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REVIEW

Antiviral strategies against influenza virus: towards new therapeutic approaches

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Abstract 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), and recently, swine flu (2009) caused millions of deaths worldwide [1]. In addition, the seasonal influenza epidemics result 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 so far been identified [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 "antigenic 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]. As depicted in Fig. 1, the IV replication cycle initiates with the attachment of HA to sialic acid (SA)-containing glycoprotein and glycolipid receptors on the cell surface [6]. The virus particle then enters the cell

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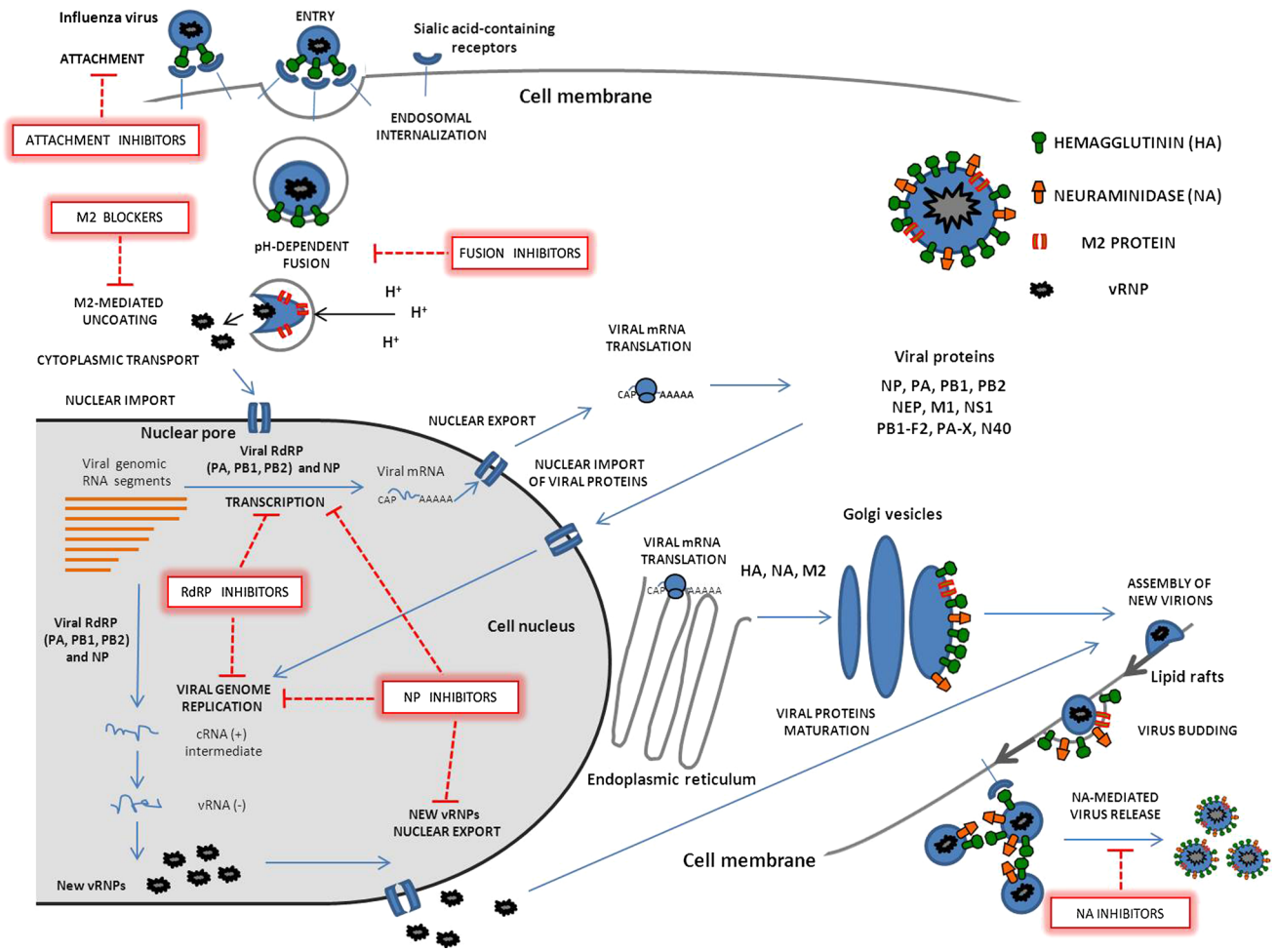

Fig. 1 Antiviral strategies targeting viral functions essential for influenza virus (IV) replication. The first step of IV infection is the interaction between viral hemagglutinin (HA) and cellular sialic acid (SA)-containing receptors, resulting in the attachment of the virion to the target cell. Attachment inhibitors, such as monoclonal antibodies (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 by endocytosis and macropinocytosis, 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., arbinol) and neutralizing mAbs directed against the stem region of HA. The activity of the viral protonic pump M2 leads to the acidification of the endosome and to viral uncoating, followed by the release of the viral ribonucleoprotein (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 RNA-dependent RNA polymerase (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. Compounds able to interfere with RdRP activities can inhibit both transcription and replication steps. Transcription, replication, correct assembly of vRNPs, and their nuclear export require the activity of viral nucleoprotein (NP), thus molecules targeting NP functions have demonstrated effective anti-influenza activity. After the assembly of new virions in the cytoplasm, they are transported at the cell membrane and then released by budding. The activity of neuraminidase (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

via clathrin-dependent endocytosis and macropinocytosis. 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 [7].

The current options to combat IV infection 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 variability of the virus and are not always protective; in addition, a rapidly emerging influenza pandemic cannot be contained by vaccination. 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, novel IV inhibitors are greatly required.

The need for developing new drugs to overcome resistance and counteract threats of sporadic outbreaks of viruses with pandemic potential 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 this review, we present an overview of recent progress in designing and developing new antiviral compounds and strategies to block critical steps of the viral life cycle by inhibiting functions of viral proteins and/or host–virus interactions. In particular, the review focuses mainly on those that in our opinion are the most novel and promising anti-influenza strategies and/or on those compounds that should deserve further attention for developing new therapeutic approaches.

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 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.

Antiviral strategies targeting the M2 ion channel

Influenza M2 is a homotetrameric protein that acts as a proton channel [8]. 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 (Fig. 1, top left) [8]. M2 is essential for viral replication and its short N-terminal extracellular domain is highly conserved in all human IAV [9]. 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: the M2 blockers amantadine and its methyl derivative rimantadine (Fig. 1, top left) [10, 11]. 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 [10]. Unfortunately, these compounds are inactive against IBV, since the amino acid sequences between the A/M2 and the M2 of IBV are different [8]. 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 [12]. Thus, new M2 blockers active against amantadine-resistant viruses are urgently needed.

Drug-resistance to amantadine and rimantadine is associated with 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 required for channel activity and proton selectivity [13]. More than 95 % of the reported transmissible IAV carry the S31N mutation in the transmembrane region of A/M2 [14, 15]. 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 (Table 1). 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 [15]. In addition to M2WJ332, some benzyl-substituted amantadine derivatives were recently found to inhibit the activity of both S31N and wild-type viruses [16]. Other frequent mutations in the A/M2 protein that confer resistance to amantadine and rimantadine are L26F and V27A [13, 17]. Recently, novel small molecules with inhibitory activity against A/M2 bearing these mutations have been reported (Table 1), including spiroadamantane 9 [18], spiran amine 8 [19], and some organosilane-based compounds [20]. 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 [21]. Importantly, passive immunotherapy with M2-7A protected mice from a lethal IV challenge [21]. Although the exact mechanism of action of M2-7A and its binding epitope have not

Table 1 New inhibitors of IV proteins and/or functions

Molecule	Biological activity	Viral target	Status	References
M2WJ332, benzyl-substituted amantadine derivatives	M2 blockers	A/M2 ion channel (S31N mutant)	Preclinical	[15, 16]
Spiroadamantane 9, Spiran amine 8	M2 blockers	A/M2 ion channel (wt, L26F and V27A mutants)	Preclinical	[18, 19]
Organosilane-based compounds	M2 blockers	A/M2 ion channel (wt and V27A mutant)	Preclinical	[20]
M2-7A Antibody	M2 blocker	M2 ion channel	Preclinical	[21]
Peramivir	Sialic acid analogue	Neuraminidase	Approved (Japan and Korea); FDA approval requested (USA)	[30]
Laninamivir	Sialic acid analogue	Neuraminidase	Approved (Japan); phase II clinical trials (USA)	[32]
Multimeric neuraminidase inhibitors	Multivalent sialic acid analogues	Neuraminidase	Preclinical	[38–40]
CH65 (neutralizing antibody)	Neutralizing activity against IAV H1N1 strains	Globular head domain of hemagglutinin	Preclinical	[45]
S139/1 and C05 (neutralizing antibodies)	Neutralizing activity against different IAV subtypes	Globular head domain of hemagglutinin	Preclinical	[46, 47]
Polyvalent synthetic sialic acid-containing inhibitors	Competitive inhibitors of the virus attachment	Hemagglutinin	Preclinical	[49–52]
Natural inhibitors containing sialic acid (e.g., serum amyloid P component)	Competitive inhibitors of the virus attachment	Hemagglutinin	Preclinical	[53]
Peptides against hemagglutinin (e.g., EB peptide, FluPep)	Inhibitors of virus attachment	Hemagglutinin	Preclinical	[54, 55]
Carbohydrate-binding agents (e.g., Cyanovirin-N, BCA)	Inhibitors of virus attachment	Specific glycosylation sites on hemagglutinin	Preclinical	[56, 60]
TBHQ, BMY-27709, 180299, Stachyflin, Thiobenzamide compounds, RO5464466	Blockers of the low pH-induced conformational change of HA	Stem region of hemagglutinin	Preclinical	[64, 66–70]
C22	Inducer of the premature conformational change of HA	Stem region of hemagglutinin	Preclinical	[65]
Arbidol	Blocker of the low pH-induced conformational change of HA	Phospholipid membrane and protein motifs of HA enriched in aromatic residues	Approved (Russia and China)	[74]
FI6v3, CR6261, CR8020, A06 and F10 (broadly neutralizing antibodies)	Inhibitors of fusogenic activity of IAV HA	Stem region of hemagglutinin	Preclinical	[75, 77–80]
CR8033 and CR8071 (neutralizing antibodies)	Inhibitors of fusogenic activity of IBV HA	Stem region of hemagglutinin	Preclinical	[76]
CR9114 (broadly neutralizing antibody)	Inhibitor of fusogenic activity of IAV and IBV HA	Stem region of hemagglutinin	Preclinical	[76]
Favipiravir	Nucleoside analogue	RdRP	Phase III clinical trials	[88]

Table 1 continued

Molecule	Biological activity	Viral target	Status	References
N-Hydroxytetramic acid compounds, 4-substituted 2,4-dioxobutanoic acids, flutimide, hydroxypyridinone compounds, marchantins	PA endonuclease inhibitors	RdRP	Preclinical	[94–96, 99, 100]
Compounds 1 and 5, AL18, Benzafurazan compounds	Inhibitors of PA/PB1 subunit interaction	RdRP	Preclinical	[109, 112, 114]
Benzbromarone, diclazuril, and trenbolone acetate	Inhibitors of PA/PB1 subunit interaction	RdRP	Approved as uricosuric agent (EU, Asia); approved as antiprotozoal (coccidiostat); preclinical, respectively	[113]

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

Another 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 from the adjacent oligosaccharide moiety of the cellular receptors recognized by HA, playing a key role in promoting IV infectivity (for a review, see [22]). Indeed, NA is responsible for virus penetration through mucosal secretions, helping the virus to access the target cells by mucus degradation [23]. 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 (Fig. 1, bottom right) [22].

The most successful NA inhibitors are represented by synthetic analogues of SA. Their mechanism of action 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 since NA active site is highly conserved [24, 25]. However, these inhibitors are effective against influenza infection only if administrated within 36-48 h of symptoms onset [25]. 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 %) [27]. Although zanamivir is well tolerated and has few adverse effects, the route of administration of this drug can represent a problem especially for children and elderly patients that might not be able to inhale zanamivir suitably [28]. To circumvent these issues, an intravenous formulation of zanamivir has been developed and is currently under 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 $\geq$1 year old [29]. More recently, two other SA analogues, peramivir and laninamivir (Table 1), 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 [30]; 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 [31]. Laninamivir (R-125489) is an SA analogue structurally similar to zanamivir. Laninamivir is administrated as an octanoyl prodrug, laninamivir octanoate (LO; CS-8958, R-118958), and holds great promise for its long-acting inhibitory activity [32]. 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 [33]. Some mutations were found to confer resistance to certain NA inhibitors but preserve the susceptibility to others [33]; for example, the main mutation conferring resistance to oseltamivir (H274Y) also confers resistance to peramivir, but not to zanamivir [34]. Currently, no mutation associated with resistance to laninamivir has been identified [33]. Based on NA crystallographic structure and knowledge of the binding mode of these NA inhibitors with the enzyme active site [35], 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 has been well summarized in other recent reviews [35, 36].

An alternative and interesting approach to increase the efficacy of the approved NA drugs is provided by the use of multivalent inhibitors and by conjugating the compounds to a biocompatible polymer [37, 38]. Indeed, several reports have shown that such multivalent presentation of NA inhibitors results in a dramatically higher antiviral potency than that obtained with the monomeric drug [38–40]. 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 [38]. In addition, a single dose of zanamivir dimers resulted in an in vivo longer-lasting activity against IV compared to monomeric zanamivir [40].

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 (see below). HA is essential for the interaction of the virus with cells by binding to SA receptors on host cells and is also involved in the low pH-induced membrane fusion between the viral envelope and the endosomal membrane (Fig. 1, top left) [22].

A possible mechanism of inhibition of viral infection is to block the interaction between viral surface molecules and cellular receptors and the subsequent 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 viral entry [41–43]. In the case of IV, the entry step can be blocked mainly by two strategies: (1) by preventing the binding between the viral HA and the terminal SA of glycoproteins and glycolipids present on the cell membrane; and (2) by blocking the process of fusion between the viral envelope and endosome membrane, necessary for the release of the vRNP into the cytoplasm (Fig. 1, top left).

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 [22], thus representing the main target of these inhibitors. The main limit of this antiviral strategy is the hyper-variability of the globular head of HA. In fact, several of the antibodies that target regions of globular head of HA are often able to neutralize a limited range of IV strains [44, 45]. 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 (Table 1), which is able to neutralize several strains of IV in vitro, by inserting its heavy-chain CDR3 loop into the receptor-binding pocket [45]. Recent studies reported a few examples of RBS-directed antibodies with heterosubtypic anti-influenza activity. In particular, two mAbs, S139/1 and C05 (Table 1), showed neutralization activity against strains from multiple IV subtypes both in vitro and in mice [46, 47].
2. *Decoy receptor or SA-containing inhibitors.* This class of agents can be subdivided in: (1) polyvalent synthetic SA-containing inhibitors; and (2) natural inhibitors containing SA. Both these types of inhibitors act

as receptor mimics and compete with sialylated receptors on the target cells for the binding to HA and thus block the virus attachment (Table 1). In addition, their binding to the virion surface can lead to virus particle aggregation, causing a reduction of virus infectivity. Regarding 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 [48]. Some of the polyvalent SA-containing inhibitors have also exhibited protective effects against IV infection in mice [49, 50]. 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 [51, 52]. A second type of inhibitors are constituted by SA-containing natural molecules, such as glycoproteins or proteoglycans. These molecules bind HA and hence create a steric obstacle to the polyvalent interaction of the virus with cells, thus blocking the process of virus adsorption 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 also have therapeutic effects in mice [53].

3. *Peptides against HA*. Some peptides exhibiting potent and broad-spectrum anti-influenza activity recently emerged as inhibitors of viral attachment [54, 55]. Similar to antibodies, these peptides specifically bind the HA protein and prevent IV adsorption 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*. This class of inhibitors is 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 [56]. In fact, removal of these glycans from HA causes a decrease of viral sensitivity to CV-N [56]. Interestingly, CV-N showed antiviral activity not only against IAV and IBV but also against a broad range of enveloped viruses, such as HIV [57], Ebola virus [58], human herpesvirus 6 [57, 58], and hepatitis C virus (HCV) [59]. The same mechanism seems to be used by a lectin from green alga *Boodlea coacta* (BCA) [60]. Indeed, BCA exhibited a strong inhibition of HA activity by specifically interacting with 1–2-linked mannose at the nonreducing terminus of HA [60]. In addition, BCA showed activity against HIV [60]. The antiviral activity of these molecules is closely associated with the degree of glycosylation on the globular head of HA. Indeed, amino acid substitutions in the HA that cause the loss of glycosylation sites are related to a reduced sensitivity to lectin-based inhibitors [61].

Another possible strategy for 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 (Fig. 1, top left). 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 [62]. 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 [63]. 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 [64–70]. Most of them prevent the fusogenic activity of the virus by blocking the low pH-induced conformational change of HA [64, 66–70]. 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 [70]. In contrast, other compounds, such as C22, inhibit membrane fusion by destabilizing the structure of HA, resulting in a premature and ineffective conformational change [65, 71]. However, these small molecules show a high propensity to select 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), which exhibits a broad spectrum of 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 [71, 72]. 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

[73]. Recently, biochemical studies showed that ARB interacts both with cell membrane phospholipids and with aromatic residues of viral glycoproteins on the surface of enveloped viruses [74]. 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 have reported antibodies that recognize conserved epitopes of the stem region of HA and show a broad activity against different subtypes and groups of IAV both in vitro and in animal models [75–81]. Among these, CR9114 is the only antibody capable of binding and neutralizing all group 1 and group 2 IAV tested, and also different IBV strains both in vitro and in mice [76]. Besides the fact that this antibody may represent a candidate therapeutic agent against IV, these findings suggest that the epitope recognized by CR9114 is highly conserved, not only among the different IAV subtypes but also among IBV. This observation heralds the prospect of a possible universal influenza vaccine against IAV and IBV based on this epitope, able to overcome the genetic variability of IV and to elicit a potent and cross-protective host immunity against all IAV and IBV strains [76].

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 (Fig. 1, left). 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 [82]. 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 [82]. The three polymerase subunits interact with each other, in particular the N-terminus of PB1 interacts with the C-terminus of PA [83–85], while the C-terminus of PB1 binds the N-terminus of PB2 [83, 86]; in addition, a weak transient interaction has been proposed for PA and PB2 [87]. 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. The best-characterized anti-influenza nucleoside analogue is favipiravir (T-705; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide; Table 1), which is a pyrazine derivative first identified in 2002 [88]. T-705 has been shown to inhibit influenza A, B, and C viruses in vitro and to be more effective than oseltamivir in protecting mice infected with IAV [88]. By cellular kinases, T-705 is converted to the active form, ribofuranosyl triphosphate, which acts as a nucleoside inhibitor of IV RdRP [89]. Importantly, fapiravir does not inhibit the synthesis of cellular RNA or DNA [90]. 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 IV strains resistant to current antiviral drugs [90, 91]. Besides IV, T-705 inhibits a number of other RNA viruses, whereas it exhibits no inhibitory effect against DNA viruses. Remarkably, very limited resistance to favipiravir has been reported [90]. 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 [91]. In the near future, we may therefore see the introduction of favipiravir in the clinical practice 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 [92, 93]. In past years, a number of inhibitors (e.g., flutimide and L-735882; Table 1) of PA endonuclease activity have been discovered by Roche, Merck, and other pharmaceutical companies using a structure–activity relationships (SAR) approach [94–96], but most of them have not been developed further, likely because none had sufficiently potent antiviral activity to justify clinical studies. 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 [92, 93]. Along this line, two groups have reported co-crystal structures of the PA endonuclease domain with known or predicted inhibitors [97, 98], 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 [99]. Using a different approach, Iwai et al. [100] screened 33 different types of phytochemicals using a PA endonuclease inhibition assay in vitro and identified marchantins as PA inhibitors. In particular, marchantin E docked well into the endonuclease active site and inhibited the growth of both IAV and IBV.

The interactions between the PA and PB1 as well as the PB1 and PB2 subunits have been shown to be essential for polymerase function [101, 102]. In addition, the subunit 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 [103, 104]. 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 [105, 106]. Recently, two crystal structures of a truncated form of PA bound to a PB1-derived peptide have been published [107, 108]. Importantly, these structures showed that relatively few residues drive binding of PB1 to PA, suggesting the potential for small molecule-mediated inhibition. Along this line, an in silico screening of 3 million small-molecule structures using one of these crystal structures [107] led to the identification of two compounds (compounds 1 and 5; Table 1) able to interfere with the interaction between PB1 and PA both in vitro and in cells, as well as transcription by the RdRP [109]. 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 [109]. In addition, the design and synthesis of analogues of another of the hits—compound 10—initially selected in the in silico screening aimed at searching for inhibitors of PA/PB1 interaction led recently to the identification of other compounds endowed with similar antiviral properties [110]. Interestingly, a compound called AL18, previously shown to inhibit subunit interactions of human cytomegalovirus DNA polymerase [111], was found to also block the PA/PB1 interaction as well as the replication of IAV and IBV [112]. In a similar, but more restricted, screening, Fukuoka and colleagues performed a docking simulation using a drug database of ~4,000 compounds and selected candidate compounds targeting the PA/PB1 interface [113]. Among these, benzbromarone, diclazuril, and trenbolone acetate (Table 1) 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 benzofurazan compounds that also showed inhibition of viral replication at micromolar concentrations [114]. However, these compounds 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 the PB1 and PB2 subunits, recently emerged with the publication of the crystal structure of the PB1/PB2 binding interface [102]. 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 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 [115]. However, to date, no small molecule targeting this protein–protein interaction has yet been reported, and indeed the flat PB1/PB2 interface may pose a significant challenge to the development of 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 [116]. Surprisingly, the authors showed that this peptide interacts with PB1 rather than PB2. Furthermore, mutational analyses and computational modeling suggested that the $PB1_{731–757}$ 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 drug discovery.

Antiviral strategies targeting the nucleoprotein

The nucleoprotein (NP) of influenza virus is one of the most abundant viral proteins in the infected cell. It plays a major role in the vRNP assembly by interacting with both RNA and viral polymerase subunits, but there is also evidence of its involvement in vRNP nuclear export and cytoplasmic transport (Fig. 1, left) [117, 118]. Recombinant NP also forms dimers and oligomers in the absence of RNA [119]; however, recent findings revealed that this process seems to be less efficient under these conditions

[120]. During viral infection, the oligomerization of NP in ring structures and its interaction with single-stranded viral genomic RNA segments is essential for vRNP assembly. In the working model, NP trimers interact with RNA leaving the nucleotides exposed to the solvent [121]. As a result of the interaction of NP and the heterotrimeric RdRP with viral RNA, the latter is twisted into a double-helical structure with the polymerase complex at one end and an RNA loop at the other [121, 122]. The interaction between the two NP-RNA filaments of the double helix is guaranteed by NP–NP contacts. In recent years, the interactions of NP with itself [123–127], with viral RNA [128], with viral RdRP, and with cellular factors have been investigated for the development of new anti-IV strategies, demonstrating the significant potential of NP as an antiviral target. 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 [129, 130]. 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 [126]. In addition, the phosphorylation at Ser-165 seems to regulate the oligomerization status and RNA binding activity of NP in infected cells [131]. Small molecules able to interfere with correct protein–protein interactions between NP monomers or to stabilize NP in the monomeric form were independently identified by several groups and block IV replication at different steps (Fig. 1) [123, 125–127, 131]. Among these, nucleozin (NCZ) is the most studied. Since NP has two sites for self-interaction [132], it is widely accepted that such compounds bind at least two different sites on NP and act either by 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 [133]: 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 (Fig. 1) [133]. Interestingly, naproxen, a clinically-approved inhibitor of inducible cyclooxygenase-2 (COX-2), was very recently identified through a structure-based in silico screening as the first inhibitor of the interaction between NP and RNA [128]. 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 cytokine storm, could be particularly useful for the treatment of infections with emerging highly pathogenic viruses characterized by an exacerbated inflammatory response.

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 (Fig. 3, bottom) [134, 135]. Actually, NS1 IFN-antagonistic properties seem to be strain-specific [135]. NS1 subverts the cellular IFN response by different strategies: (1) it cooperatively binds to viral dsRNA [136], thus protecting it from the recognition of two cytoplasmic "sentinels" of viral infection, i.e., protein-kinase RNA-activated [137] and 2′-5′-oligo(A) synthetase/RNaseL [138], 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; (2) it binds to cellular mRNA processing factors, such as cellular polyadenylation specificity factor 30 (CPSF30) and polyA-binding protein II (PABP2) [139, 140], thus inhibiting 3′-mRNAs (such as IFN mRNAs) processing and nuclear/cytoplasmic trafficking; (3) it interacts with the ubiquitin-ligase TRIM25 and blocks RIG-I activation, thus preventing subsequent IFN cascade activation [140]; (4) it interacts with cellular IFN-inducible hGBP1 to antagonize its antiviral activity [141]; and, finally, (5) it targets IκB kinase (IKK) to block NF-kB activation [142]. 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 [143]. In fact, NS1 dimerization is essential for RNA binding, since mutations that block dimers formation also affect the RNA binding ability of NS1 [144].

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 [145]. 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 [146]. A derivative, JJ3297, resulted

to be dependent on cellular RNAseL functions for antiviral activity [147]. These compounds provide the proof-of-principle that NS1 activity can be blocked by small molecules that could also be used in combination with IFN agonists to enhance their antiviral activity. Another target of NS1-based antiviral strategies recently proposed 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 [148] and by high-throughput screening (HTS) developed to search for inhibitors of the binding of recombinant NS1 to a viral RNA construct in vitro [149]. 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 [150, 151]. The activity of such compounds suggests that the block of NS1 binding to viral RNA could also be a feasible anti-influenza strategy.

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, to reduce the development of drug-resistance, and to allow administration of 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 [152] and oseltamivir + T-705 [153] 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 [154]. 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 [155]. 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. In addition, besides combinations of therapeutic agents directed against viral targets, combinations with compounds directed against cellular targets have also been proposed (see below).

New host-based anti-influenza virus strategies

IV replication is strictly host-dependent. In fact, a plethora of cellular proteins play an important role in viral replication [156–160]. In recent years, several studies have 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 [161]), proteomic approaches [162], and yeast two-hybrid screenings [159, 163]. Antiviral drugs targeting cellular functions are expected to have some advantages over inhibitors directed against viral targets, in particular for genetically variable viruses such as IAV. In fact, inhibitors of cellular proteins and/or pathways should be less prone to induce the selection of resistant viral strains; on the other hand, perturbing the cellular environment to disrupt viral functions could have adverse side effects that should be carefully considered. We will describe cellular proteins and/or pathways that represent attractive anti-influenza targets due to their active engagement in the viral cycle and some already identified small-molecular-weight inhibitors that are currently under preclinical or clinical investigation as anti-IV agents.

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 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 (Fig. 2, top left). The proteolytic activation of HA by cellular proteases is essential for IV propagation and may thus 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 protease family, are found in human airway epithelium, and have been proposed as activators of HAs which have a monobasic cleavage site and are expressed by low pathogenic avian viruses and all human H1, H2, and H3 subtypes [164–166]. Processing of HAs with such a monobasic cleavage site restricts the infection to the respiratory airway epithelia in mammals and to the respiratory and intestinal epithelia in birds. In contrast, HAs expressed by highly pathogenic avian strains, such as H5 and H7 strains, contain multibasic cleavage sites that are recognized and cleaved by intracellular proteases, such as furin and proprotein convertase 5 and 6, and by specific transmembrane proteases such as TMPRSS13 [167, 168].

Given the essential role of cellular proteases in the activation of HA, protease inhibitors have been investigated as potential anti-influenza agents [169]. As an example, aprotinin, which is a 58-amino-acid single-chain globular polypeptide of bovine origin, demonstrated anti-influenza

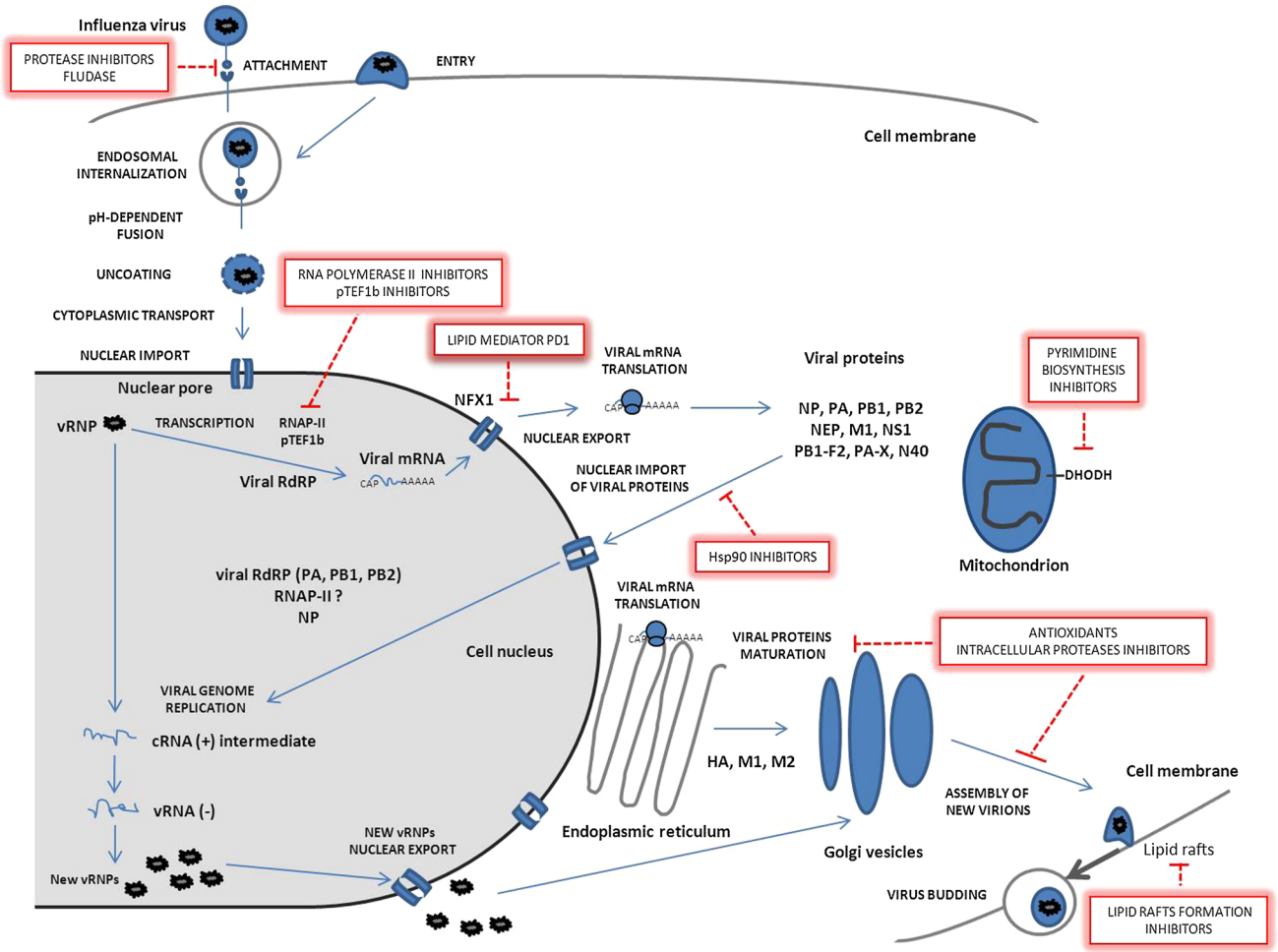

Fig. 2 Antiviral strategies involving host factors engaged in IV replication. IV infection can be blocked by inhibitors of transmembrane proteases responsible of HA activation and by Fludase. After the nuclear import of vRNPs, the first step is the transcription of the viral genome into viral mRNA and this process is catalyzed by RdRP with the involvement of cellular proteins such as RNA polymerase II (RNAP-II) and transcription elongation factor pTEF1b. Inhibitors of both RNAP-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. The soluble lipid mediator PD1 is able to inhibit IV replication by interfering with the nuclear export of viral transcripts mediated by the cellular proteins NFX1 and Nup62. In the cytosol, viral mRNAs are 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. Also, inhibitors of mitochondrial dihydroorotate dehydrogenase (DHODH), an enzyme of the pyrimidine biosynthetic pathway, possess anti-influenza activity. The newly synthesized vRNPs have then to be exported into the cytoplasm to be assembled together with the other viral proteins into the new virions. Antioxidants able to restore the cellular redox potential and intracellular protease inhibitors can interfere with the correct processing and maturation of HA. Finally, compounds that interfere with the proper formation of lipid rafts at the plasma membrane can block IV budding

activity in human adenoid epithelial cells [170], in a mouse model of influenza infection [171], and also in humans [172]. Aprotinin is used in clinical practice for the prevention of post-operative bleeding; however, very recently, the use of this protease inhibitor for the treatment of seasonal and swine-originated pandemic influenza has been approved in Russia [173]. In these cases, aerosolized aprotinin can be administered via inhalation at lower doses that those used in the surgical practice. Zhirnov et al. [173] demonstrated that, in the presence of aprotinin, IV prevalently contains uncleaved HA0 and the resulting viral progeny is less infectious. Other protease inhibitors demonstrated anti-influenza activity; for example, a recent screening with the catalytic domain of TMPRSS2 and known trypsin-like serine proteases inhibitors led to the identification of a sulfonylated 3-amindinophenylalanylamide derivative able to block IV propagation in cultured human adenocarcinoma-derived epithelial cells Calu-3 [174]. Finally, as mentioned above, the HAs of highly pathogenic avian IV strains, such as H5 and H7 subtypes [175], contain multibasic cleavage

sites and are activated during the transit from ER to the plasma membrane by furin and other cellular proteases located in the trans-Golgi network (Fig. 2, bottom). Thus, the inhibition of furin or furin-like proprotein convertases might also represent an anti-IV strategy [176, 177].

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 (Fig. 2, top left), have been demonstrated to be effective inhibitors of IV infection [178, 179]. A new candidate drug currently under clinical development as an inhibitor of influenza and parainfluenza infections is DAS181 (also known as Fludase), a recombinant fusion protein that prevents IV attachment by enzymatically removing SA from the receptors of the human epithelia airway [178]. DAS181 is composed of the catalytic domain of *Actinomyces viscosus* sialidase fused with the respiratory epithelium-anchoring domain of human protein amphiregulin [180, 181]. The advantages of *A. viscosus* sialidase over other bacterial sialidases are broad substrate specificity, higher specific activity, and good tolerance by the human immune system [178]. 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 [181, 182]. Importantly, this compound is also effective against strains resistant to the existing antiviral drugs, for example against oseltamivir-resistant H1N1 clinical isolates [181]. Moreover, a recent phase II clinical study of inhaled DAS181 showed a significant decrease of viral load in influenza-infected patients [183].

Budding and release of new virus particles occur at specific domains of the plasma membrane, called lipid rafts (Fig. 1, bottom right) [184]. Lipid rafts are dynamic microdomains of the cell membrane characterized by a higher percentage of cholesterol, sphingolipids, and phospholipids containing saturated fatty acids. Viral HA and NA 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 [184]. It was reported that cholesterol depletion [185] or the expression of an IFN-inducible cellular protein, viperin [186], disrupt plasma membrane by perturbing the correct formation of lipid rafts. 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 [186]. Furthermore, inhibition of FPPS, by chemical agents, siRNA, or viperin expression, also prevents IAV release by perturbing the correct formation of lipid rafts (Fig. 2, bottom right) [186]. However, pamidronate, a clinically-approved FPPS inhibitor, did not show efficacy in vivo in protecting mice from lethal influenza [187]. Thus, FPPS inhibitors may not be suitable anti-influenza agents despite their ability to prevent replication in vitro.

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

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 (Fig. 1, left). 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 (Fig. 2, left) [188]. Inhibitors of RNAP-II and Cdk9 that showed anti-influenza activity are listed in Table 2.

The nuclear translocation of vRNP components to promote new vRNP assembly and export also depends on a number of host factors such as importins and molecular chaperones that may represent antiviral targets. 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 [189]. Inhibitors of Hsp90, such as geldanamycin and 17-AAG, affect IV replication in cell culture, and the possible mechanism could be the block of the nuclear import of PB1 and PB2 and the induction of their degradation [190]. Other host factors involved in the nuclear export of vRNP represent targets of possible new anti-influenza strategies (Fig. 2, center). In Table 2 are listed a series of inhibitors of cellular proteins that also showed anti-IV activity by interfering with vRNP trafficking.

Antiviral strategies involving host signaling pathways

Many host cell signaling pathways are affected during IV infection. Among these, there are some tyrosine kinase receptors, the downstream mitogen-activated protein kinases (MAPKs) pathway, the phophatidylinositol-3-kinase/Akt/mTOR pathway, and the NF-κB pathway. The activation of host signaling pathways is essential for productive IV infection, since small molecules that inhibit any step of signal transduction are able to block virus propagation by affecting different events in IV life cycle as will be described below.

The MAPKs signaling pathways are activated in response to a variety of stimuli, including infection by IV, and detrimental effects on virus replication are observed

Table 2 Compounds with anti-IV activity that act by inhibiting host functions and/or cellular pathways

Compound	Biological activity(ies)	Target/ anti-IV activity	Status	References
α-Amanitin, actinomycin D	Cellular RNA pol II inhibitors	Viral RNA transcription	Preclinical	[188]
5,6-Dichloro-1-beta-D-ribo-furanosyl-benzimidazole	CDK9 inhibitor	Viral RNA transcription elongation	Preclinical	[239]
Flavopiridol	CDK9 inhibitor	Viral RNA transcription elongation	Preclinical	[240]
Leptomycin B	Cellular CRM1/exportin inhibitor	vRNP nuclear export	Preclinical	[241]
GSK 650394	Cellular SGK1 kinase	vRNP nuclear export	Preclinical	[242]
AG879, A9	Cellular tyrosine kinase receptors inhibitors	vRNA synthesis, vRNP nuclear export, viral particle release	Preclinical	[199]
UO126	Raf/MEK/ERK signaling inhibitor	vRNP trafficking and ?	Preclinical	[200]
PD-0325901, AZD-6244, AZD-8330, RDEA-119	Raf/MEK/ERK signaling inhibitors	vRNP trafficking and ?	Phase I or further clinical trials as anticancer agents	[208]
N-acetylcysteine, SB203580	p38 inhibitors	vRNP trafficking and ?	Preclinical	[203]
Glycyrrhizin, glutathione, curcumin, resveratol	Antioxidants	Multi-step inhibition of redox-sensitive viral functions, HA maturation	Preclinical	[195, 205–207]
Nitazoxanide	Anti-protozoal, broad-spectrum antiviral	HA terminal glycosylation and?	Approved as anti-parasitic drug	[209]
Wortmannin, LY294002, mTOR inhibitors	PI3K/Akt signaling inhibitors	Undetermined	Preclinical	[210–212]
ON108110	Multi-targeted kinase inhibitor	Undetermined	Preclinical	[243]
Acetylsalicylic acid	NF-κB pathway inhibitor, COX inhibitor	Multi-step inhibition of redox-sensitive viral functions	Approved as analgesic/anti-inflammatory agent	[215]
Pyrrolidine dithiocarbamate	NF-κB pathway inhibitor	Undetermined	Preclinical	[219]
Nimesulide	COX-2 inhibitor	Undetermined	Approved as anti-inflammatory agent	[224]
Naproxen	COX inhibitor	NP/RNA binding	Approved as anti-inflammatory agent	[128]
A3	DHODH inhibitor	Inhibition of viral components biosynthesis	Preclinical	[226]
Protectin D1	Cellular lipid mediator	vRNA nuclear export inhibitor	Preclinical	[231]
Compound 3	IFN response inducer	Global inhibitor of viral replication	Preclinical	[235]
ASN2	IFN response inducer	Global inhibitor of viral replication, PB1 inhibitor	Preclinical	[236]
5′-triphosphate RNAs	RIG-I agonist	Global inhibitor of viral replication	Preclinical	[129, 237, 238]

if the activation is blocked [191]. 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 (Fig. 3, center) [192–194]. Of particular interest, MAPKs inhibition can be obtained both pharmacologically (by using inhibitors) and by restoring the physiological intracellular reduced state in the infected cells after the oxidative stress caused by IV infection [195]. In fact, MAPKs pathway, like other cellular signaling pathways activated by IV, such as phosphoinositide 3-kinase (PI3K) and NF-κB-mediated signaling, is strictly dependent on host cell redox state [196–198]. A list of receptor tyrosine kinases and MAPKs signaling inhibitors that block influenza virus

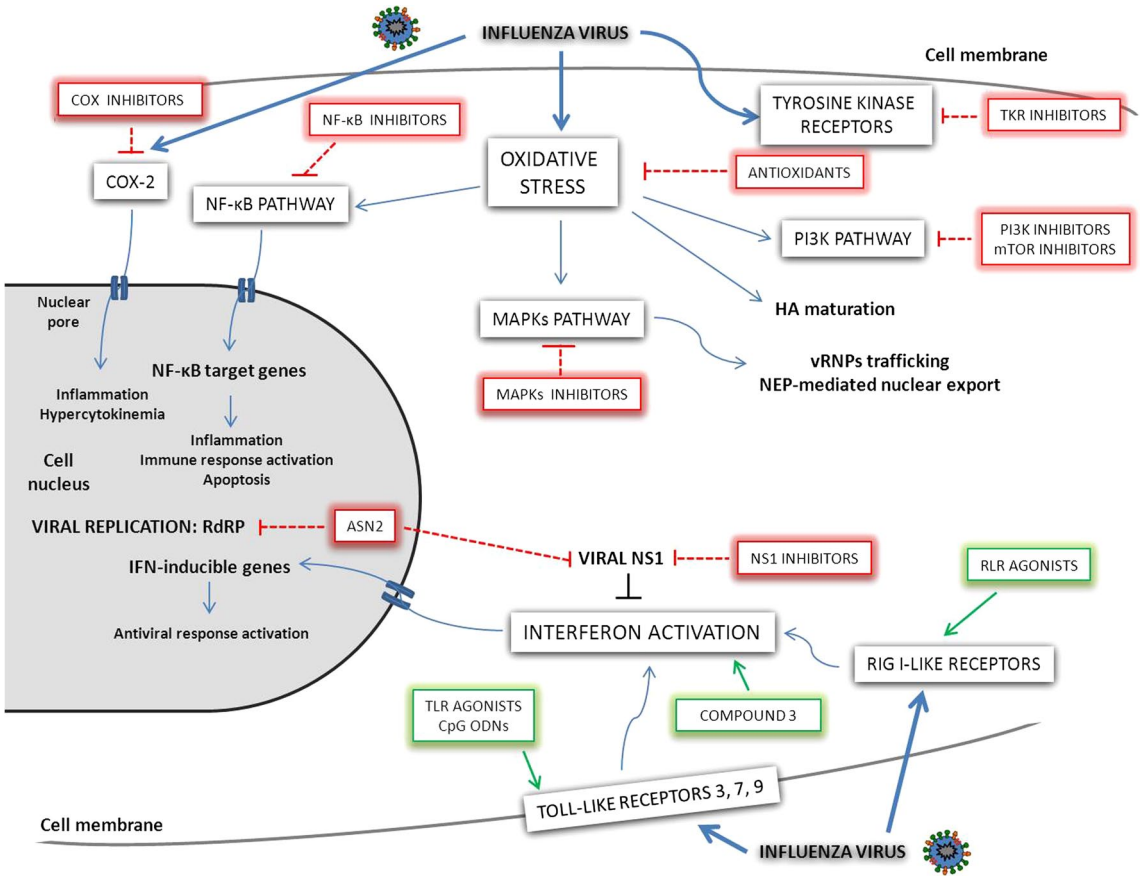

Fig. 3 Antiviral strategies involving host signaling pathways and antiviral defense mechanisms. IV infection triggers the direct activation of a series of cellular signaling pathways such as some tyrosine kinase receptors (TKR) pathways, pro-inflammatory pathways, and the interferon (IFN)-activated antiviral response. Also the oxidative stress that follows viral infection activates several redox-sensitive cellular pathways, such as NF-κB and MAPKs pathways. Furthermore, COX-2 inhibitors have been shown to inhibit both IV replication and viral-induced inflammation and cytokine production. Antioxidants that interfere with redox-sensitive cellular processes also inhibit viral functions such as vRNPs trafficking and nuclear export, as well as HA maturation, thus blocking viral replication. The antiviral response activated by IV is dependent on IFN cascade activation and initiates with the recognition of viral patterns by the Toll-like receptors TLR-3, -7, and -9 on the cell membrane and by cytoplasmic RIG-I-like receptors (RLRs), which act as sensors of viral invasion. TLRs and RLRs agonists as well as CpG oligonucleotides (ODNs) exhibit 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

replication can be found in Table 2. Along this line, molecules that block IV replication and the pro-inflammatory cascade are represented, for example, by kinase inhibitors, such as the tyrosine kinase receptor inhibitors AG879 and A9 [199], the MEK-specific inhibitor UO126 [191, 200, 201], and antioxidant compounds, such as p38 inhibitor SB203580 and *N*-acetylcysteine [202, 203], glycyrrizin [204], glutathione, and its derivatives [205, 206], and curcumin and resveratol derivatives [207]. All these compounds are potential new host-based anti-influenza agents that could be used in combination with "old" anti-influenza drugs such as oseltamivir, as prospected in [208].

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 that needs an oxidative environment, proteolytic cleavage, and is finally inserted into the plasma membrane. Thus, compounds that restore the cellular redox state, such as glutathione, are also able to exert anti-IV activity by two distinct mechanisms: (1) by interference with the activation of redox-sensitive signaling pathways exploited by the virus, such as MAPKs and PI3K pathways (Fig. 3), and (2) by blocking the maturation of viral HA protein (Fig. 1, bottom right) [194, 195]. Also, thiazolides such as nitazoxanide are potential broad-spectrum antivirals that block HA terminal glycosylation and impair the trafficking of HA precursors from ER to Golgi [209]. The exact mechanism of action of these

compounds 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 PI3K/Akt/mTOR is also activated 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; however, compounds targeting PI3K or downstream effectors, such as PI3K inhibitors wortmannin [210] and LY294002 [211] or mTOR inhibitors [212], 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 [213], the inhibition of this host cell pathway as a new anti-influenza approach clearly deserves further investigation.

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 (Fig. 3, left) [214]. The activation of NF-κB pathway is critical for IV productive replication [215–217] 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 IKK, which is responsible of its derepression and transcriptional activation [218]. 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 [215] and pyrrolidine dithiocarbamate [219], as well as antioxidant agents (see above and [220]), have been reported to block IV replication and propagation. Of particular interest is the NF-κB inhibitor SC75741 [221]. This compound is an efficient blocker of NF-κB module at non-toxic concentrations and also an inhibitor of IAV and IBV replication in MDCK and A549 cells [221, 222]. The effect of SC75741 on virus replication is to prevent efficient nuclear export of vRNP as a result of the lack of NF-κB transcriptional activity, i.e., via inhibition of virus-induced pro-apoptotic genes expression essential for vRNP export. Importantly, no resistant viruses were selected even after 8 passages in the presence of SC75741, thus this inhibitor showed a very low potential in selecting viral resistant variants [221]. Furthermore, SC75741 demonstrated its efficacy in reducing viral replication and cytokine expression in mice infected with highly pathogenic avian IV strains H5N1 and H7N7 [208]. This inhibitor did not show long-term inhibitory effects on cell proliferation [221] and, although more pre-clinical studies are needed, it might represent another candidate drug that targets cellular functions to block IV replication.

The cellular COX pathway is also engaged during IV infection (Fig. 3, left), in particular in the case of human infection with highly pathogenic avian strains such as H5N1 [223]. 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, COX-2 could also represent a promising cellular target for new anti-IV strategies [224].

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 approaches.

Antiviral strategies involving host cell metabolism

IV uses cellular constituents to produce its own viral RNAs, proteins, and lipid envelope. However, the virus needs NTPs, amino acids, and membrane components from the host cell to obtain new building blocks to be recycled in the synthesis of viral components. The degradation of cellular nucleic acids is not the only strategy whereby IV enriches the NTPs pool for viral RNA synthesis [225]; in fact, de novo synthesis of pyrimidine and uracil salvage pathways are also engaged during IV infection, depending on the cell type [226]. 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; Fig. 2, right) [226]. DHODH has also emerged as a target of another class of inhibitors, the quinolone carboxylic acid derivatives, which were reported to inhibit IAV replication in infected cells [227]. 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 are able to reverse the cellular mRNA export block mediated by viral NS1 protein [227]. 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 [228].

Lipid mediators and lipid metabolic pathways became hot topics in the last year because of the discovery of their

role in the modulation of the inflammatory response upon infection by IAV [229, 230]. In particular, the lipid soluble mediator protectin D1 (PD1) is a docosahexaenoic acid-derived protectin D1 isomer product of the 12/15-lipoxigenase that is reduced in lungs of infected mice during infection with IAV [231]. Previously, PD1 was reported to have anti-inflammatory and pro-resolving activity [232, 233]. Morita et al. found that PD1 has potent and specific anti-IV activity in cells and in a mouse model of severe influenza infection with low and highly pathogenic viruses [231]. Mechanistically, PD1 selectively blocks the nuclear export of viral RNA molecules by inhibiting their recruitment to cellular NFX1 protein that binds Nup62 (Fig. 2, center). Importantly, PD1 did not show evident detrimental effects on the transport of cellular mRNAs. Other features make PD1 one of the most promising drug candidate to treat severe influenza infection, such as (1) the activity when also added 48 h post-infection, the time at which the current anti-IV drugs have demonstrated limited efficacy, thus highlighting its potential as both prophylactic and therapeutic agent; (2) the synergic activity with peramivir against IV infection in mice; and (3) the observation that the exogenous administration of PD1 is feasible and attenuates the lung response to viral injuries in infected mice. Antiviral molecules like PD1 and also the aforementioned COX inhibitors, nimesulide and naproxen (clinically approved drugs), belong to an emerging new class of anti-IV agents that couple specific antiviral activity with the ability to block the pro-inflammatory response activated upon infection and responsible for the immunopathogenesis of IV. For all these reasons, the further clinical development of PD1, given its potency and late-stage efficacy, should be greatly encouraged.

Antiviral strategies involving host cell antiviral response

Infection by 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 (Fig. 3, bottom). One of the most important cellular defense strategies against IV infection is the activation of type I and III IFN pathways [234]. 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 [234]) with NS1 representing the major player (see above).

Given the pivotal role of IFN pathway in the early anti-IV cellular response, its activation or enhancement represents an important option for antiviral intervention. Compounds 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 (Fig. 3, bottom). Indeed, treatment with poly(I·C), CpG oligodeoxynucleotides, 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 [235]. 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 (Fig. 3, bottom) [235]. Furthermore, a novel small molecule, ASN2, which inhibits viral RdRP subunit PB1, was reported to have additional IFN-inducing properties (Fig. 3) [236]. Short synthetic RNA molecules with an exposed 5′-triphosphate (5′ppp) moiety are substrate for RIG-I and have been reported to be able to induce a potent antiviral response that results in inhibition of IAV replication [129, 237, 238]. In particular, a synthetic 5′ppp-RNA was recently shown not only to inhibit IV replication in A549 cells but also, when intravenously administered, to trigger an antiviral state in infected mice that protected from lethal IV infection [238].

In conclusion, the induction of cellular antiviral responses 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

In the last few years, the need to overcome resistance and counteract threats of unforeseeable outbreaks of pandemic viruses has greatly fueled the search for new anti-influenza drugs. Recent research efforts mainly include (1) the improvement of existing drugs directed against M2 and NA; (2) the development of inhibitors against other antiviral targets, in particular RdRP, NP, and NS1; (3) 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 (4) modulation of cell metabolism and host antiviral response. In particular, in the last few years, the targeting of cellular factors involved in IV replication has received much attention because such an antiviral approach could counteract viral drug resistance, as resistance against host-targeted antivirals would likely not emerge as rapidly as it has for virus-targeted inhibitors. In addition, as 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. Indeed, the possibility of blocking different viral and/or cellular targets may allow the generation of an "antiviral cocktail" that could reduce the probability of generating escape mutants.

Many of the inhibitors described here are still very far from being used in human therapy. Although some of them possess drug-like properties and promising efficacy/toxicity profiles, many preclinical and clinical studies usually undertaken in the drug discovery process [244] will be necessary to determine whether any of these compounds can have the potential to be developed into useful therapeutic strategies. Nevertheless, 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.

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