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Review

Antivirals — current trends in fighting influenza

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Influenza virus infection is a major source of morbidity and mortality worldwide. Due to the variable effectiveness of existing vaccines, especially in the early stages of an epidemic, antiviral drugs represent the first line of defense against the virus. Currently, there are two major classes of anti-influenza drugs approved by the FDA for clinical use: M2 protein inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (zanamivir and oseltamivir). However, increasing resistance to these available influenza antivirals among circulating influenza viruses highlights the need to develop alternative approaches for the prevention and/or treatment of influenza. This review presents an overview of currently available drugs for influenza treatment as well as summarizes some new antiviral strategies that are now being tested covering agents targeting both the viral proteins and the host-virus interaction. We discuss their mechanisms of action, resistance and the therapeutic potential as new antiviral drug for use in future influenza pandemics. Additionally, combination therapy based on these drugs is also described.

Key words: influenza virus, antivirals, novel anti-influenza drugs, antiviral therapy, inhibitors

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INTRODUCTION

Influenza is considered to be a major cause of morbidity and mortality among the leading global infections (Miller *et al*., 2009). Seasonal influenza affects annually up to 20% of the world's population and 250 000–500 000 deaths are reported every year. Antigenic mutation or reassortment can result in new highly virulent influenza strains which arise unexpectedly to cause new epidemics or worldwide pandemics. The swine-origin H1N1 virus from the 2009 pandemic and the H5N1 and H7N9 avian influenza viruses are examples of animal viruses infecting and causing the disease in humans. Currently, two main strategies are used to control influenza infections: vaccination and antiviral treatment. Current influenza vaccines need to be updated seasonally and provide only partial protection in some risk groups. Additionally, in case of an epidemic or pandemic caused by a new strain, development of an effective vaccine requires a certain time period (6 months using the conventional egg-based method) and can be too slow to be effective against the spread of infection. Additionally, vaccines may cause insufficient protection for some immunocompromised patients. Therefore, the use of antiviral drugs against influenza virus could represent the first line of defense, especially in the beginning of a new pandemic until a suitable, effective vaccine becomes available.

Evidence from past influenza seasons and the 2009 H1N1 pandemic has shown that antiviral treatment can have clinical and public health benefits if initiated sufficiently soon after the illness onset. The chemotherapy may shorten the duration of symptoms and reduce the risk of complications and death. Antiviral treatment should be started ideally within 48 hours after the onset of symptoms. It is recommended to be administered as early as possible for any patient with confirmed or suspected influenza who is hospitalized, has severe, complicated, or progressive illness, or is at an increased risk of influenza complications.

This review summarizes drugs that are currently available to prevent influenza virus infection, and describes recent developments in the field of new drug development against this virus. The aim of this review is to discuss new, most promising antiviral agents effective against a large variety of influenza strains and to examine their therapeutic potential. Moreover, combination therapies based on two or more different antivirals with the potential for greater potency and clinical efficiency will be also discussed.

CURRENT ANTI-INFLUENZA DRUGS

Broad spectrum antiviral drugs effective against multiple strains of influenza virus are necessary to control the outbreaks and spread of new infections in the early phases as well as to alleviate the disease symptoms in infected individuals. However, despite intense research for new antivirals only few compounds representing two classes of influenza virus inhibitors are currently available for prophylaxis in exposed individuals and for treatment of severe influenza.

M2 ion channel inhibitors

Historically the first identified inhibitor of influenza virus was amantadine (Davies *et al*., 1964). Amantadine is an amine derivative of adamantane, showing antiviral properties against influenza A virus. Two adamantane derivatives, amantadine and rimantadine, have been licensed as first generation anti-influenza drugs and are currently commercially available as Symmetrel (Endo Pharmaceuticals) and Flumadine (Forest Pharmaceuti-

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Abbreviations: ARB, arbidol; HA, hemagglutinin; NA, neuraminidase; NP, nucleoprotein; RdRP, RNA-dependent RNA polymerase; RNPs, ribonucleoproteins; SA, sialic acid; siRNAs, short interfering RNAs; vRNPs, viral ribonucleoprotein complexes; WT, wild type

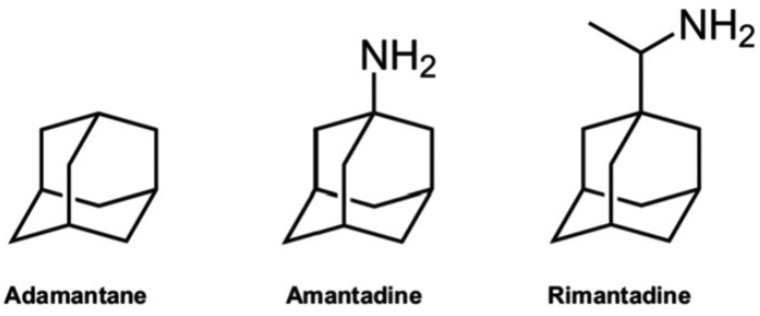

Figure 1. Adamantane and its amine derivatives amantadine and rimantadine

cals), respectively (Fig. 1). Both compounds are M2 ion channel inhibitors. M2 is a transmembrane protein of influenza virus and its tetrameric bundle structure forms ion channels in the viral envelope, mediating selective transport of protons from the late endosome into the viral particle. The resulting acidification of the virus interior facilitates hemagglutinin-mediated membrane fusion and dissociation of M1 matrix protein from the ribonucleoprotein complex leading to uncoating of viral nucleocapsids followed by the transport of the viral genome into the nucleus of the infected cell. Amine derivatives of adamantane block M2 ion channels most probably by intercalating into the interior of the channel (Leonov *et al.,* 2011), thus blocking the influx of H+ ions and subsequent infection steps. Although adamantanes show strong anti-influenza activity at micromolar concentrations, rapid emergence of drug-resistant virus variants restricts the use of both drugs for prevention and treatment. M2-inhibitor resistance arises as a result of single amino acid substitutions in the M2 protein. The most common substitution S31M is found in more than 95% of resistant viruses, but other single substitutions in M2 have also been reported (Bright *et al*., 2005; Krumbholz *et al*., 2009; Wang *et al*., 2013a). Additionally, compensatory mutations S141N and G185A in hemagglutinin (HA) protein HA1, conferring resistance to adamantanes, have been found in 2009 pandemic A (H1N1) virus strains (Choi *et al*., 2009). Those virus variants retain full virulence and transmissibility and are resistant to both amantadine and rimantadine (Hayden, 2006). As a result, the circulating global pool of influenza viruses contains adamantane-resistant variants at the level of 93%, as found for viruses isolated in the USA in years 2005-2006 (Bright *et al*., 2006). In the subsequent years 2007-2009, 98.9% of influenza A (H3N2) and 4.7% of A (H1N1) viruses were resistant to adamantanes (CDC, 2011) and in the 2009 global pandemic caused by a new influenza A (H1N1) strain all isolates tested in WHO Collaborating Centers turned out to be adamantine-resistant (WHO 2010). Due to the high global prevalence of resistant influenza A virus strains which are easily transmitted, and the occurrence of side effects and specificity against influenza A strains only, the use of amantadine and rimantadine is currently suspended in favor of new-generation influenza drugs, neuraminidase inhibitors.

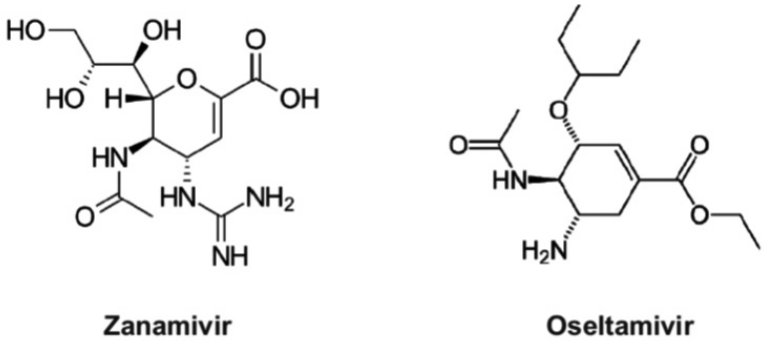

Figure 2. Viral neuraminidase inhibitors zanamivir and oseltamivir

Inhibitors of neuraminidase

While the M2 inhibitors act on the cell entry step, the second generation drugs inhibitors of neuraminidase (NA) impair the release of virus particles from infected cells in the late stage of infection. Influenza virus neuraminidase is one of the two major surface glycoprotein antigens anchored in the viral envelope with a glycoside hydrolase (sialidase) activity critical for the release of progeny virions from infected cells (Nicholls *et al*., 2012). Newly synthesized influenza virions remain attached to the host cell membrane via an interaction of hemagglutinin, the second major surface glycoprotein of influenza virus, with cellular receptors bearing sialic acid (SA). By cleaving off the terminal SA from the host cell receptors NA enables the release of progeny viruses from the membranes of infected cells, preventing self-aggregation of virions and facilitating their movement through the mucus of the respiratory epithelia, and also precluding re-infection of the same cells (Matrosovich *et al*., 2004; Suzuki *et al*., 2005). Blocking of the NA activity results in clustering of the newly formed viruses on the cell surface restricting their transmission to neighboring cells, thus impeding the spread of the infection in the organism. The structure of the neuraminidase active site is highly conserved between influenza A and B strains and all NA subtypes of influenza (Yen *et al*., 2006), therefore NA inhibitors show a broad spectrum of activity.

The development of NA inhibitors began in the 1970s, when the first structural analogues of sialic acid were synthesized (Schulman & Palese, 1975). Currently, several licensed drugs are available of which zanamivir (trademark Relenza, GlaxoSmithKline) and oseltamivir (trademark Tamiflu, Roche) are the most widely used ones (Fig. 2) (von Itzstein *et al*., 1993; 1994; 2007; von Itzstein & Thomson, 2009). Both are synthetic analogues of sialic acid (SA) and are administered as pro-drugs that are metabolized *in vivo* into their active forms which by competition with the natural ligand bind to the active site of NA blocking the enzyme activity. The two drugs differ in their route of administration and bioavailability (Thomson & von Itzstein, 2012). Osaltamivir is available as an oral formulation and 80% of the drug dose shows systemic distribution while the inhaled formulation of zanamivir has low bioavailability with only 2% of the dose entering systemic circulation, therefore it is administered topically directly into the airways and, consequently, is not recommended for complicated systemic infections (Davies, 2010; Alves Galvao *et al*., 2012).

Although the target site for the NA inhibitors is highly conserved, again a number of resistant mutations arise as a consequence of drug-induced selective pressure (Samson *et al.*, 2013). The mutations include substitutions in both NA and HA that reduce either the affinity of NA for the inhibitor or the affinity of HA for the sialylated glycoproteins. The most common mutation conferring resistance to oseltamivir (H274Y in the NA gene) does not affect the sensitivity to zanamivir (Pizzorno *et al.*, 2011). The binding of oseltamivir to NA induces a local conformational alteration in the protein, therefore mutations blocking such a rearrangement prevent the binding of the drug in the enzyme active site. Zanamivir on the other hand binds without any conformational rearrangements, therefore the mutant viruses remain zanamivir-sensitive. In contrast to the resistance to adamantanes, neuraminidase inhibitor resistance develops over a longer time period and occurs with a relatively low frequency (Samson *et al.*, 2013). This is due to the fact that the resistance mutations significanltly affect viral infectivity and ability to replicate in the host organism (Herlocher *et al.*, 2004; Yen *et al.*, 2005). Until several years ago a wide spread of oseltamivir-resistant N1-containing viruses associated with the H275Y mutation was unlikely due to the associated loss in fitness observed in seasonal H1N1 viruses (Ives *et al.*, 2002; Herlocher *et al.*, 2004). However, this changed in 2009, when efficiently transmitted and infectious oseltamivir-resistant A (H1N1) viruses were detected and characterized in Europe and USA (Ciancio *et al.*, 2009; Gooskens *et al.*, 2009, Dharan *et al.*, 2009). It has been shown that several other mutations outside the active pocket of NA can restore functional cooperation between HA and NA (Bloom *et al.*, 2010) giving rise to resistant viral variants transmitted very efficiently from person to person and able to replace susceptible viruses even in the absence of drug pressure. Additionally, double adamantane/oseltamivir-resistant strains have been described among the 2008-2009 seasonal A (H1N1) viruses (Sheu *et al.*, 2010). The increasing emergence of multiple drug-resistant influenza strains emphasizes the need for constant development of improved antiviral drugs and strategies, including drug combinations, rational use of currently available antivirals, and monitoring influenza virus drug resistance. When resistance remains unrecognized, a prolonged use of ineffective therapy can lead to selection of highly resistant viral populations.

MAJOR CLASSES OF FUTURE POTENTIAL ANTI-INFLUENZA DRUGS

The potential anti-influenza drugs are usually classified according to their target in the viral life-cycle. The antiviral strategies cover agents targeting the viral proteins and/or the host-virus interaction.

Anti-influenza virus strategies targeting the virus

Hemagglutinin inhibitors

Hemagglutinin is a homotrimeric type 1 membrane glycoprotein. HA comprises a membrane-distal globular head domain which contains the receptor binding site, and a stem structure with the fusion peptide responsible for intraendosomal membrane fusion. In the first step of the infection cycle, HA molecules bind specifically to host-cell receptors decorated with sialic acid and enable the entry of the virus into the cell by fusion of the viral and cell membranes (Steinhauer, 2010).

Synthetic receptor mimics, such as sialyl-containing macromolecules, which compete with the naturally occurring sialylated receptors, have been described as another group of antiviral compounds blocking the attachment. Gangliosides such as SPG (sialylparagloboside), GD1a, GM3, and GM4 (Terabayashi *et al.*, 2006), pentadecapeptides (Matsubara *et al.*, 2010) and liposomes delivering the glycan sialylneolacto-*N*-tetraose c (LSTc) (Hendricks *et al.*, 2013) are examples of such compounds. They are recognized by the receptor-binding sites of HA, preventing the interaction of the virus with cells and in consequence blocking viral adsorption to target cells.

Clathrin-independent endocytosis is the next step during the influenza virus entry process following the binding to the host-cell receptors. The low pH inside the late endosome provokes an extensive and irreversible conformational change of HA, enabling the fusion of the viral and endosomal membranes. Small molecules that bind the stem region of HA and prevent the low pH-induced conformational changes required for the fusion represent another class of antiviral compounds. *tert*-Butylhydroquinone was the first compound belonging to this group discovered in 1993 (Bodian *et al.*, 1993). This compound was shown to bind to HA and stabilize its nonfusogenic conformation thereby blocking viral activity. Next, several other compounds with a similar mechanism of action have been reported. For example, 4c, BMY-27709, CL-385319, RO5464466 and stachyflin have been identified as specific fusion inhibitors active against different influenza strains, but their subtype-dependent activities as well as low barrier for resistance caused discontinuation of further studies (Luo *et al.*, 1997; Plotch *et al.*, 1999; Yoshimoto *et al.*, 1999; Vanderlinden *et al.*, 2010; Zhu *et al.*, 2011).

Additionally, a small molecule arbidol (ARB), which interacts with HA to stabilize it against the low pH transition to its fusogenic state and consequently inhibits the HA-mediated membrane fusion during influenza virus infection, has been discovered (Leneva *et al.*, 2009; Teissier *et al.*, 2011) (Fig. 3). This compound exhibits a broad spectrum activity and has been reported to inhibit replication of influenza A and B virus as well as other viruses such as hepatitis B, hepatitis C, parainfluenza virus, rhinovirus and RSV. Arbidol has been approved as an antiviral treatment for influenza A and B infection in Russia and China. Up till now, arbidol-resistant influenza viruses have not been isolated in the clinic, however, ARB-resistant mutants selected from the most sensitive reassortant had single amino acid substitutions in the HA2 subunit which caused an increase of the pH of fusion and the associated conformational change in HA were observed (Leneva *et al.*, 2009). Recent studies have shown that ARB interacts with the polar head groups of phospholipids of the cell membrane as well as with aromatic residues of viral glycoproteins abundant on the surface of enveloped viruses (Teissier *et al.*, 2011).

Virus-neutralizing antibodies raised during the influenza virus infection are yet another example of HA-binding inhibitors. A large number of neutralizing antibodies, which are predominantly directed toward the globular head of HA, have been generated and shown to inhibit virus replication in both cell-culture and animal models (Martinez *et al.*, 2009). However, most of these antibodies neutralize only the HA subtype they were generated against and only a few closely related HAs. In recent years, monoclonal antibodies that bind to the conserved receptor binding site of HA have been developed and these antibodies can neutralize many different influenza strains (reviewed in this issue by Kalenik

et al.). CH65 was the first human monoclonal antibody directed against HA1 derived from plasma of a person immunized with the 2007 influenza vaccine. It has been shown that this antibody is able to neutralize several influenza strains *in vitro* (Whittle *et al.*, 2011). Recent studies have reported other examples of neutralizing antibodies with heterosubtypic anti-influenza activity. The more broadly acting monoclonal antibody C05, which was isolated from a phage-display library constructed from bone marrow of a patient after seasonal influenza infection, was shown to neutralize H1, H2, H3, H9 and H12 virus strains (Ekiert *et al.*, 2012). Another cross-reactive monoclonal antibody S139/1 showed neutralization activity against H1, H2, H3, H13 and H16 virus strains (Yoshida *et al.*, 2009).

Broad-neutralizing antibodies targeting the stem region of HA, which is highly conserved among all different influenza subtypes, are another possible antiviral approach. Such antibodies interfere with the conformational changes required for fusion and do not prevent binding to the host cell, as do antibodies described earlier directed to the globular head of HA. Several such antibodies: 1C9, C179, F10, CR6261, CR8020 and FI6 have been described in the literature (Okuno *et al.*, 1993; Prabhu *et al.*, 2009; Ekiert *et al.*, 2009; Corti *et al.*, 2011; Ekiert *et al.*, 2011). All these antibodies showed broad neutralizing activities against different subtypes and groups of influenza A virus both *in vitro* and *in vivo* in mouse models. Another antibody named PN-SIA28 was shown to neutralize all tested isolates belonging to phylogenetic group 1, including H1N1, H2N2, H5N1 and H9N2 subtypes, and several isolates belonging to group 2, including H3N2 isolates from the first period of the 1968 pandemic (Clementi *et al.*, 2011). Therefore, PN-SIA28 has been shown to neutralize isolates belonging to subtypes responsible for all the reported pandemics, as well as other subtypes with pandemic potential. Recently, the human monoclonal antibody CR9114 has been isolated (Dreyfus *et al.*, 2012) that, in contrast to the previously described Abs binds a conserved epitope in the HA stem and neutralizes all influenza A viruses from groups 1 and 2, and also different strains of influenza B viruses. Additionally, the CR9114 antibody protects against lethal challenge with influenza A and B viruses, which makes it an attractive candidate for both development of monoclonal antibody-based treatments and a universal flu vaccine for all influenza A and B viruses.

Novel M2 ion channel blockers

Nowadays, most circulating influenza strains are adamantane-resistant. Many attempts have been undertaken to develop new M2 inhibitors which would interfere with amantadine-resistant viruses. 3-(2-adamantyl)pyrrolidines (Stamatiou *et al.*, 2001) and other pyrrolidines, azetidines and aziridines (Zoidis *et al.*, 2006) were synthesized and evaluated for antiviral activity against H2N2 and H3N2 influenza A virus. However, none of those compounds proved to be better than the parent compounds, therefore other molecules structurally related to adamantadine have been searched for. Di-, tri- and tetrazole derivatives of adamantane have been found to exhibit a much higher antiviral activity against a rimantadine-resistant influenza strain and significantly less cytotoxicity than the currently used rimantadine (Zarubaev *et al.*, 2010). Drug-resistance to amantadine and rimantadine is associated with mutations located mostly in the transmembrane region of the A/M2 protein. Spiro-adamantane inhibitors with potent activity against V27A and L26P mutant A/M2 protein were recently developed (Wang *et al.*, 2011). Additionally, several imidazole and guanazole derivatives of pinanamine have been synthesized by Zhao and coworkers (2012) however, these compounds have shown weak inhibition of the predominant amantadine-resistant M2 mutant — A/M2-S31N. Recently, a small molecule called M2WJ332 has been discovered by Wang *et al.*, (2013a) and shown to inhibit the A/M2-S31N variant with a potency greater than that of amantadine against wild type (wt) A/M2. Moreover, some benzyl-substituted amantadine derivatives have been found to possess antiviral activity against both S31N and wt viruses, demonstrating the potential of S31N as a target for designing novel M2 inhibitors that address the problem of drug-resistant influenza A infections (Wang *et al.*, 2013b).

In addition, an imine compound 8e (Zhao *et al.*, 2011) and a spiro compound 4b (Kolocouris *et al.*, 1996), belonging to polycyclic amine compounds have been reported to be nearly 200-fold more potent against wild-type virus than amantadine. At present, no adamantan(amin)e derivative M2 blocker is active against both wild type and all circulating amantadine-resistant strains. Due to this fact further search is under way in order to find such a universal and potent antiviral drug.

Finally, a neutralizing antibody against the highly conserved M2 ion channel, believed to offer broad protection against influenza A viruses, has been proposed as a potent antiviral agent (Wei *et al.*, 2011). This M2-7A antibody, which specifically binds to M2-containing cell membrane as well as to influenza A virion, has been shown to inhibit the replication of both amantadine-sensitive and resistant influenza A viruses *in vitro* and to protect mice from a lethal influenza virus challenge.

Novel neuraminidase inhibitors

NA represents a promising drug target both for the prophylaxis and treatment of influenza infections, mostly due to the fact that the structure of the neuraminidase active site is highly conserved among influenza A and B strains. Moreover, resistance to neuraminidase inhibitors develops slower than to other anti-influenza drugs. However, following the extensive application of neuraminidase inhibitors such as oseltamivir and zanamivir for influenza treatment, a number of mutations have been identified in the NA of viruses from clinical isolates. In parallel, numerous mutations in the NA of viruses grown *in vitro* in the presence of NA inhibitors have been discovered.

In response to the need for new anti-influenza drugs, two other NA inhibitors named peramivir and laninamivir have been developed (Sidwell & Smee, 2002; Koyama *et al.*, 2009) (Fig. 3). Peramivir (RWJ-270201, BCX-1812) is a cyclopentane compound that potently and selectively inhibits influenza virus NA. Notably, it retains activity against various zanamivir- and oseltamivir-resistant influenza A and B viruses (Mishin *et al.*, 2005). Intravenous form of peramivir has been approved for influenza treatment under the trade name Rapiacta in Japan and as Peramiflu in South Korea; phase III studies with this drug administered intravenously are currently undertaken in the USA.

Various other cyclopentane-based compounds like cyclopentane amide as well as pyrrolidine derivatives named A-192558 and A-315675 have been synthesized and characterized in order to optimize further the antiviral and NA inhibitory properties (Wang *et al.*, 2001; Kati *et al.*, 2002). Both compounds effectively inhibited different strains of influenza A and B viruses and a potent inhibitory effect of A-315675 against oseltamivir-resistant

influenza viruses of the N1 and N2 subtypes was also confirmed (Abed *et al.*, 2008).

Laninamivir (R-125489), structurally similar to zanamivir, is administered orally as an octanoyl prodrug, laninamivir octanoate (CS-8958). It inhibits the NA activity of various influenza A and B viruses, including N1-N9 subtypes and oseltamivir-resistant viruses with long-retention profile (Koyama *et al.*, 2009; Kiso *et al.*, 2010). Laninamivir was approved for influenza treatment in Japan in 2010 under the name "Inavir" and is currently being studied for the treatment and prophylaxis of influenza in phase III clinical trials in the USA.

New-generation NA inhibitors are currently under investigation to increase the efficacy of the approved NA drugs. The modifications include for example multimeric forms of zanamivir or addition of the biodegradable polymer poly-L-glutamine (Watson *et al.*, 2004; Weight *et al.*, 2011; Lee *et al.*, 2012). Such multimeric forms of zanamivir are significantly more active than the monomeric form and, more importantly, show outstanding long-lasting protective activity when tested in mouse influenza infectivity experiments. Dimeric derivatives of zanamivir conjugates with various linking groups have been shown to be 100-fold more potent than zanamivir both *in vitro* and *in vivo* (Macdonald *et al.*, 2005). Moreover, these compounds show long persistence in lungs with a long-lasting antiviral activity that allows for a single dosing regimen.

Plant extracts could be a source of new-generation anti-influenza compounds targeting the enzymatic activity of viral NA. It has been found that flavonols isolated from *Rhodiola rosea* (Jeong *et al.*, 2009) as well as extracts from tropical medicinal plants used as herbal medicines by traditional healers to treat flu symptoms exhibit antiviral action against different strains of influenza virus (Rajasekaran *et al.*, 2013). Such extracts could serve as candidates for the development of safe and less toxic drugs challenging the NA of drug-resistant viruses in an attempt to safeguard human health and global economy.

Inhibitors of influenza virus RNA-dependent RNA polymerase

Transcription and replication of the influenza virus genome is carried out by the viral RNA-dependent RNA polymerase (RdRP). This holoenzyme is composed of three subunits PB1, PB2, and PA, which, in addition to the RNA polymerase activity responsible for the elongation of nascent RNA chains, also has endonuclease activity critical for the synthesis of viral mRNAs. It utilizes the process known as "cap-snatching" to generate short capped primers that initiate the synthesis of viral mRNAs (Fodor, 2013). The capped primers are cleaved off from the host mRNAs by the endonuclease activity of RdRP within the nucleus of an infected cell (for details see: Bartlam *et al.*, 2010). Since the influenza RNA polymerase is highly conserved among influenza strains, it is a good target for the development of new specific antivirals. Two classes of influenza RNA polymerase inhibitors have been discovered: nucleoside analogues blocking RNA chain elongation, and compounds targeting the endonuclease cap-snatching activity.

Favipiravir (T-705) is the best characterized nucleoside analogue with a potent anti-influenza virus activity *in vitro* and *in vivo* (Furuta *et al.*, 2002) (Fig. 3). T-705 is metabolized intracellularly to form T-705 ribofuranosyl triphosphate which acts as a purine analogue and inhibits viral RNA synthesis. It has been shown that T-705 inhibits influenza A, B and C strains, including amantadine and oseltamivir resistant variants. The drug has also been shown to have an antiviral effect on the highly pathogenic avian H5N1 and recently emerged H7N9 viruses, with efficacy already confirmed in animal studies (Furuta *et al.*, 2013). Additionally, the viral resistance to favipiravir has been reported to be very low (Furuta *et al.*, 2009). Favipiravir (trade name: AVIGAN) obtained the approval in Japan in March 2014 as a pharmaceutical product

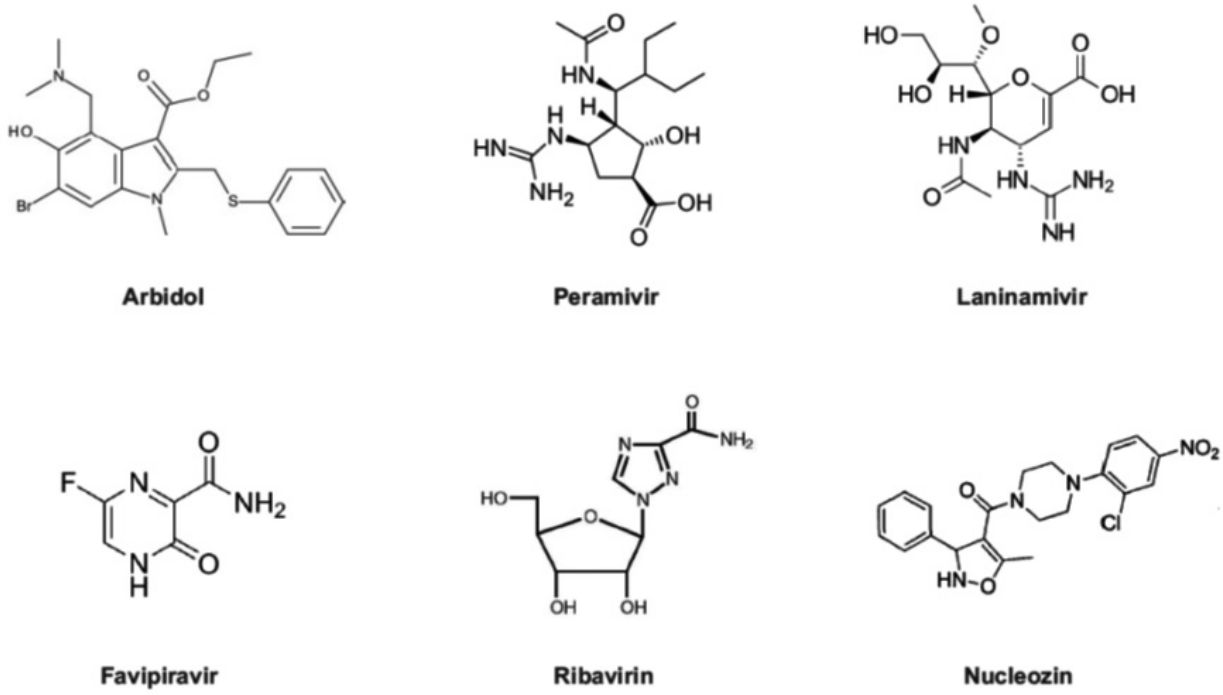

Figure 3. Recently developed anti-influenza drugs

available to establish preparedness against the possible outbreak of novel or re-emerging influenza virus infections for which NA inhibitors or other influenza drugs could be ineffective or not sufficiently effective. Clinical tests are also being conducted in the United States.

Ribavirin is another nucleoside analogue with anti-influenza activity (Fig. 3). In addition, its amidine prodrug viramidine with a marked potential as an anti-influenza agent has also been discovered (Sidwell *et al.*, 2005). Ribavirin in the oral route is routinely used in hepatitis C virus treatment. Additionally, the intravenous form of ribavirin has been registered for the treatment of haemorrhagic fever with renal syndrome. Ribavirin has been shown to be active against both human and avian influenza viruses and no drug resistance has been reported. The only disadvantage of therapy using ribavirin is the development of haemolytic anaemia, which is, however, reversible after cessation of treatment.

Several compounds targeting the endonuclease cap-snatching activity of the RdRP complex have been discovered in recent years. This class of antivirals include for example 4-substituted 2,4-dioxobutanoic acid derivatives (Tomassini *et al.*, 1994), *N*-hydroxamic acid/*N*-hydroxy-imide derivatives (Cianci *et al.*, 1996), cap analogues (Lv *et al.*, 2011), and short capped oligonucleotides (Tado *et al.*, 2001). In addition, flutimide, a 2,6-diketopiperizine, isolated from extracts of the fungus *Delitschia confertaspora,* has been shown to inhibit specifically the cap-dependent endonuclease activity of influenza viral RNA polymerase (Tomassini *et al.*, 1996). This compound has been shown to inhibit the replication of influenza A and B viruses in cell culture.

Due to the fact that the interaction between the PA and PB1 subunits of RdRP is highly conserved, it represents an attractive strategy for the development of new drugs. It is supposed that compounds blocking the interaction between PA and PB1 will have broad antiviral efficacy against most, if not all, influenza strains. Using the crystal structure of the primary protein-protein interface between the PB1 and PA subunits of the influenza A virus polymerase, an *in silico* screen to identify potential small molecule inhibitor has led to the identification of two compounds (1 and 5) which were shown to inhibit the interaction between PB1 and PA and transcription by the full viral ribonucleoprotein complex (Muratore *et al.*, 2012a). Moreover, compound 1 was shown to act as a potent replication inhibitor of a variety of influenza A virus strains in Madin-Darby canine kidney (MDCK) cells, including H3N2 and H1N1 seasonal and 2009 pandemic strains as well as an oseltamivir-resistant isolate. In parallel, the AL18 compound, which was previously reported as potent inhibitor of human cytomegalovirus DNA polymerase, was serendipitously found to block the interaction between the PB1 and PA polymerase subunits of influenza A virus (Muratore *et al.*, 2012b). As a consequence, AL18 effectively inhibited influenza A virus polymerase activity and the overall replication of influenza A and B viruses.

ELISA-based screening was used to design and identify a novel class of influenza A (H1N1) inhibitors that efficiently disrupt the PA-PB1 interaction (Kessler *et al.*, 2013). However, only a limited number of compounds were tested, therefore Pagano and colleagues (2014) decided to synthesize a large library of benzofuran derivatives in order to test their antiviral activity against influenza A virus H1N1. It was shown that most of the new derivatives inhibited the viral RNA polymerase complex through blocking the interaction between the PA and PB1 subunits and thus inhibiting viral replication. However, due to their substantial toxicity, these compounds can only be a starting point in the identification and development of novel therapeutic antiviral agents against influenza virus.

Antiviral strategies targeting viral nucleoprotein

The influenza virus nucleoprotein (NP), is one of the most abundant viral proteins produced during infection and has both structural and regulatory functions in virus replication. NP, the main structural component of the viral ribonucleoprotein complexes (vRNPs), has a crucial role in vRNPs nuclear import, and in viral RNA transcription and nuclear export (Portela & Digard 2002). The conserved amino acid sequence of NP makes it an attractive target for the development of new anti-influenza strategies that could be broadly active across different virus strains.

Short interfering RNAs (siRNAs) specific for nucleotide sequences encoding highly conserved regions of NP protein have been found to reduce influenza viral production *in vitro* and *in vivo* (Ge *et al.*, 2004; Tompkins *et al.*, 2004; Zhou *et al.*, 2007). Treatment with siRNAs significantly reduced lung virus titers in infected mice and protected animals from a lethal challenge with highly pathogenic avian influenza A viruses of the H5 and H7 subtypes. Moreover, several small molecules targeting NP oligomerization have been found recently by several groups. Ingavirin inhibiting NP oligomerization and subsequent nuclear import of newly synthesized NP was reported by Semenova and colleagues (2010). Another molecule, nucleozin (Fig. 3), identified to stabilize interactions between NP monomers and thereby promoting the formation of non-functional NP aggregates is currently under intensive studies. The inhibitory mechanism of nucleozin has both early- and late-acting effects on the influenza virus life cycle (Amorin *et al.*, 2013). Nucleozin has been reported to inhibit viral RNA and protein synthesis as well as the production of infectious progeny. It blocks the cytoplasmic trafficking of ribonucleoproteins (RNPs) exported from the nucleus, promoting the formation of large perinuclear aggregates of RNPs along with the cellular protein Rab11. This effect leads to the reduction of production of virus particles, which are often much smaller than normal. Recently, the synthesis of some structural analogues of nucleozin has been reported (Gerritz *et al.*, 2011; Cheng *et al.*, 2012).

Host-based anti-influenza virus strategies

Most antiviral strategies available to date are directed toward influenza viral proteins. However, there are many cellular factors which play a crucial role in influenza virus propagation and represent attractive targets for development of new drugs hampering the virus-cell interactions. The development of inhibitors against host rather than viral proteins has some advantages by limiting the emergence of drug-resistant viral strains. However, the disturbance of cellular function may cause adverse side effects which need to be carefully studied.

The entry process of the influenza virus, which is crucial for the establishment of viral infection, is a good target for drug development. The proposed compounds interfere with virus attachment to host cell receptors, internalization, endocytosis, fusion of viral and endosomal membranes, and uncoating and import of the viral genome into the nucleous (Eyer & Hruska, 2013).

Inhibitors of virus attachment

The use of sialidases removing sialic acid from the surface of epithelial cells hampers the binding of the globular head domain of HA during the virus attachment step. Indeed, it was shown many years ago that following enzymatic removal of sialic acid from their surface cells are less susceptible to infection with influenza virus (Gottschalk, 1959). Recently, a new anti-influenza virus agent, DAS181 (Fludase), which is a recombinant fusion protein composed of a sialidase catalytic domain derived from *Actinomyces viscosus* and a cell-surface-anchoring domain, has been reported (Malakhov *et al*., 2006). It has been shown to remove effectively α2-6- and α2-3-linked sialic acids from receptors used by both human and avian influenza viruses, preventing viral attachment and replication. DAS181, which is currently in phase II clinical trials, displays antiviral activity against a broad variety of influenza A and B viruses. Fludase has been shown to suppress the replication of highly pathogenic avian influenza A H5N1, H1N1 and recently H7N9 virus, in cell culture and in a murine model (Belser *et al*., 2007; Triana-Baltzer *et al*., 2009; Marjuki *et al*., 2014). Since DAS181 targets the host cell, it is supposed to generate low drug resistance.

Inhibitors of endocytosis and fusion

The entry of influenza viruses into cells is mediated by endocytosis. A productive entry of influenza virus requires the low-pH environment of the late endosome for fusion and release of the virus into the cytoplasm and transport of the viral genome into the nucleus. Virus endocytosis could be inhibited by specific membrane fluidity modulators which restrict the movement of membrane molecules. The glycolipids fattiviracin (Harada *et al*., 2007) and glycyrrhizin (Wolkerstorfer *et al*., 2009) with a broad antiviral activity against diverse enveloped viruses have both been reported to display also an anti-influenza virus activity inhibiting the endocytic uptake of the virus by decreasing the fluidity of the cell membrane. Glycyrrhizin is under clinical studies in Japan to define its toxicity profile. The aryl methyldiene rhodamine derivative LJ001 is another interesting compound affecting membrane fluidity by intercalating into viral as well as cellular membranes (Wolf *et al*., 2010).

Inhibition of endosome acidification is an alternative strategy to prevent viral fusion. Two macrolide antibiotics, bafilomycin A1 and concanamycin A, were shown some time ago to inhibit vacuolar proton ATPase responsible for the acidification of the endosomes and to prevent the entry of influenza A and B viruses into cells (Guinea & Carrasco, 1995; Ochiai *et al*., 1995). Moreover, compounds such as diphyllin and saliphenylhalamide have been reported to produce a similar antiviral effect by targeting this vacuolar proton pump (Huss & Wieczorek, 2009; Konig *et al*., 2010). Chloroquine, a compound which elevates the endosomal pH, used for several years in malaria treatment, has also been shown to inhibit influenza entry in *in vitro* studies (Ooi *et al*., 2006). Unfortunately, chloroquine was unable to prevent infection with influenza virus in a double-blind, placebo-controlled efficacy trial, which eliminates it as an efficient agent for clinical use (Paton *et al*., 2011).

Combination therapy

Combination therapies using extant antivirals with different modes of action are promising as they achieve greater potency and clinical efficacy, allow administration of lower doses of the drugs, resulting in decreased side effects, as well as reduce the development of antiviral resistance. Additionally, combination therapy could be of help if the treatment is applied later in the course of infection or in immunocompromised patients. Drug combinations have become a routine treatment for human immunodeficiency virus and hepatitis C virus infections. Several *in vitro* and *in vivo* studies have demonstrated enhanced activity of anti-influenza agents when used in combination, although the clinical relevance for most combinations remains to be determined (Madren *et al*., 1995; Govorkova *et al*., 2004; Ilyushina *et al*., 2007; Nguyen *et al*., 2009; 2012; Tarbet *et al*., 2012; 2013). An additive and synergistic effect in tissue culture and in influenza A virus infections in mice was observed for a combination of rimantadine or amantadine with ribavirin (Wilson *et al*., 1980), oseltamivir, peramivir or zanamivir with rimantadine (Govorkova *et al*., 2004), oseltamivir with amantadine (Ilyushina *et al*., 2007), oseltamivir with ribavirin (Ilyushina *et al*., 2008), and oseltamivir, peramivir or zanamivir with favipravir (Tarbet *et al*., 2013). A double-blind, randomized, placebo-controlled trial to compare a therapy with zanamivir in combination with rimantadine versus rimantadine alone was also conducted for treating influenza in hospitalized adults (Ison *et al*., 2003). That clinical study confirmed a higher efficacy of the combination of zanamivir and rimantadine which was well-tolerated by patients.

Another clinical study using triple-combination antiviral drug therapy (amantadine, ribavirin, and oseltamivir) versus oseltamivir monotherapy was conducted by Kim and colleagues (2011) in patients with severe A/H1N1 2009 (pH1N1) influenza in Korea to evaluate the efficacy and safety of combination antiviral therapy. That study revealed that the 14-day mortality was statistically significantly lower in patients treated with the combination therapy (17% *versus* 35% for oseltamivir alone), but there was no significant difference in survival at 90 days. Several other combination therapies based on approved as well as new drugs are currently under investigation. Some of them appear to be promising in the therapy and management of human influenza virus infections.

CONCLUSIONS

The continuous threat of a new influenza pandemic urges scientists and medical practitioners alike to improve their understanding of the biology of influenza virus and to search for new antiviral strategies. Within the past few years, several new drugs interfering with different viral or host factors have been reported. Novel treatment options seem to have a higher selection barrier of resistant strains and a broader antiviral activity against both influenza A and B infections. Several drugs are currently in different stages of preclinical up to advanced clinical development and some of them will probably be available in near future, either alone or in combination, for the treatment of influenza virus infections.

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