Faynboym AM, Foreman PK, Horowitz M, Horowitz L, Webby R, Palese P, Lerner RA, Bhatt RR (2010) Protection from the 2009 H1N1 pandemic influenza by an antibody from combinatorial survivor-based libraries. PLoS Pathog 6 (7):e1000990

96. 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 (2009) Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat Struct Mol Biol 16 (3):265-273

97. 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 (2008) Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. PLoS One 3 (12):e3942

98. Baba M, Snoeck R, Pauwels R, de Clercq E (1988) 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 32 (11):1742-1745

99. Hosoya M, Balzarini J, Shigeta S, De Clercq E (1991) 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 35 (12):2515-2520

100. Ito M, Baba M, Sato A, Pauwels R, De Clercq E, Shigeta S (1987) Inhibitory effect of dextran sulfate and heparin on the replication of human immunodeficiency virus (HIV) in vitro. Antiviral Res 7 (6):361-367

101. Luscher-Mattli M, Gluck R (1990) Dextran sulfate inhibits the fusion of influenza virus with model membranes, and suppresses influenza virus replication in vivo. Antiviral Res 14 (1):39-50

102. Krumbiegel M, Dimitrov DS, Puri A, Blumenthal R (1992) Dextran sulfate inhibits fusion of influenza virus and cells expressing influenza hemagglutinin with red blood cells. Biochim Biophys Acta 1110 (2):158-164

103. Kim M, Yim JH, Kim SY, Kim HS, Lee WG, Kim SJ, Kang PS, Lee CK (2012) In vitro inhibition of influenza A virus infection by marine microalga-derived sulfated polysaccharide p-KG03. Antiviral Res 93 (2):253-259

104. Leibbrandt A, Meier C, Konig-Schuster M, Weinmullner R, Kalthoff D, Pflugfelder B, Graf P, Frank-Gehrke B, Beer M, Fazekas T, Unger H, Prieschl-Grassauer E, Grassauer A (2010) Iota-carrageenan is a potent inhibitor of influenza A virus infection. PLoS One 5 (12):e14320

105. Cole AM, Hong T, Boo LM, Nguyen T, Zhao C, Bristol G, Zack JA, Waring AJ, Yang OO, Lehrer RI (2002) Retrocyclin: a primate peptide that protects cells from infection by T- and M-tropic strains of HIV-1. Proc Natl Acad Sci U S A 99 (4):1813-1818

106. Leikina E, Delanoe-Ayari H, Melikov K, Cho MS, Chen A, Waring AJ, Wang W, Xie Y, Loo JA, Lehrer RI, Chernomordik LV (2005) Carbohydrate-binding molecules inhibit viral fusion and entry by crosslinking membrane glycoproteins. Nat Immunol 6 (10):995-1001

107. Liang QL, Zhou K, He HX (2010) Retrocyclin 2: a new therapy against avian influenza H5N1 virus in vivo and vitro. Biotechnol Lett 32 (3):387-392

108. Yasin B, Wang W, Pang M, Cheshenko N, Hong T, Waring AJ, Herold BC, Wagar EA, Lehrer RI (2004) Theta defensins protect cells from infection by herpes simplex virus by inhibiting viral adhesion and entry. J Virol 78 (10):5147-5156

109. Fodor E (2013) The RNA polymerase of influenza a virus: mechanisms of viral transcription and replication. Acta Virol 57 (2):113-122

110. Gonzalez S, Zurcher T, Ortin J (1996) Identification of two separate domains in the influenza virus PB1 protein involved in the interaction with the PB2 and PA subunits: a model for the viral RNA polymerase structure. Nucleic Acids Res 24 (22):4456-4463

111. Ohtsu Y, Honda Y, Sakata Y, Kato H, Toyoda T (2002) Fine mapping of the subunit binding sites of influenza virus RNA polymerase. Microbiol Immunol 46 (3):167-175

112. Perez DR, Donis RO (1995) A 48-amino-acid region of influenza A virus PB1 protein is sufficient for complex formation with PA. J Virol 69 (11):6932-6939

113. Poole EL, Medcalf L, Elton D, Digard P (2007) Evidence that the C-terminal PB2-binding region of the influenza A virus PB1 protein is a discrete alpha-helical domain. FEBS Lett 581 (27):5300-5306

114. Hemerka JN, Wang D, Weng Y, Lu W, Kaushik RS, Jin J, Harmon AF, Li F (2009) Detection and characterization of influenza A virus PA-PB2 interaction through a bimolecular fluorescence complementation assay. J Virol 83 (8):3944-3955

115. Tisdale M, Ellis M, Klumpp K, Court S, Ford M (1995) Inhibition of influenza virus transcription by 2'-deoxy-2'-fluoroguanosine. Antimicrob Agents Chemother 39 (11):2454-2458

116. Sidwell RW, Huffman JH, Khare GP, Allen LB, Witkowski JT, Robins RK (1972) Broad-spectrum antiviral activity of Virazole: 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide. Science 177 (50):705-706

117. Eriksson B, Helgstrand E, Johansson NG, Larsson A, Misiorny A, Noren JO, Philipson L, Stenberg K, Stening G, Stridh S, Oberg B (1977) Inhibition of influenza virus ribonucleic acid polymerase by ribavirin triphosphate. Antimicrob Agents Chemother 11 (6):946-951

118. Chan-Tack KM, Murray JS, Birnkrant DB (2009) Use of ribavirin to treat influenza. N Engl J Med 361 (17):1713-1714

119. Riner A, Chan-Tack KM, Murray JS (2009) Original research: Intravenous ribavirin--review of the FDA's Emergency Investigational New Drug Database (1997-2008) and literature review. Postgrad Med 121 (3):139-146

120. Sidwell RW, Bailey KW, Wong MH, Barnard DL, Smee DF (2005) In vitro and in vivo influenza virus-inhibitory effects of viramidine. Antiviral Res 68 (1):10-17

121. Furuta Y, Takahashi K, Fukuda Y, Kuno M, Kamiyama T, Kozaki K, Nomura N, Egawa H, Minami S, Watanabe Y, Narita H, Shiraki K (2002) In vitro and in vivo activities of anti-influenza virus compound T-705. Antimicrob Agents Chemother 46 (4):977-981

122. Furuta Y, Takahashi K, Kuno-Maekawa M, Sangawa H, Uehara S, Kozaki K, Nomura N, Egawa H, Shiraki K (2005) Mechanism of action of T-705 against influenza virus. Antimicrob Agents Chemother 49 (3):981-986

123. Furuta Y, Takahashi K, Shiraki K, Sakamoto K, Smee DF, Barnard DL, Gowen BB, Julander JG, Morrey JD (2009) T-705 (favipiravir) and related compounds: Novel broad-spectrum inhibitors of RNA viral infections. Antiviral Res 82 (3):95-102

124. Furuta Y, Gowen BB, Takahashi K, Shiraki K, Smee DF, Barnard DL (2013) Favipiravir (T-705), a novel viral RNA polymerase inhibitor. Antiviral Res 100 (2):446-454

125. Dias A, Bouvier D, Crepin T, McCarthy AA, Hart DJ, Baudin F, Cusack S, Ruigrok RW (2009) The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature 458 (7240):914-918

126. Yuan P, Bartlam M, Lou Z, Chen S, Zhou J, He X, Lv Z, Ge R, Li X, Deng T, Fodor E, Rao Z, Liu Y (2009) Crystal structure of an avian influenza polymerase PA(N) reveals an endonuclease active site. Nature 458 (7240):909-913

127. Parkes KE, Ermert P, Fassler J, Ives J, Martin JA, Merrett JH, Obrecht D, Williams G, Klumpp K (2003) Use of a pharmacophore model to discover a new class of influenza endonuclease inhibitors. J Med Chem 46 (7):1153-1164

128. Tomassini J, Selnick H, Davies ME, Armstrong ME, Baldwin J, Bourgeois M, Hastings J, Hazuda D, Lewis J, McClements W, et al. (1994) Inhibition of cap (m7GpppXm)-dependent endonuclease of influenza virus by 4-substituted 2,4-dioxobutanoic acid compounds. Antimicrob Agents Chemother 38 (12):2827-2837

129. Tomassini JE, Davies ME, Hastings JC, Lingham R, Mojena M, Raghoobar SL, Singh SB, Tkacz JS, Goetz MA (1996) A novel antiviral agent which inhibits the endonuclease of influenza viruses. Antimicrob Agents Chemother 40 (5):1189-1193

130. DuBois RM, Slavish PJ, Baughman BM, Yun MK, Bao J, Webby RJ, Webb TR, White SW (2012) Structural and biochemical basis for development of influenza virus inhibitors targeting the PA endonuclease. PLoS Pathog 8 (8):e1002830

131. Kowalinski E, Zubieta C, Wolkerstorfer A, Szolar OH, Ruigrok RW, Cusack S (2012) Structural analysis of specific metal chelating inhibitor binding to the endonuclease domain of influenza pH1N1 (2009) polymerase. PLoS Pathog 8 (8):e1002831

132. Bauman JD, Patel D, Baker SF, Vijayan RS, Xiang A, Parhi AK, Martinez-Sobrido L, Lavoie EJ, Das K, Arnold E (2013) Crystallographic Fragment Screening and Structure-Based Optimization Yields a New Class of Influenza Endonuclease Inhibitors. ACS Chem Biol, in press

133. Iwai Y, Murakami K, Gomi Y, Hashimoto T, Asakawa Y, Okuno Y, Ishikawa T, Hatakeyama D, Echigo N, Kuzuhara T (2011) Anti-influenza activity of marchantins, macrocyclic bisbibenzyls contained in liverworts. PLoS One 6 (5):e19825

134. Guilligay D, Tarendeau F, Resa-Infante P, Coloma R, Crepin T, Sehr P, Lewis J, Ruigrok RW, Ortin J, Hart DJ, Cusack S (2008) The structural basis for cap binding by influenza virus polymerase subunit PB2. Nat Struct Mol Biol 15 (5):500-506

135. Giannecchini S, Clausi V, Nosi D, Azzi A (2009) Oligonucleotides derived from the packaging signal at the 5' end of the viral PB2 segment specifically inhibit influenza virus in vitro. Arch Virol 154 (5):821-832

136. Perez DR, Donis RO (2001) Functional analysis of PA binding by influenza a virus PB1: effects on polymerase activity and viral infectivity. J Virol 75 (17):8127-8136

137. Sugiyama K, Obayashi E, Kawaguchi A, Suzuki Y, Tame JR, Nagata K, Park SY (2009) Structural insight into the essential PB1-PB2 subunit contact of the influenza virus RNA polymerase. EMBO J 28 (12):1803-1811

138. Loregian A, Palu G (2005) Disruption of protein-protein interactions: towards new targets for chemotherapy. J Cell Physiol 204 (3):750-762

139. Palu G, Loregian A (2013) Inhibition of herpesvirus and influenza virus replication by blocking polymerase subunit interactions. Antiviral Res 99 (3):318-327

140. Ghanem A, Mayer D, Chase G, Tegge W, Frank R, Kochs G, Garcia-Sastre A, Schwemmle M (2007) Peptide-mediated interference with influenza A virus polymerase. J Virol 81 (14):7801-7804

141. Wunderlich K, Mayer D, Ranadheera C, Holler AS, Manz B, Martin A, Chase G, Tegge W, Frank R, Kessler U, Schwemmle M (2009) Identification of a PA-binding peptide with inhibitory activity against influenza A and B virus replication. PLoS One 4 (10):e7517

142. He X, Zhou J, Bartlam M, Zhang R, Ma J, Lou Z, Li X, Li J, Joachimiak A, Zeng Z, Ge R, Rao Z, Liu Y (2008) Crystal structure of the polymerase PA(C)-PB1(N) complex from an avian influenza H5N1 virus. Nature 454 (7208):1123-1126

143. Obayashi E, Yoshida H, Kawai F, Shibayama N, Kawaguchi A, Nagata K, Tame JR, Park SY (2008) The structural basis for an essential subunit interaction in influenza virus RNA polymerase. Nature 454 (7208):1127-1131

144. Muratore G, Goracci L, Mercorelli B, Foeglein A, Digard P, Cruciani G, Palu G, Loregian A (2012) Small molecule inhibitors of influenza A and B viruses that act by disrupting subunit interactions of the viral polymerase. Proceedings of the National Academy of Sciences of the United States of America 109 (16):6247-6252

145. Loregian A, Coen DM (2006) Selective anti-cytomegalovirus compounds discovered by screening for inhibitors of subunit interactions of the viral polymerase. Chem Biol 13 (2):191-200

146. Muratore G, Mercorelli B, Goracci L, Cruciani G, Digard P, Palu G, Loregian A (2012) Human cytomegalovirus inhibitor AL18 also possesses activity against influenza A and B viruses. Antimicrob Agents Chemother 56 (11):6009-6013

147. Fukuoka M, Minakuchi M, Kawaguchi A, Nagata K, Kamatari YO, Kuwata K (2012) Structure-based discovery of anti-influenza virus A compounds among medicines. Biochimica et Biophysica Acta 1820 (2):90-95

148. Kessler U, Castagnolo D, Pagano M, Deodato D, Bernardini M, Pilger B, Ranadheera C, Botta M (2013) Discovery and synthesis of novel benzofurazan derivatives as inhibitors of influenza A virus. Bioorg Med Chem Lett 23 (20):5575-5577

149. Chase G, Wunderlich K, Reuther P, Schwemmle M (2011) Identification of influenza virus inhibitors which disrupt of viral polymerase protein-protein interactions. Methods 55 (2):188-191

150. Li C, Ba Q, Wu A, Zhang H, Deng T, Jiang T (2013) A peptide derived from the C-terminus of PB1 inhibits influenza virus replication by interfering with viral polymerase assembly. FEBS J 280 (4):1139-1149

151. Cheng H, Wan J, Lin MI, Liu Y, Lu X, Liu J, Xu Y, Chen J, Tu Z, Cheng YS, Ding K (2012) 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 55 (5):2144-2153

152. Hung HC, Liu CL, Hsu JT, Horng JT, Fang MY, Wu SY, Ueng SH, Wang MY, Yaw CW, Hou MH (2012) Development of an anti-influenza drug screening assay targeting nucleoproteins with tryptophan fluorescence quenching. Anal Chem 84 (15):6391-6399

153. 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 (2010) Identification of influenza A nucleoprotein as an antiviral target. Nat Biotechnol 28 (6):600-605

154. Shen YF, Chen YH, Chu SY, Lin MI, Hsu HT, Wu PY, Wu CJ, Liu HW, Lin FY, Lin G, Hsu PH, Yang AS, Cheng YS, Wu YT, Wong CH, Tsai MD (2011) E339...R416 salt bridge of nucleoprotein as a feasible target for influenza virus inhibitors. Proc Natl Acad Sci U S A 108 (40):16515-16520

155. 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 (2010) High-throughput identification of compounds targeting influenza RNA-dependent RNA polymerase activity. Proc Natl Acad Sci U S A 107 (45):19151-19156

156. Lejal N, Tarus B, Bouguyon E, Chenavas S, Bertho N, Delmas B, Ruigrok RW, Di Primo C, Slama-Schwok A (2013) Structure-based discovery of the novel antiviral properties of naproxen against the nucleoprotein of influenza A virus. Antimicrob Agents Chemother 57 (5):2231-2242

157. Lin L, Liu Q, Berube N, Detmer S, Zhou Y (2012) 5'-Triphosphate-short interfering RNA: potent inhibition of influenza A virus infection by gene silencing and RIG-I activation. J Virol 86 (19):10359-10369

158. Tompkins SM, Lo CY, Tumpey TM, Epstein SL (2004) Protection against lethal influenza virus challenge by RNA interference in vivo. Proc Natl Acad Sci U S A 101 (23):8682-8686

159. Chenavas S, Estrozi LF, Slama-Schwok A, Delmas B, Di Primo C, Baudin F, Li X, Crepin T, Ruigrok RW (2013) Monomeric nucleoprotein of influenza A virus. PLoS Pathog 9 (3):e1003275

160. Amorim MJ, Kao RY, Digard P (2013) Nucleozin targets cytoplasmic trafficking of viral ribonucleoprotein-Rab11 complexes in influenza A virus infection. J Virol 87 (8):4694-4703

161. Wang Z, Liu X, Zhao Z, Xu C, Zhang K, Chen C, Sun L, Gao GF, Ye X, Liu W (2011) Cyclophilin E functions as a negative regulator to influenza virus replication by impairing the formation of the viral ribonucleoprotein complex. PLoS One 6 (8):e22625

162. Verhelst J, Parthoens E, Schepens B, Fiers W, Saelens X (2012) Interferon-inducible protein Mx1 inhibits influenza virus by interfering with functional viral ribonucleoprotein complex assembly. J Virol 86 (24):13445-13455

163. Hale BG, Randall RE, Ortin J, Jackson D (2008) The multifunctional NS1 protein of influenza A viruses. J Gen Virol 89 (Pt 10):2359-2376

164. Ayllon J, Hale BG, Garcia-Sastre A (2012) Strain-specific contribution of NS1-activated phosphoinositide 3-kinase signaling to influenza A virus replication and virulence. J Virol 86 (9):5366-5370

165. Marc D, Barbachou S, Soubieux D (2013) The RNA-binding domain of influenzavirus non-structural protein-1 cooperatively binds to virus-specific RNA sequences in a structure-dependent manner. Nucleic Acids Res 41 (1):434-449

166. Bergmann M, Garcia-Sastre A, Carnero E, Pehamberger H, Wolff K, Palese P, Muster T (2000) Influenza virus NS1 protein counteracts PKR-mediated inhibition of replication. J Virol 74 (13):6203-6206

167. Min JY, Krug RM (2006) The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2'-5' oligo (A) synthetase/RNase L pathway. Proc Natl Acad Sci U S A 103 (18):7100-7105

168. Ramos I, Carnero E, Bernal-Rubio D, Seibert CW, Westera L, Garcia-Sastre A, Fernandez-Sesma A (2013) Contribution of double-stranded RNA and CPSF30 binding domains of influenza virus NS1 to the inhibition of type I interferon production and activation of human dendritic cells. J Virol 87 (5):2430-2440

169. Gack MU, Albrecht RA, Urano T, Inn KS, Huang IC, Carnero E, Farzan M, Inoue S, Jung JU, Garcia-Sastre A (2009) Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. Cell Host Microbe 5 (5):439-449

170. Zhu Z, Shi Z, Yan W, Wei J, Shao D, Deng X, Wang S, Li B, Tong G, Ma Z (2013) Nonstructural protein 1 of influenza A virus interacts with human guanylate-binding protein 1 to antagonize antiviral activity. PLoS One 8 (2):e55920

171. Gao S, Song L, Li J, Zhang Z, Peng H, Jiang W, Wang Q, Kang T, Chen S, Huang W (2012) Influenza A virus-encoded NS1 virulence factor protein inhibits innate immune response by targeting IKK. Cell Microbiol 14 (12):1849-1866

172. Santos A, Pal S, Chacon J, Meraz K, Gonzalez J, Prieto K, Rosas-Acosta G (2013) SUMOylation affects the interferon blocking activity of the influenza A nonstructural protein NS1 without affecting its stability or cellular localization. J Virol 87 (10):5602-5620

173. Wang W, Riedel K, Lynch P, Chien CY, Montelione GT, Krug RM (1999) RNA binding by the novel helical domain of the influenza virus NS1 protein requires its dimer structure and a small number of specific basic amino acids. RNA 5 (2):195-205

174. Basu D, Walkiewicz MP, Frieman M, Baric RS, Auble DT, Engel DA (2009) Novel influenza virus NS1 antagonists block replication and restore innate immune function. J Virol 83 (4):1881-1891

175. Jablonski JJ, Basu D, Engel DA, Geysen HM (2012) Design, synthesis, and evaluation of novel small molecule inhibitors of the influenza virus protein NS1. Bioorg Med Chem 20 (1):487-497

176. Walkiewicz MP, Basu D, Jablonski JJ, Geysen HM, Engel DA (2011) Novel inhibitor of influenza non-structural protein 1 blocks multi-cycle replication in an RNase L-dependent manner. J Gen Virol 92 (Pt 1):60-70

177. Ai H, Zheng F, Zhu C, Sun T, Zhang L, Liu X, Li X, Zhu G, Liu H (2010) Discovery of novel influenza inhibitors targeting the interaction of dsRNA with the NS1 protein by structure-based virtual screening. Int J Bioinform Res Appl 6 (5):449-460

178. Maroto M, Fernandez Y, Ortin J, Pelaez F, Cabello MA (2008) Development of an HTS assay for the search of anti-influenza agents targeting the interaction of viral RNA with the NS1 protein. J Biomol Screen 13 (7):581-590

179. Cho EJ, Xia S, Ma LC, Robertus J, Krug RM, Anslyn EV, Montelione GT, Ellington AD (2012) Identification of influenza virus inhibitors targeting NS1A utilizing fluorescence polarization-based high-throughput assay. J Biomol Screen 17 (4):448-459

180. You L, Cho EJ, Leavitt J, Ma LC, Montelione GT, Anslyn EV, Krug RM, Ellington A, Robertus JD (2011) Synthesis and evaluation of quinoxaline derivatives as potential influenza NS1A protein inhibitors. Bioorg Med Chem Lett 21 (10):3007-3011

181. Ilyushina NA, Hoffmann E, Salomon R, Webster RG, Govorkova EA (2007) Amantadine-oseltamivir combination therapy for H5N1 influenza virus infection in mice. Antivir Ther 12 (3):363-370

182. Smee DF, Hurst BL, Wong MH, Bailey KW, Tarbet EB, Morrey JD, Furuta Y (2010) Effects of the combination of favipiravir (T-705) and oseltamivir on influenza A virus infections in mice. Antimicrob Agents Chemother 54 (1):126-133

183. Ilyushina NA, Bovin NV, Webster RG, Govorkova EA (2006) Combination chemotherapy, a potential strategy for reducing the emergence of drug-resistant influenza A variants. Antiviral Res 70 (3):121-131

184. Ison MG, Gnann JW, Jr., Nagy-Agren S, Treannor J, Paya C, Steigbigel R, Elliott M, Weiss HL, Hayden FG (2003) Safety and efficacy of nebulized zanamivir in hospitalized patients with serious influenza. Antivir Ther 8 (3):183-190

185. 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 (2010) Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication. Nature 463 (7282):818-822

186. 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 (2010) Human host factors required for influenza virus replication. Nature 463 (7282):813-817

187. Muller KH, Kakkola L, Nagaraj AS, Cheltsov AV, Anastasina M, Kainov DE (2012) Emerging cellular targets for influenza antiviral agents. Trends Pharmacol Sci 33 (2):89-99

188. Shaw ML (2011) The host interactome of influenza virus presents new potential targets for antiviral drugs. Rev Med Virol 21 (6):358-369

189. Jorba N, Juarez S, Torreira E, Gastaminza P, Zamarreno N, Albar JP, Ortin J (2008) Analysis of the interaction of influenza virus polymerase complex with human cell factors. Proteomics 8 (10):2077-2088

190. Shapira SD, Gat-Viks I, Shum BO, Dricot A, de Grace MM, Wu L, Gupta PB, Hao T, Silver SJ, Root DE, Hill DE, Regev A, Hacohen N (2009) A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection. Cell 139 (7):1255-1267

191. Tafforeau L, Chantier T, Pradezynski F, Pellet J, Mangeot PE, Vidalain PO, Andre P, Rabourdin-Combe C, Lotteau V (2011) Generation and comprehensive analysis of an influenza virus polymerase cellular interaction network. J Virol 85 (24):13010-13018

192. Bottcher E, Matrosovich T, Beyerle M, Klenk HD, Garten W, Matrosovich M (2006) Proteolytic activation of influenza viruses by serine proteases TMPRSS2 and HAT from human airway epithelium. J Virol 80 (19):9896-9898

193. Bottcher E, Freuer C, Steinmetzer T, Klenk HD, Garten W (2009) MDCK cells that express proteases TMPRSS2 and HAT provide a cell system to propagate influenza viruses in the absence of trypsin and to study cleavage of HA and its inhibition. Vaccine 27 (45):6324-6329

194. Bottcher-Friebertshauser E, Freuer C, Sielaff F, Schmidt S, Eickmann M, Uhlendorff J, Steinmetzer T, Klenk HD, Garten W (2010) Cleavage of influenza virus hemagglutinin by airway proteases TMPRSS2 and HAT differs in subcellular localization and susceptibility to protease inhibitors. J Virol 84 (11):5605-5614

195. Chaipan C, Kobasa D, Bertram S, Glowacka I, Steffen I, Tsegaye TS, Takeda M, Bugge TH, Kim S, Park Y, Marzi A, Pohlmann S (2009) Proteolytic activation of the 1918 influenza virus hemagglutinin. J Virol 83 (7):3200-3211

196. Kido H, Okumura Y, Yamada H, Le TQ, Yano M (2007) Proteases essential for human influenza virus entry into cells and their inhibitors as potential therapeutic agents. Curr Pharm Des 13 (4):405-414

197. Ovcharenko AV, Zhirnov OP (1994) Aprotinin aerosol treatment of influenza and paramyxovirus bronchopneumonia of mice. Antiviral Res 23 (2):107-118

198. Zhirnov OP, Klenk HD, Wright PF (2011) Aprotinin and similar protease inhibitors as drugs against influenza. Antiviral Res 92 (1):27-36

199. Sielaff F, Bottcher-Friebertshauser E, Meyer D, Saupe SM, Volk IM, Garten W, Steinmetzer T (2011) Development of substrate analogue inhibitors for the human airway trypsin-like protease HAT. Bioorg Med Chem Lett 21 (16):4860-4864

200. Meyer D, Sielaff F, Hammami M, Bottcher-Friebertshauser E, Garten W, Steinmetzer T (2013) Identification of the first synthetic inhibitors of the type II transmembrane serine protease TMPRSS2 suitable for inhibition of influenza virus activation. Biochem J 452 (2):331-343

201. Kido H, Yokogoshi Y, Sakai K, Tashiro M, Kishino Y, Fukutomi A, Katunuma N (1992) Isolation and characterization of a novel trypsin-like protease found in rat bronchiolar epithelial Clara cells. A possible activator of the viral fusion glycoprotein. J Biol Chem 267 (19):13573-13579

202. Murakami M, Towatari T, Ohuchi M, Shiota M, Akao M, Okumura Y, Parry MA, Kido H (2001) Mini-plasmin found in the epithelial cells of bronchioles triggers infection by broad-spectrum influenza A viruses and Sendai virus. Eur J Biochem 268 (10):2847-2855

203. Towatari T, Ide M, Ohba K, Chiba Y, Murakami M, Shiota M, Kawachi M, Yamada H, Kido H (2002) Identification of ectopic anionic trypsin I in rat lungs potentiating pneumotropic virus infectivity and increased enzyme level after virus infection. Eur J Biochem 269 (10):2613-2621

204. Fritz H, Wunderer G (1983) Biochemistry and applications of aprotinin, the kallikrein inhibitor from bovine organs. Arzneimittelforschung 33 (4):479-494

205. Zhirnov OP, Matrosovich TY, Matrosovich MN, Klenk HD (2011) Aprotinin, a protease inhibitor, suppresses proteolytic activation of pandemic H1N1v influenza virus. Antivir Chem Chemother 21 (4):169-174206. Zhirnov OP, Ovcharenko AV, Bukrinskaya AG (1985) Myxovirus replication in chicken embryos can be suppressed by aprotinin due to the blockage of viral glycoprotein cleavage. J Gen Virol 66 (Pt 7):1633-1638

207. Pan HY, Yamada H, Chida J, Wang S, Yano M, Yao M, Zhu J, Kido H (2011) Up-regulation of ectopic trypsins in the myocardium by influenza A virus infection triggers acute myocarditis. Cardiovasc Res 89 (3):595-603

208. Zhirnov OP, Ikizler MR, Wright PF (2002) Cleavage of influenza a virus hemagglutinin in human respiratory epithelium is cell associated and sensitive to exogenous antiproteases. J Virol 76 (17):8682-8689

209. Hosoya M, Shigeta S, Ishii T, Suzuki H, De Clercq E (1993) Comparative inhibitory effects of various nucleoside and nonnucleoside analogues on replication of influenza virus types A and B in vitro and in ovo. J Infect Dis 168 (3):641-646

210. Jie Z, Cai Y, Yang W, Jin M, Zhu W, Zhu C (2003) Protective effects of alpha 1-antitrypsin on acute lung injury in rabbits induced by endotoxin. Chin Med J (Engl) 116 (11):1678-1682

211. Stieneke-Grober A, Vey M, Angliker H, Shaw E, Thomas G, Roberts C, Klenk HD, Garten W (1992) Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J 11 (7):2407-2414

212. Becker GL, Lu Y, Hardes K, Strehlow B, Levesque C, Lindberg I, Sandvig K, Bakowsky U, Day R, Garten W, Steinmetzer T (2012) Highly potent inhibitors of proprotein convertase furin as potential drugs for treatment of infectious diseases. J Biol Chem 287 (26):21992-22003

213. Becker GL, Sielaff F, Than ME, Lindberg I, Routhier S, Day R, Lu Y, Garten W, Steinmetzer T (2010) Potent inhibitors of furin and furin-like proprotein convertases containing decarboxylated P1 arginine mimetics. J Med Chem 53 (3):1067-1075

214. 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 (2006) Sialidase fusion

protein as a novel broad-spectrum inhibitor of influenza virus infection. Antimicrob Agents Chemother 50 (4):1470-1479

215. Triana-Baltzer GB, Sanders RL, Hedlund M, Jensen KA, Aschenbrenner LM, Larson JL, Fang F (2011) Phenotypic and genotypic characterization of influenza virus mutants selected with the sialidase fusion protein DAS181. J Antimicrob Chemother 66 (1):15-28

216. Triana-Baltzer GB, Gubareva LV, Klimov AI, Wurtman DF, Moss RB, Hedlund M, Larson JL, Belshe RB, Fang F (2009) Inhibition of neuraminidase inhibitor-resistant influenza virus by DAS181, a novel sialidase fusion protein. PLoS One 4 (11):e7838

217. Nicholls JM, Aschenbrenner LM, Paulson JC, Campbell ER, Malakhov MP, Wurtman DF, Yu M, Fang F (2008) Comment on: concerns of using sialidase fusion protein as an experimental drug to combat seasonal and pandemic influenza. J Antimicrob Chemother 62 (2):426-428

218. Moss RB, Hansen C, Sanders RL, Hawley S, Li T, Steigbigel RT (2012) A phase II study of DAS181, a novel host directed antiviral for the treatment of influenza infection. J Infect Dis 206 (12):1844-1851

219. Rust MJ, Lakadamyali M, Zhang F, Zhuang X (2004) Assembly of endocytic machinery around individual influenza viruses during viral entry. Nat Struct Mol Biol 11 (6):567-573

220. Matlin KS, Reggio H, Helenius A, Simons K (1981) Infectious entry pathway of influenza virus in a canine kidney cell line. J Cell Biol 91 (3 Pt 1):601-613

221. Yoshimura A, Kuroda K, Kawasaki K, Yamashina S, Maeda T, Ohnishi S (1982) Infectious cell entry mechanism of influenza virus. J Virol 43 (1):284-293

222. Eierhoff T, Hrincius ER, Rescher U, Ludwig S, Ehrhardt C (2010) The epidermal growth factor receptor (EGFR) promotes uptake of influenza A viruses (IAV) into host cells. PLoS Pathog 6 (9):e1001099

223. Forgac M (2007) Vacuolar ATPases: rotary proton pumps in physiology and pathophysiology. Nat Rev Mol Cell Biol 8 (11):917-929

224. Hinton A, Bond S, Forgac M (2009) V-ATPase functions in normal and disease processes. Pflugers Arch 457 (3):589-598

225. Muller KH, Kainov DE, El Bakkouri K, Saelens X, De Brabander JK, Kittel C, Samm E, Muller CP (2011) The proton translocation domain of cellular vacuolar ATPase provides a target for the treatment of influenza A virus infections. Br J Pharmacol 164 (2):344-357

226. Guinea R, Carrasco L (1995) Requirement for vacuolar proton-ATPase activity during entry of influenza virus into cells. J Virol 69 (4):2306-2312

227. Bowman BJ, Bowman EJ (2002) Mutations in subunit C of the vacuolar ATPase confer resistance to bafilomycin and identify a conserved antibiotic binding site. J Biol Chem 277 (6):3965-3972

228. Bowman EJ, Graham LA, Stevens TH, Bowman BJ (2004) The bafilomycin/concanamycin binding site in subunit c of the V-ATPases from Neurospora crassa and Saccharomyces cerevisiae. J Biol Chem 279 (32):33131-33138

229. Chen HW, Cheng JX, Liu MT, King K, Peng JY, Zhang XQ, Wang CH, Shresta S, Schooley RT, Liu YT (2013) Inhibitory and combinatorial effect of diphyllin, a v-ATPase blocker, on influenza viruses. Antiviral Res 99 (3):371-382

230. Khor R, McElroy LJ, Whittaker GR (2003) The ubiquitin-vacuolar protein sorting system is selectively required during entry of influenza virus into host cells. Traffic 4 (12):857-868

231. Widjaja I, de Vries E, Tscherne DM, Garcia-Sastre A, Rottier PJ, de Haan CA (2010) Inhibition of the ubiquitin-proteasome system affects influenza A virus infection at a postfusion step. J Virol 84 (18):9625-9631

232. Kulka RG, Raboy B, Schuster R, Parag HA, Diamond G, Ciechanover A, Marcus M (1988) A Chinese hamster cell cycle mutant arrested at G2 phase has a temperature-sensitive ubiquitin-activating enzyme, E1. J Biol Chem 263 (30):15726-15731

233. Dudek SE, Luig C, Pauli EK, Schubert U, Ludwig S (2010) The clinically approved proteasome inhibitor PS-341 efficiently blocks influenza A virus and vesicular stomatitis virus propagation by establishing an antiviral state. J Virol 84 (18):9439-9451

234. Haasbach E, Pauli EK, Spranger R, Mitzner D, Schubert U, Kircheis R, Planz O (2011) Antiviral activity of the proteasome inhibitor VL-01 against influenza A viruses. Antiviral Res 91 (3):304-313

235. Nagata K, Kawaguchi A, Naito T (2008) Host factors for replication and transcription of the influenza virus genome. Rev Med Virol 18 (4):247-260

236. Zhang J, Li G, Ye X (2010) Cyclin T1/CDK9 interacts with influenza A virus polymerase and facilitates its association with cellular RNA polymerase II. J Virol 84 (24):12619-12627

237. Li OT, Poon LL (2011) DNA intercalator stimulates influenza transcription and virus replication. Virol J 8:120

238. Tamm I, Folkers K, Horsfall FL, Jr. (1953) Inhibition of influenza virus multiplication by alkyl derivatives of benzimidazole. II. Measurement of inhibitory activity by hemagglutination titrations. J Exp Med 98 (3):229-243

239. Canduri F, Perez PC, Caceres RA, de Azevedo WF, Jr. (2008) CDK9 a potential target for drug development. Med Chem 4 (3):210-218

240. Wang S, Fischer PM (2008) Cyclin-dependent kinase 9: a key transcriptional regulator and potential drug target in oncology, virology and cardiology. Trends Pharmacol Sci 29 (6):302-313

241. Randjelovic J, Eric S, Savic V (2013) Computational study and peptide inhibitors design for the CDK9 - cyclin T1 complex. J Mol Model 19 (4):1711-1725

242. Bier K, York A, Fodor E (2011) Cellular cap-binding proteins associate with influenza virus mRNAs. J Gen Virol 92 (Pt 7):1627-1634

243. Twu KY, Noah DL, Rao P, Kuo RL, Krug RM (2006) The CPSF30 binding site on the NS1A protein of influenza A virus is a potential antiviral target. J Virol 80 (8):3957-3965

244. Amorim MJ, Read EK, Dalton RM, Medcalf L, Digard P (2007) Nuclear export of influenza A virus mRNAs requires ongoing RNA polymerase II activity. Traffic 8 (1):1-11

245. Naito T, Momose F, Kawaguchi A, Nagata K (2007) Involvement of Hsp90 in assembly and nuclear import of influenza virus RNA polymerase subunits. J Virol 81 (3):1339-1349

246. Chase G, Deng T, Fodor E, Leung BW, Mayer D, Schwemmle M, Brownlee G (2008) Hsp90 inhibitors reduce influenza virus replication in cell culture. Virology 377 (2):431-439

247. Chen J, Huang S, Chen Z (2010) Human cellular protein nucleoporin hNup98 interacts with influenza A virus NS2/nuclear export protein and overexpression of its GLFG repeat domain can inhibit virus propagation. J Gen Virol 91 (Pt 10):2474-2484

248. Watanabe K, Takizawa N, Katoh M, Hoshida K, Kobayashi N, Nagata K (2001) Inhibition of nuclear export of ribonucleoprotein complexes of influenza virus by leptomycin B. Virus Res 77 (1):31-42

249. Watanabe K, Takatsuki H, Sonoda M, Tamura S, Murakami N, Kobayashi N (2011) Anti-influenza viral effects of novel nuclear export inhibitors from Valerianae Radix and Alpinia galanga. Drug Discov Ther 5 (1):26-31

250. Watanabe K, Takizawa N, Noda S, Tsukahara F, Maru Y, Kobayashi N (2008) Hsc70 regulates the nuclear export but not the import of influenza viral RNP: A possible target for the development of anti-influenza virus drugs. Drug Discov Ther 2 (2):77-84

251. Alamares-Sapuay JG, Martinez-Gil L, Stertz S, Miller MS, Shaw ML, Palese P (2013) Serum- and glucocorticoid-regulated kinase 1 is required for nuclear export of the ribonucleoprotein of influenza A virus. J Virol 87 (10):6020-6026

252. Yang SH, Sharrocks AD, Whitmarsh AJ (2013) MAP kinase signalling cascades and transcriptional regulation. Gene 513 (1):1-13

253. Pleschka S, Wolff T, Ehrhardt C, Hobom G, Planz O, Rapp UR, Ludwig S (2001) Influenza virus propagation is impaired by inhibition of the Raf/MEK/ERK signalling cascade. Nat Cell Biol 3 (3):301-305

254. Kujime K, Hashimoto S, Gon Y, Shimizu K, Horie T (2000) p38 mitogen-activated protein kinase and c-jun-NH2-terminal kinase regulate RANTES production by influenza virus-infected human bronchial epithelial cells. J Immunol 164 (6):3222-3228

255. Marjuki H, Alam MI, Ehrhardt C, Wagner R, Planz O, Klenk HD, Ludwig S, Pleschka S (2006) Membrane accumulation of influenza A virus hemagglutinin triggers nuclear export of the viral genome via protein kinase Calpha-mediated activation of ERK signaling. J Biol Chem 281 (24):16707-16715

256. Nencioni L, Iuvara A, Aquilano K, Ciriolo MR, Cozzolino F, Rotilio G, Garaci E, Palamara AT (2003) Influenza A virus replication is dependent on an antioxidant pathway that involves GSH and Bcl-2. FASEB J 17 (6):758-760

257. Nencioni L, De Chiara G, Sgarbanti R, Amatore D, Aquilano K, Marcocci ME, Serafino A, Torcia M, Cozzolino F, Ciriolo MR, Garaci E, Palamara AT (2009) Bcl-2 expression and p38MAPK activity in cells infected with influenza A virus: impact on virally induced apoptosis and viral replication. J Biol Chem 284 (23):16004-16015

258. Go YM, Kang SM, Roede JR, Orr M, Jones DP (2011) Increased inflammatory signaling and lethality of influenza H1N1 by nuclear thioredoxin-1. PLoS One 6 (4):e18918

259. Nencioni L, Sgarbanti R, De Chiara G, Garaci E, Palamara AT (2007) Influenza virus and redox mediated cell signaling: a complex network of virus/host interaction. New Microbiol 30 (4):367-375

260. Shin YK, Liu Q, Tikoo SK, Babiuk LA, Zhou Y (2007) Influenza A virus NS1 protein activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway by direct interaction with the p85 subunit of PI3K. J Gen Virol 88 (Pt 1):13-18

261. Droebner K, Pleschka S, Ludwig S, Planz O (2011) Antiviral activity of the MEK-inhibitor U0126 against pandemic H1N1v and highly pathogenic avian influenza virus in vitro and in vivo. Antiviral Res 92 (2):195-203

262. Ludwig S, Wolff T, Ehrhardt C, Wurzer WJ, Reinhardt J, Planz O, Pleschka S (2004) MEK inhibition impairs influenza B virus propagation without emergence of resistant variants. FEBS Lett 561 (1-3):37-43

263. De Flora S, Grassi C, Carati L (1997) Attenuation of influenza-like symptomatology and improvement of cell-mediated immunity with long-term N-acetylcysteine treatment. Eur Respir J 10 (7):1535-1541

264. Geiler J, Michaelis M, Naczk P, Leutz A, Langer K, Doerr HW, Cinatl J, Jr. (2010) N-acetyl-L-cysteine (NAC) inhibits virus replication and expression of pro-inflammatory molecules in A549 cells infected with highly pathogenic H5N1 influenza A virus. Biochem Pharmacol 79 (3):413-420

265. Michaelis M, Geiler J, Naczk P, Sithisarn P, Leutz A, Doerr HW, Cinatl J, Jr. (2011) Glycyrrhizin exerts antioxidative effects in H5N1 influenza A virus-infected cells and inhibits virus replication and pro-inflammatory gene expression. PLoS One 6 (5):e19705

266. Cai J, Chen Y, Seth S, Furukawa S, Compans RW, Jones DP (2003) Inhibition of influenza infection by glutathione. Free Radic Biol Med 34 (7):928-936

267. Sgarbanti R, Nencioni L, Amatore D, Coluccio P, Fraternale A, Sale P, Mammola CL, Carpino G, Gaudio E, Magnani M, Ciriolo MR, Garaci E, Palamara AT (2011) Redox regulation of the influenza hemagglutinin maturation process: a new cell-mediated strategy for anti-influenza therapy. Antioxid Redox Signal 15 (3):593-606

268. Fioravanti R, Celestino I, Costi R, Cuzzucoli Crucitti G, Pescatori L, Mattiello L, Novellino E, Checconi P, Palamara AT, Nencioni L, Di Santo R (2012) Effects of polyphenol compounds on influenza A virus replication and definition of their mechanism of action. Bioorg Med Chem 20 (16):5046-5052

269. Haasbach E, Hartmayer C, Planz O (2013) Combination of MEK inhibitors and oseltamivir leads to synergistic antiviral effects after influenza A virus infection in vitro. Antiviral Res 98 (2):319-324

270. Kunzelmann K, Beesley AH, King NJ, Karupiah G, Young JA, Cook DI (2000) Influenza virus inhibits amiloride-sensitive Na+ channels in respiratory epithelia. Proc Natl Acad Sci U S A 97 (18):10282-10287

271. Ludwig S, Planz O, Pleschka S, Wolff T (2003) Influenza-virus-induced signaling cascades: targets for antiviral therapy? Trends Mol Med 9 (2):46-52

272. Root CN, Wills EG, McNair LL, Whittaker GR (2000) Entry of influenza viruses into cells is inhibited by a highly specific protein kinase C inhibitor. J Gen Virol 81 (Pt 11):2697-2705

273. Mahmoudian S, Auerochs S, Grone M, Marschall M (2009) Influenza A virus proteins PB1 and NS1 are subject to functionally important phosphorylation by protein kinase C. J Gen Virol 90 (Pt 6):1392-1397

274. Mitzner D, Dudek SE, Studtrucker N, Anhlan D, Mazur I, Wissing J, Jansch L, Wixler L, Bruns K, Sharma A, Wray V, Henklein P, Ludwig S, Schubert U (2009) Phosphorylation of the influenza A virus protein PB1-F2 by PKC is crucial for apoptosis promoting functions in monocytes. Cell Microbiol 11 (10):1502-1516

275. Reinhardt J, Wolff T (2000) The influenza A virus M1 protein interacts with the cellular receptor of activated C kinase (RACK) 1 and can be phosphorylated by protein kinase C. Vet Microbiol 74 (1-2):87-100

276. Hoffmann HH, Palese P, Shaw ML (2008) Modulation of influenza virus replication by alteration of sodium ion transport and protein kinase C activity. Antiviral Res 80 (2):124-134

277. Rossignol JF, La Frazia S, Chiappa L, Ciucci A, Santoro MG (2009) Thiazolides, a new class of anti-influenza molecules targeting viral hemagglutinin at the post-translational level. J Biol Chem 284 (43):29798-29808

278. Ehrhardt C, Marjuki H, Wolff T, Nurnberg B, Planz O, Pleschka S, Ludwig S (2006) Bivalent role of the phosphatidylinositol-3-kinase (PI3K) during influenza virus infection and host cell defence. Cell Microbiol 8 (8):1336-1348

279. Ehrhardt C, Wolff T, Ludwig S (2007) Activation of phosphatidylinositol 3-kinase signaling by the nonstructural NS1 protein is not conserved among type A and B influenza viruses. J Virol 81 (21):12097-12100

280. Hale BG, Jackson D, Chen YH, Lamb RA, Randall RE (2006) Influenza A virus NS1 protein binds p85beta and activates phosphatidylinositol-3-kinase signaling. Proc Natl Acad Sci U S A 103 (38):14194-14199

281. Ayllon J, Garcia-Sastre A, Hale BG (2012) Influenza A viruses and PI3K: are there time, place and manner restrictions? Virulence 3 (4):411-414

282. Zhou Z, Jiang X, Liu D, Fan Z, Hu X, Yan J, Wang M, Gao GF (2009) Autophagy is involved in influenza A virus replication. Autophagy 5 (3):321-328

283. Shin YK, Liu Q, Tikoo SK, Babiuk LA, Zhou Y (2007) Effect of the phosphatidylinositol 3-kinase/Akt pathway on influenza A virus propagation. J Gen Virol 88 (Pt 3):942-950

284. Murray JL, McDonald NJ, Sheng J, Shaw MW, Hodge TW, Rubin DH, O'Brien WA, Smee DF (2012) Inhibition of influenza A virus replication by antagonism of a PI3K-AKT-mTOR pathway member identified by gene-trap insertional mutagenesis. Antivir Chem Chemother 22 (5):205-215

285. Kurtz JE, Ray-Coquard I (2012) PI3 kinase inhibitors in the clinic: an update. Anticancer Res 32 (7):2463-2470

286. Martinez-Gil L, Alamares-Sapuay JG, Ramana Reddy MV, Goff PH, Premkumar Reddy E, Palese P (2013) A small molecule multi-kinase inhibitor reduces influenza A virus replication by restricting viral RNA synthesis. Antiviral Res 100 (1):29-37

287. Bonizzi G, Karin M (2004) The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol 25 (6):280-288

288. Mazur I, Wurzer WJ, Ehrhardt C, Pleschka S, Puthavathana P, Silberzahn T, Wolff T, Planz O, Ludwig S (2007) Acetylsalicylic acid (ASA) blocks influenza virus propagation via its NF-kappaB-inhibiting activity. Cell Microbiol 9 (7):1683-1694

289. Nimmerjahn F, Dudziak D, Dirmeier U, Hobom G, Riedel A, Schlee M, Staudt LM, Rosenwald A, Behrends U, Bornkamm GW, Mautner J (2004) Active NF-kappaB signalling is a prerequisite for influenza virus infection. J Gen Virol 85 (Pt 8):2347-2356

290. Wurzer WJ, Ehrhardt C, Pleschka S, Berberich-Siebelt F, Wolff T, Walczak H, Planz O, Ludwig S (2004) NF-kappaB-dependent induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL is crucial for efficient influenza virus propagation. J Biol Chem 279 (30):30931-30937

291. Flory E, Kunz M, Scheller C, Jassoy C, Stauber R, Rapp UR, Ludwig S (2000) Influenza virus-induced NF-kappaB-dependent gene expression is mediated by overexpression of viral proteins and involves oxidative radicals and activation of IkappaB kinase. J Biol Chem 275 (12):8307-8314

292. Ehrhardt C, Ruckle A, Hrincius ER, Haasbach E, Anhlan D, Ahmann K, Banning C, Reiling SJ, Kuhn J, Strobl S, Vitt D, Leban J, Planz O, Ludwig S (2013) The NF-kappaB inhibitor SC75741 efficiently blocks influenza virus propagation and confers a high barrier for development of viral resistance. Cell Microbiol 15 (7):1198-1211

293. Pinto R, Herold S, Cakarova L, Hoegner K, Lohmeyer J, Planz O, Pleschka S (2011) Inhibition of influenza virus-induced NF-kappaB and Raf/MEK/ERK activation can reduce both virus titers and cytokine expression simultaneously in vitro and in vivo. Antiviral Res 92 (1):45-56

294. Uchide N, Ohyama K, Bessho T, Yuan B, Yamakawa T (2002) Effect of antioxidants on apoptosis induced by influenza virus infection: inhibition of viral gene replication and transcription with pyrrolidine dithiocarbamate. Antiviral Res 56 (3):207-217

295. Uchide N, Toyoda H (2011) Antioxidant therapy as a potential approach to severe influenza-associated complications. Molecules 16 (3):2032-2052

296. Hassan IH, Zhang MS, Powers LS, Shao JQ, Baltrusaitis J, Rutkowski DT, Legge K, Monick MM (2012) Influenza A viral replication is blocked by inhibition of the inositol-requiring enzyme 1 (IRE1) stress pathway. J Biol Chem 287 (7):4679-4689

297. Lee SM, Cheung CY, Nicholls JM, Hui KP, Leung CY, Uiprasertkul M, Tipoe GL, Lau YL, Poon LL, Ip NY, Guan Y, Peiris JS (2008) Hyperinduction of cyclooxygenase-2-mediated proinflammatory cascade: a mechanism for the pathogenesis of avian influenza H5N1 infection. J Infect Dis 198 (4):525-535

298. Lee SM, Gai WW, Cheung TK, Peiris JS (2011) Antiviral effect of a selective COX-2 inhibitor on H5N1 infection in vitro. Antiviral Res 91 (3):330-334

299. Dumit VI, Dengjel J (2012) Autophagosomal protein dynamics and influenza virus infection. Front Immunol 3:43

300. Dai J, Wang G, Li W, Zhang L, Yang J, Zhao X, Chen X, Xu Y, Li K (2012) High-throughput screening for anti-influenza A virus drugs and study of the mechanism of procyanidin on influenza A virus-induced autophagy. J Biomol Screen 17 (5):605-617

301. Dai JP, Zhao XF, Zeng J, Wan QY, Yang JC, Li WZ, Chen XX, Wang GF, Li KS (2013) Drug screening for autophagy inhibitors based on the dissociation of Beclin1-Bcl2 complex using BiFC technique and mechanism of eugenol on anti-influenza A virus activity. PLoS One 8 (4):e61026

302. Mata MA, Satterly N, Versteeg GA, Frantz D, Wei S, Williams N, Schmolke M, Pena-Llopis S, Brugarolas J, Forst CV, White MA, Garcia-Sastre A, Roth MG, Fontoura BM (2011) Chemical inhibition of RNA viruses reveals REDD1 as a host defense factor. Nat Chem Biol 7 (10):712-719

303. Beloso A, Martinez C, Valcarcel J, Santaren JF, Ortin J (1992) Degradation of cellular mRNA during influenza virus infection: its possible role in protein synthesis shutoff. J Gen Virol 73 (Pt 3):575-581

304. Hoffmann HH, Kunz A, Simon VA, Palese P, Shaw ML (2011) Broad-spectrum antiviral that interferes with de novo pyrimidine biosynthesis. Proc Natl Acad Sci U S A 108 (14):5777-5782

305. McLean JE, Neidhardt EA, Grossman TH, Hedstrom L (2001) Multiple inhibitor analysis of the brequinar and leflunomide binding sites on human dihydroorotate dehydrogenase. Biochemistry 40 (7):2194-2200

306. Zhang L, Das P, Schmolke M, Manicassamy B, Wang Y, Deng X, Cai L, Tu BP, Forst CV, Roth MG, Levy DE, Garcia-Sastre A, de Brabander J, Phillips MA, Fontoura BM (2012) Inhibition of pyrimidine synthesis reverses viral virulence factor-mediated block of mRNA nuclear export. J Cell Biol 196 (3):315-326

307. Smee DF, Hurst BL, Day CW (2012) D282, a non-nucleoside inhibitor of influenza virus infection that interferes with de novo pyrimidine biosynthesis. Antivir Chem Chemother 22 (6):263-272

308. Morita M, Kuba K, Ichikawa A, Nakayama M, Katahira J, Iwamoto R, Watanebe T, Sakabe S, Daidoji T, Nakamura S, Kadowaki A, Ohto T, Nakanishi H, Taguchi R, Nakaya T, Murakami M, Yoneda Y, Arai H, Kawaoka Y, Penninger JM, Arita M, Imai Y (2013) The lipid mediator protectin D1 inhibits influenza virus replication and improves severe influenza. Cell 153 (1):112-125

309. Serhan CN, Chiang N (2008) Endogenous pro-resolving and anti-inflammatory lipid mediators: a new pharmacologic genus. Br J Pharmacol 153 Suppl 1:S200-215

310. Takeda M, Leser GP, Russell CJ, Lamb RA (2003) Influenza virus hemagglutinin concentrates in lipid raft microdomains for efficient viral fusion. Proc Natl Acad Sci U S A 100 (25):14610-14617

311. Barman S, Nayak DP (2007) Lipid raft disruption by cholesterol depletion enhances influenza A virus budding from MDCK cells. J Virol 81 (22):12169-12178

312. Wang X, Hinson ER, Cresswell P (2007) The interferon-inducible protein viperin inhibits influenza virus release by perturbing lipid rafts. Cell Host Microbe 2 (2):96-105

313. Durrant JD, Cao R, Gorfe AA, Zhu W, Li J, Sankovsky A, Oldfield E, McCammon JA (2011) Non-bisphosphonate inhibitors of isoprenoid biosynthesis identified via computer-aided drug design. Chem Biol Drug Des 78 (3):323-332

314. Lindert S, Zhu W, Liu YL, Pang R, Oldfield E, McCammon JA (2013) Farnesyl diphosphate synthase inhibitors from in silico screening. Chem Biol Drug Des 81 (6):742-748

315. Hidari KI, Suzuki Y, Suzuki T (2006) Suppression of the biosynthesis of cellular sphingolipids results in the inhibition of the maturation of influenza virus particles in MDCK cells. Biol Pharm Bull 29 (8):1575-1579

316. Tafesse FG, Sanyal S, Ashour J, Guimaraes CP, Hermansson M, Somerharju P, Ploegh HL (2013) Intact sphingomyelin biosynthetic pathway is essential for intracellular transport of influenza virus glycoproteins. Proc Natl Acad Sci U S A 110 (16):6406-6411

317. Garcia-Sastre A (2011) Induction and evasion of type I interferon responses by influenza viruses. Virus Res 162 (1-2):12-18

318. Martinez-Gil L, Ayllon J, Ortigoza MB, Garcia-Sastre A, Shaw ML, Palese P (2012) Identification of small molecules with type I interferon inducing properties by high-throughput screening. PLoS One 7 (11):e49049

319. Ortigoza MB, Dibben O, Maamary J, Martinez-Gil L, Leyva-Grado VH, Abreu P, Jr., Ayllon J, Palese P, Shaw ML (2012) A novel small molecule inhibitor of influenza A viruses that targets polymerase function and indirectly induces interferon. PLoS Pathog 8 (4):e1002668

320. Moon HJ, Lee JS, Choi YK, Park JY, Talactac MR, Chowdhury MY, Poo H, Sung MH, Lee JH, Jung JU, Kim CJ (2012) Induction of type I interferon by high-molecular poly-gamma-glutamate protects B6.A2G-Mx1 mice against influenza A virus. Antiviral Res 94 (1):98-102

321. Goulet ML, Olagnier D, Xu Z, Paz S, Belgnaoui SM, Lafferty EI, Janelle V, Arguello M, Paquet M, Ghneim K, Richards S, Smith A, Wilkinson P, Cameron M, Kalinke U, Qureshi S, Lamarre A, Haddad EK, Sekaly RP, Peri S, Balachandran S, Lin R, Hiscott J (2013) Systems analysis of a RIG-I agonist inducing broad spectrum inhibition of virus infectivity. PLoS Pathog 9 (4):e1003298

322. Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, Chen J, Jie Z, Qiu H, Xu K, Xu X, Lu H, Zhu W, Gao Z, Xiang N, Shen Y, He Z, Gu Y, Zhang Z, Yang Y, Zhao X, Zhou L, Li X, Zou S, Zhang Y, Yang L, Guo J, Dong J, Li Q, Dong L, Zhu Y, Bai T, Wang S, Hao P, Yang W, Han J, Yu H, Li D, Gao GF, Wu G, Wang Y, Yuan Z, Shu Y (2013) Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med 368 (20):1888-1897

323. Morens DM, Taubenberger JK, Fauci AS (2013) Pandemic influenza viruses--hoping for the road not taken. N Engl J Med 368 (25):2345-2348

Figure legends

Fig. 1. Antiviral strategies targeting viral functions essential for IV replication.

The first step of IV infection is the interaction between viral HA and cellular SA-containing receptors, resulting in the attachment of the virion to the target cell. Attachment inhibitors, such as mAb directed against the globular head of HA, natural and synthetic compounds containing SA, HA-binding peptides, and compounds that recognize glycosylation sites of HA, interfere with this process and block IV infection. After the internalization of the virion, HA mediates the fusion of the viral envelope with the endosomal membrane, a pH-dependent process that can be inhibited by fusion inhibitors such as small molecules that inhibit the low pH-induced conformational change of HA (e.g., ARB), neutralizing mAbs directed against the stem region of HA, and high-molecular-weight molecules that prevent the fusion by steric hindrance. The activity of the viral protonic pump M2 leads to the acidification of the endosome and to the viral uncoating, followed by the release of the vRNP into the cytoplasm. M2 inhibitors such as adamantanes block IAV (but not IBV) replication at this step. After nuclear translocation of the vRNP, the viral genomic segments are transcribed by the viral RdRP into mRNAs that are then transported into the cytoplasm and translated into viral proteins necessary for viral genome replication, also catalyzed by viral RdRP. Small molecules able to interfere with RdRP complex activities can inhibit both transcription and replication steps. Transcription, replication, correct assembly of vRNPs, and their nuclear export require the activity of viral NP. Also small molecules targeting NP functions have demonstrated effective anti-influenza activity. After the assembly of new virions, they are transported at the cell membrane and then released by budding process. The activity of NA present on the virion surface is essential for the cleavage of SA molecules from HA and to allow the release of viral particles. NA inhibitors such as zanamivir and oseltamivir block IAV and IBV replication by interfering with this step.

Fig. 2. Antiviral strategies involving host factors engaged in IV replication.

IV infection can be inhibited by inhibitors of extracellular proteases responsible of HA activation. Fludase interfers with the interaction of viral HA to cellular SA-containing receptors, while tyrosine-kinase receptors and EGFR inhibitors, as well as chemical inhibitors of endocytosis and macropinocytosis processes block the endosomal internalization of the virus particle. vATPase inhibitors have shown activity against the pH-dependent fusion step that allows viral uncoating and also proteasome inhibitors might have an inhibitory effect on the latter process. After the nuclear import of vRNPs, the first step is the transcription of the viral genome into viral mRNA and this process is catalyzed by RdRP with the involvement of the cellular RNA polymerase II and transcription elongation factor pTEF1b. Inhibitors of both RNA polymerase II and pTEF1b complex are able to interfere with this process and to block viral mRNA synthesis. Viral mRNAs are then exported into the cytoplasm and translated into viral proteins required for the viral genome replication, which takes place into the nucleus. At this stage, Hsp90 inhibitors can be effective in blocking the nuclear import of viral proteins. The newly synthesized vRNPs have then to be exported into the cytoplasm to be assembled into the new virions. Small molecule inhibitors of cellular Crm1 and SGK1 proteins involved in the vRNPs export can inhibit this step. Antioxidants able to restore the cellular redox potential and intracellular protease inhibitors interfere with the correct processing and maturation of HA; in addition, inhibitors of mitochondrial DHODH, an enzyme of the pyrimidine biosynthetic pathway, have demonstrated anti-influenza activity. Also the soluble lipid mediator PD1 is able to inhibit IV replication by interfering with the nuclear export of viral transcripts mediated by the cellular protein NFX1. Finally, compounds that interfere with proper lipid raft formation at the plasma membrane have also shown the ability to block IV budding.

Fig. 3. Antiviral strategies involving host signaling pathways, metabolism, and antiviral defense.

IV infection triggers the activation of a series of cellular signaling pathways as well as the IFN-dependent antiviral response. PKC and COX-2 pathways are activated directly upon infection, while the oxidative stress that follows viral infection activates several redox-sensitive cellular pathways, such as NF-κB, PI3K, and MAPKs. COX-2 inhibitors have demonstrated to inhibit both IV replication and viral-induced inflammation and cytokine production. PKC inhibitors are able to interfere with post-fusion events and endocytosis after virion internalization. Antioxidants that interfere with redox-sensitive cellular processes also interfere with viral functions such as vRNPs trafficking and nuclear export, as well as HA maturation, thus blocking viral replication. Furthermore, IV replication in the host cell activates the UPR in the ER, which can be counteracted by chemical chaperones that alleviate ER stress and have shown anti-influenza activity. The antiviral response activated by IV is dependent on IFN cascade activation and initiates with the recognition of viral patterns by the TLR-3, -7, and -9 on the cell membrane and by cytoplasmic RLRs, which act as sensors of viral invasion. TLRs and RLRs agonists as well as CpG ODNs have shown anti-influenza activity by stimulating the IFN response. The viral NS1 protein antagonizes this antiviral response, thus antiviral strategies either targeted against this protein or involving the activation of IFN pathway have inhibitory effects on IV replication.