Recent Developments in Anti-influenza A Virus Drugs and Use in Combination Therapies

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Abstract: The pandemic potential of influenza viruses has engaged a large portion of the antiviral drug discovery research community in the development of numerous antiviral agents, with the ultimate goal to supplement effective immunization when new strains arise, especially after an antigenic shift. Antiviral agents against influenza A targets different replication steps of the virus life cycles. Some of the agents are analogues of biomolecules required during virus infection and others are inspired from natural plant extracts. In this review, we summarize their mechanisms of action during the influenza life cycle *in vitro* and the efficacies of combinational therapies with these agents against the influenza virus infections *in vivo*.

Keywords: Anti-influenza A virus drugs, Ion channel, Hemagglutinin, RNA polymerase, Neuraminidase, Membrane fusion, Drug resistance, Combination therapy.

1. INFLUENZA A VIRUS

Influenza A virus is a negative-strand RNA virus that possesses 8 genome segments in the virion. Each genome segment encodes one or two proteins, as well as short 5' and 3' flanking sequences. Segments 1-3 encode the virus polymerase complex: basic polymerase 1 (PB1, segment 2), which catalyzes nucleotide addition (and which also encodes a proapoptotic mitochondrial protein that is translates in a different reading frame, PB1-F2 [1]); basic polymerase 2 (PB2, segment 1), which controls the recognition of host-cell RNA; and acidic protein (PA, segment 3), which might possess an endonuclease activity [2]. Segments 4 and 6 encode surface envelope glycoproteins that are highly antigenic. The hemagglutinin (HA) protein, binds sialic-acid covalently linked to the terminal galactose of an oligosaccharide on a glycoprotein or glycolipid and promotes entry into host cells. The neuraminidase (NA) protein facilitates budding of progeny virions from infected cells by cleaving sialic acid from galactose. Segment 5 encodes a nucleoprotein (NP), which binds to viral RNA and delivers them into the nucleus after virus membrane fusion. Segment 7 encodes two proteins that share a short overlapping region: the matrix protein M1 encodes the main component of the viral capsid, and M2, which is an integral membrane protein, functions as an ion channel. Segment 8, the smallest segment of the viral genome, encodes a nonstructural protein (NS1), which affects cellular and viral mRNA transport, splicing and translation [3] and NS2 protein, a minor component of the virion, the function of which is to make a complex with M1 protein and interact with a nuclear export factor CRM1 that exports vRNA from the nucleus [4]. A mature virion of influenza A virus is composed of the nucleocapsid, a surrounding layer of M1, and the membrane envelope, which contains the HA, NA and M2 proteins.

2. HUMAN PATHOGENIC INFLUENZA A VIRUS

In general, influenza B and C viruses infect humans and cause mild illness in children [5]. In contrast, influenza A virus infects birds, horses, pigs, and humans. Wild-waterfowl and shorebirds are the natural hosts of influenza A viruses, which are occasionally transmitted to other species and may then cause devastating outbreaks in domestic poultry or give rise to human influenza pandemics [6]. The type A viruses are the most virulent human pathogens among the three influenza types and cause the most severe disease. The type A virus can be subdivided into different serotypes based on the antibody response to viral antigens. The main immuno-responsive antigens of the virus are hemagglutinin (HA, 16 variants) and neuraminidase (NA, 9 variants). The serotypes that have been confirmed in humans are mainly H1N1 (Spanish flu in 1918, and Swain Flu in 2009), H2N2 (Asian Flu in 1957), H3N2 (Hong Kong Flu in 1968), and H5N1 (Bird Flu in 2004).

3. INFLUENZA A VIRUS INFECTION

The HA forms a trimer of identical subunits, each monomer consists of HA1 and HA2 that are processed by proteolytic cleavage of a single precursor HA0 [7]. The site of cleavage is at a single arginine residue within the consensus motif Q(E)-T/X-R. It is the substrate for trypsin-like endoproteases secreted from the bronchial epithelial cells [8]. This cleavage is essential for activation of membrane fusion between the virus and cell. In the case of the highly pathogenic avian influenza A virus, the processing occurs by ubiquitous cellular proteases, which selectively recognize the multi-basic consensus cleavage site motif, R-X-K/R-R. This motif is readily cleaved by ubiquitously processing protease such as furin in the trans-Golgi network [8].

The trimer of HA for most influenza A viruses recognizes sialic acid that covalently binds to galactose coupled with an α-2, 6 galactose linked receptors. In contrast, the HA of avian pathogenic H5N1 influenza virus binds to sialic acid coupled with an α-2, 3-galactose linked receptors. Once the virus attaches to the host cells, it is incorporated into the endosome *via* endocytosis. The low pH condition in the endosome triggers an influx of protons into the virion through the M2 ion channels, which induces a conformational change in the HA protein, leading to the fusion of the viral membrane and endosome membrane [9]. The low pH also triggers the dissociation of the viral ribonucleocapsid (vRNPs) from the M1 matrix protein [10].

After the vRNPs are released into the cytoplasm, they are imported into the nucleus as a result of recognition of the nuclear localization signal (NLS) by karyopherin proteins [11]. In the nucleus, the viral RNA-dependent RNA polymerase (PA, PB1, PB2) uses the negative-sense vRNA as a template to synthesize two different positive-sense RNA species: one species of RNA is mRNA templates for viral protein synthesis, and the other type is complementary RNA (cRNA) intermediates from which the RNA polymerase subsequently transcribes more copies of negative-sense, genomic vRNA. The newly synthesized vRNAs form stable

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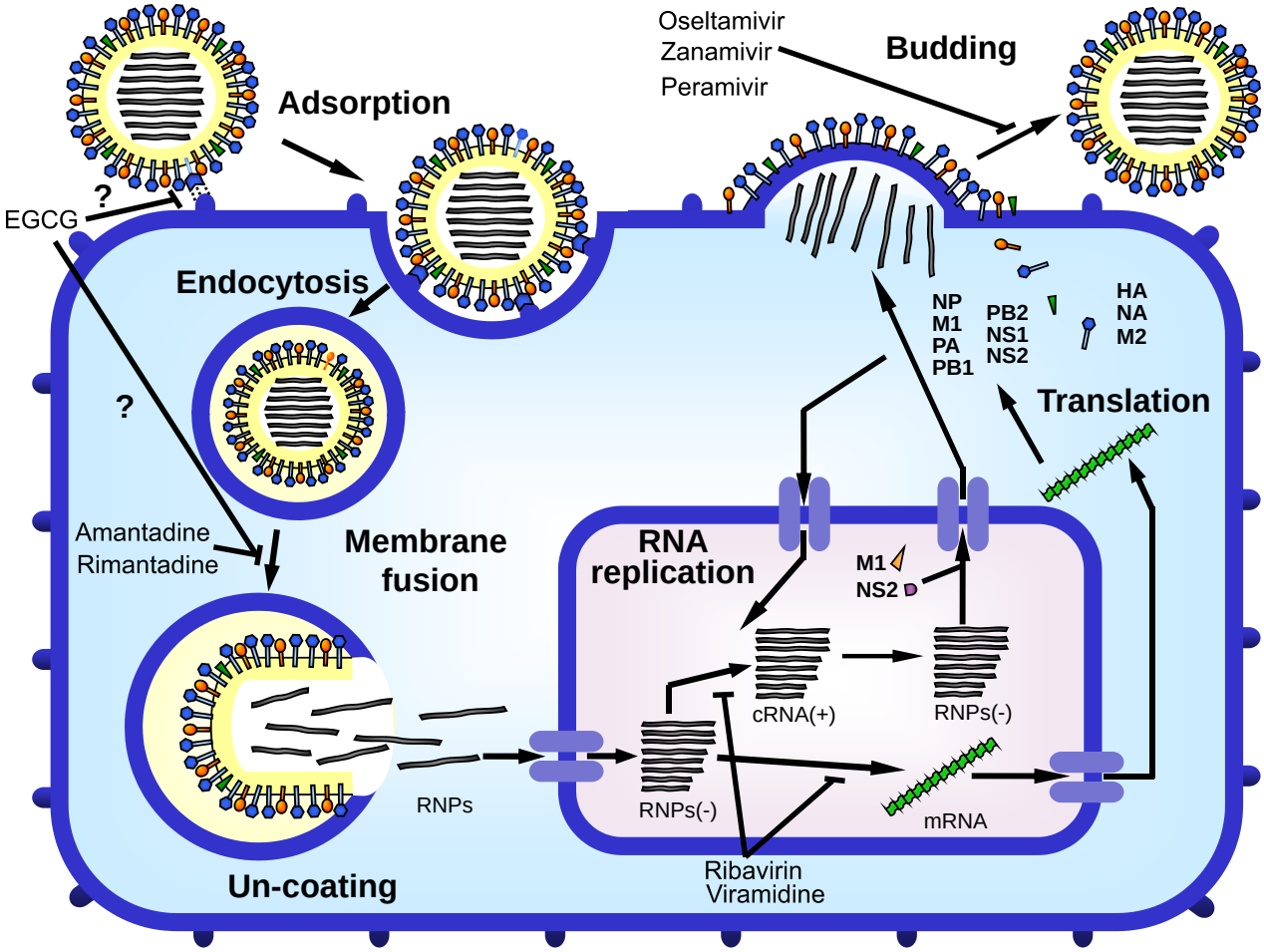


Fig. (1). Influenza virus life cycle in host cells and mechanism of action of antiviral agents.

complexes with nucleoproteins in the nucleus and are transported to the cytoplasm by an M1-NS2 complex [4]. The vRNPs and other viral proteins reach the apical surface of the cell membrane to be incorporated into new virion. The NA of the progeny virus cleaves the glycoside linkage between sialic acid and galactose of cellular glycoproteins enabling the completed virions to bud from the cellular membrane.

4. ANTIVIRAL AGENTS

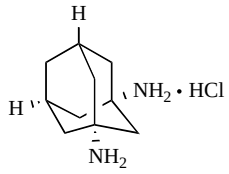
Currently, there are a number of anti-influenza virus agents such as fusion inhibitors, transcription and genome replication inhibitors, and neuraminidase inhibitors. Here we summarize their mechanisms of action during the replication cycle of virus infection and *in vivo* efficacies (Fig. **1**).

4.1. Ion Channel Blocker

The symmetric tricyclic amines amantadine (Fig. **2a**) and rimantadine (Fig. **2b**) have been known to block the interior channel of the tetrameric M2 proton pump. Based on X-ray [12] and solid-state NMR studies [13], amantadine interacts with the Ser31 cluster, which is located near the entrance of the channel and inhibits the migration of protons (H^+) into the interior by physical obstruction. In contrast, a solution NMR study suggested that rimantadine binds in the vicinity of the Trp41 gate, which is located near the C-terminal region of the tetrameric M2 transmembrane domain and stabilizes the channel in the closed state [14]. These agents prevent the acidification of the virus particle within the endosome and inhibit the virus un-coating process. Thus, they can inhibit all influenza A types viruses, but not influenza B viruses because the latter group does not use an M2 ion channel to complete virus replication. As has previously been stated, many current circulating strains of influenza A viruses are now resistant to varying degrees to amantadine and rimantadine, making these two drugs much less useful clinical entities. The fact that resistance to such agents arose rather quickly, in addition to experiences with HIV drug resistance development, emphasize the point that when treating rapidly evolving virus species with drugs, combination therapy opposed to monotherapy would be the most prudent course of action. Both prophylactic and therapeutic efficacy of amantadine and rimantadine have been demonstrated in mouse models for influenza A infections (See Hayden and Gubareva for review, [15]) as well in the human clinical experience [16]. However, it is well known that currently circulating strains of influenza A viruses are almost always resistant to amantadine and rimantadine [17, 18]. Nevertheless, it has been shown that the combination of amantadine with oseltamivir required 15-fold less oseltamivir than the oseltamivir monotherapy dosing regimen to confer complete protection against lethal aerosol influenza virus infection [19]. To emphasize the wisdom of using combinational therapy rather than monotherapy to treat influenza virus infections, the combination mentioned above was even effective against an amantadine-resistant A/PR/8/34 (H1N1) virus. In addition, amantadine in combination with oseltamivir was shown to promote enhanced survival of infected mice over treatment with agent alone in mice inoculated with neurotropic recombinant A/Vietnam/1203/04 (H5N1) virus [20]. Finally, it has also been shown in a robust lethal mouse model for influenza A using low pathogenic H5N1 duck virus, that various combinations of known anti-influenza drugs and

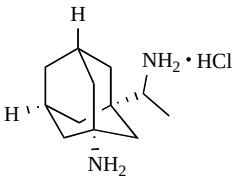
amantadine, were superior in preventing mortality compared to the use of individual drugs alone [21].

(**a**)



Amantadine

(**b**)



Rimantadine

Fig. (2). Ion channel blockers; (**a**) amantadine and (**b**) rimantadine.

The most fascinating concept has been the use of triple combination therapy to treat influenza infections. It was found that in mouse models using A/New Caledonia/20/99 (H1N1), A/Sydney/05/97 (H3N2), and A/Duck/MN/1525/81 (H5N1) as challenge viruses, the synergy of the triple combination was 2- to 13-fold greater than the synergy of any double combination depending on the influenza virus subtype [22]. Whether the use of combinations can prevent the development of resistance to individual agents used in combination remains to be demonstrated. Current dogma suggests that development of resistant would be a mathematically remote event if two agents in a given combination were to target independent regions within an active site for ligand binding or a functional domain of a protein (i.e., in the M2 channel protein).

4.2. Adsorption and Membrane Fusion Inhibitors

Epigallocatechin gallate (Fig. **3a**), a major catechin component of the green tea plant (*Camellia sinensis*), possesses the most potent antiviral activity among tea polyphenols [23]. Reports on the anti-influenza activity of EGCG found that it inhibited virus adsorption [24], as well as acidification of endosomes and lysosomes [25]. Such virus inhibition activity is different from other current NA or proton pump inhibitors, suggesting that EGCG can be developed into a new class of antiviral compounds that are effective against current drug resistant influenza strains. However, relatively high concentrations of EGCG were required to observe significant antiviral activity, probably due to the compound's poor lipid membrane permeability [26].

In contrast, EGCG-fatty acid monoesters showed improved antiviral activities against influenza A/PR8/34/(H1N1) virus infection in Mardin-Derby Canine Kidney (MDCK) cells [27]. The antiviral activities of EGCG-monoesters were increased in an alkyl length dependent manner [27]. Palmitoyl monoesters of EGCG (EGCG-C16; Fig. **3b**) are the most potent inhibitors and the half maximal effective concentration (EC_{50}) against A/PR8/34/(H1N1) was 20 nM which is about 20-fold less than natural EGCG [27]. These results indicated that the improved viral membrane permeability of EGCG might increase the anti-influenza virus activity. EGCG-C16 also inhibited a series of human influenza viruses, an experimental strain A/Puerto Rico/8/34/ (H1N1), vaccine strains (A/Beijing/262/95/ (H1N1), A/Panama/2007/99/ (H3N2), and B/Yamanashi/166/98), drug-resistant strains (Yokohama/77/2008/ (H1N1) OPR: oseltamivir phosphate-resistant (OPR), Yokohama/63/2007/ (H1N1) AR: amantadine-resistant (AR), A/Yokohama/91/2008/ (H1N1) OPR/AR: (OPR/AR) and avian pathogenic influenza (A/Duck/Hong Kong/342/78/ (H5N2)) *in vitro* [28]. Because EGCG-C16 inactivated influenza B virus does not use an M2 ion channel to complete virus replication, the target was expected to be another membrane protein such as HA and NA [28]. Furthermore, EGCG-C16 completely inhibited avian influenza virus infection against embryonated chicken eggs, while oseltamivir phosphate and zanamivir could not inhibit the virus infection [28]. Further studies are necessary to discuss the mechanism of action; however, EGCG-fatty acid monoesters have the potential to be a novel type of anti-influenza agent.

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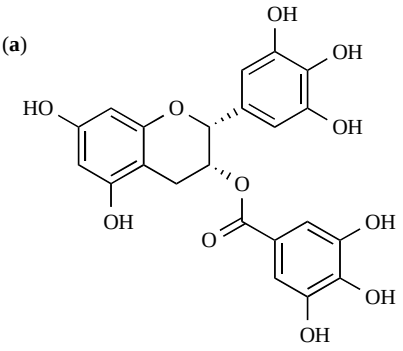
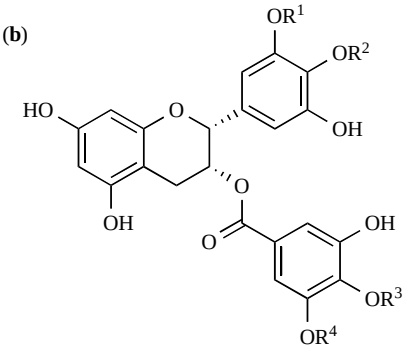


Epigallocatechin gallate (EGCG)

(**b**)



EGCG-C16

One of R^{1-4} is $-C(=O)(CH_2)_{14}CH_3$, others are -H

Fig. (3). Adsorption and membrane fusion inhibitors.

4.3. Transcription and Genome Replication Inhibitors

Ribavirin (Fig. **4a**) and viramidine (Fig. **4b**) are guanosine analogues that lower intracellular GTP levels through the inhibition of inosine 5'-monophosphate dehydrogenase (IMPDH), an enzyme involved in the *de novo* synthesis of guanine nucleotides. They also inhibit transcription and elongation by interfering with RNA-dependent RNA polymerase. Ribavirin has consistently been shown to inhibit seasonal isolates of H1N1 and H3N2 viruses, influenza B viruses and pandemic H1N1 influenza A viruses [29-32] at 75 mg/mg/kg/d (maximum tolerated dose), usually twice a day within 48 h of the initiation of infection, although ribavirin could be administered to mice infected with the pandemic strain A/Mexico/4108/2009 at 40 mg/kg/d if the treatment were started at the time of infection [32]. Viramidine was found to be less potent, but was better tolerated [30]. In addition, Sidwell *et al.*. (2005) found that ribavirin was significantly efficacious in preventing mortality in mice infected with influenza A/duck/MN/1525/81 (H5N1) virus in a lethal mouse model when administered at 75 mg/kg/d twice a day for 5 days. In another *in vivo* study, 90% of BALB/c mice treated orally with ribavirin at 75 mg/kg/day, starting 4 h before virus inoculation and given for 8 days, were protected

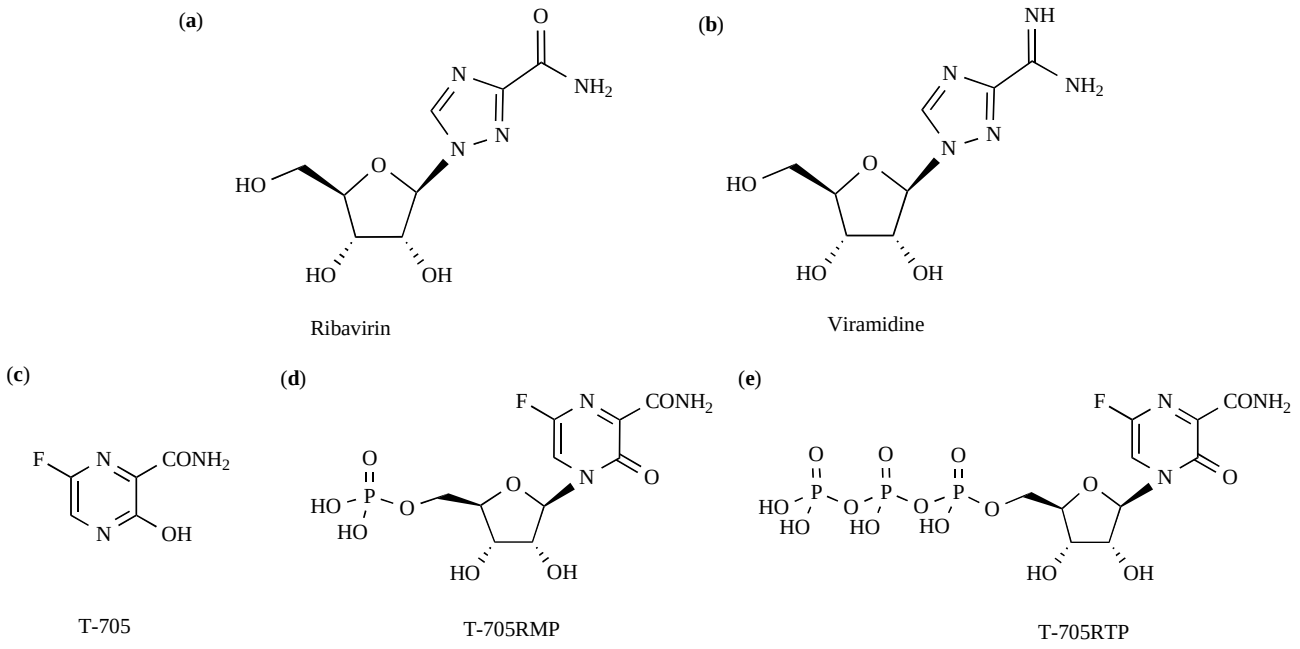


Fig. (4). Transcription and genome replication inhibitors; (**a**) ribavirine, (**b**) viramidine, (**c**) T-705, (**d**) T-705RMP, and (**e**) T-705RTP.

from the lethal effects of A/Vietnam/1203/04 (H5N1) influenza virus infection [33]. In addition to using ribavirin as monotherapy, it has also been used successfully in combination with oseltamivir to treat lethal influenza A infections of mice [22, 23, 33-35]. In an H5N1 study using the combination of ribavirin at 37.5 mg/kg/day and oseltamivir at 1 mg/kg/day and the combination of ribavirin at 37.5 mg/kg/day and oseltamivir at 10 mg/kg/day, mice were synergistically protected against mortality caused by A/Vietnam/1203/04 (H5N1) and A/Turkey/15/06 (H5N1) viruses, respectively. The combinations also significantly inhibited virus replication in mouse organs, and ameliorated the cytokine response. More importantly, the oseltamivir-ribavirin combinations were as efficacious or more compared to the monotherapies used at the same concentrations. It has been established that ribavirin cannot be used for treating respiratory virus infections, mainly due to its toxicity and its teratogenic potential. Several points may be used in arguing in favor of using ribavirin for treating severe influenza virus infections [32]. First, it probably will not be necessary to use the drug for sustained periods of time, thus greatly reducing toxicity and exposure to health care personnel. Second, it now has a proven safety record for treating patients with HCV and thus the timeline of adverse events is well known. The combination studies mentioned above demonstrate that ribavirin could be used at lower concentrations than would be normally used if given in combination with another drug, thereby also reducing toxicity. Third, ribavirin is not likely to induce resistance, and when used in combination with other drugs, it may be that drug resistance development to those drugs will also be reduced, since they may be used at lower doses than those associated with resistance induction.

Favipiravir (T-705; Fig. **4c**) is a pyrazine derivative that has broad RNA virus inhibitory activities including against influenza A, B, and C [36]. It is initially converted to the monophosphate (T-705RMP; Fig. **4d**) by host-derived phosphoribosyl transferase, and subsequently converted to its triphosphate (T-705RTP, Fig. **4**) by nucleotide kinase [37]. T-705RTP inhibits influenza virus RNA polymerase activity in a GTP-competitive manner [37]. In contrast to ribavirin, T-705 does not inhibit cellular DNA or RNA synthesis and has little effect on IMPDH. The level of IMPDH inhibition by T-705RMP is about 150-fold weaker than that by ribavirin MP [37].

Furuta *et al.*. (2002) were the first to report on *in vivo* inhibition using anti-influenza mouse model. In an H1N1 seasonal influenza A model, favipiravir, administered p.o. four times daily at a dose of 100 mg/kg/d prevented mortality. Treatment could be delayed to 25 h post virus exposure with complete prevention of mortality [38]. The compound has also been shown to inhibit virulent as well as less virulent strains of influenza A H5N1 viruses and pandemic H1N1 influenza viruses [39, 40]. Remarkably, in an H5N1 model using a low virulence duck virus, treatment with favipiravir at 300 mg/kg/d could be delayed for 60 h post-virus exposure and still completely prevent mortality in mice [41]. At that dose, the compound was still well tolerated in control mice. Most importantly, favipiravir has also been shown to inhibit oseltamivir-resistant H5N1 avian influenza viruses [40]. When used in combination with oseltamivir against influenza A/Victoria/3/75 (H3N2) virus in mice, combining ineffective doses of both compounds (25 mg/kg/day of favipiravir and 25 mg/kg/day of oseltamivir) resulted in 90% survival and improved body weight during infection, whereas only 10-11% of mice receiving the monotherapies survived [42]. Similar results were achieved against H1N1 and low pathogenic avian H5N1 viruses. Thus, favipiravir represents a new, highly effective broad-spectrum influenza virus inhibitory compound that targets an entirely different part of the influenza replication cycle from currently approved drugs.

4.4. Neuraminidase Inhibitors

Zanamivir (GG167; Fig. **5d**), oseltamivir carboxylate (GS4071; Fig. **5f**), oseltamivir (GS4104; Fig. **5g**), and peramivir (BCX-1812; RWJ-270201; Fig. **5h**), are neuraminic acid (Neu5Ac; Fig. **5a**) analogues that are designed to bind a conserved region of the catalytic site of neuraminidase (NA) [See the review in ref. 43]. At the last step of influenza virus infection, the NA cleaves the glycoside linkage between Neu5Ac and galactose of glycoproteins on virus and cell membranes. This event enables the progeny virions to leave the infected cells and spread to other host cells. The active site of NA is conserved among 9 NA subtypes of influenza A and B viruses; thus, these NA inhibitors inhibit a broad spectrum of viral infectivity.

2-Deoxy-2,3-didehydro-N-acetylneuraminic acid (DANA; Fig. **5b**), is a dehydrated form of Neu5Ac. It was developed as a neuraminidase inhibitor by Meindle *et al.* (1974) [44]. Based on previous mechanistic studies, the double bond in the cyclohexene template mimics the transition state of the sialosyl carbon [45].

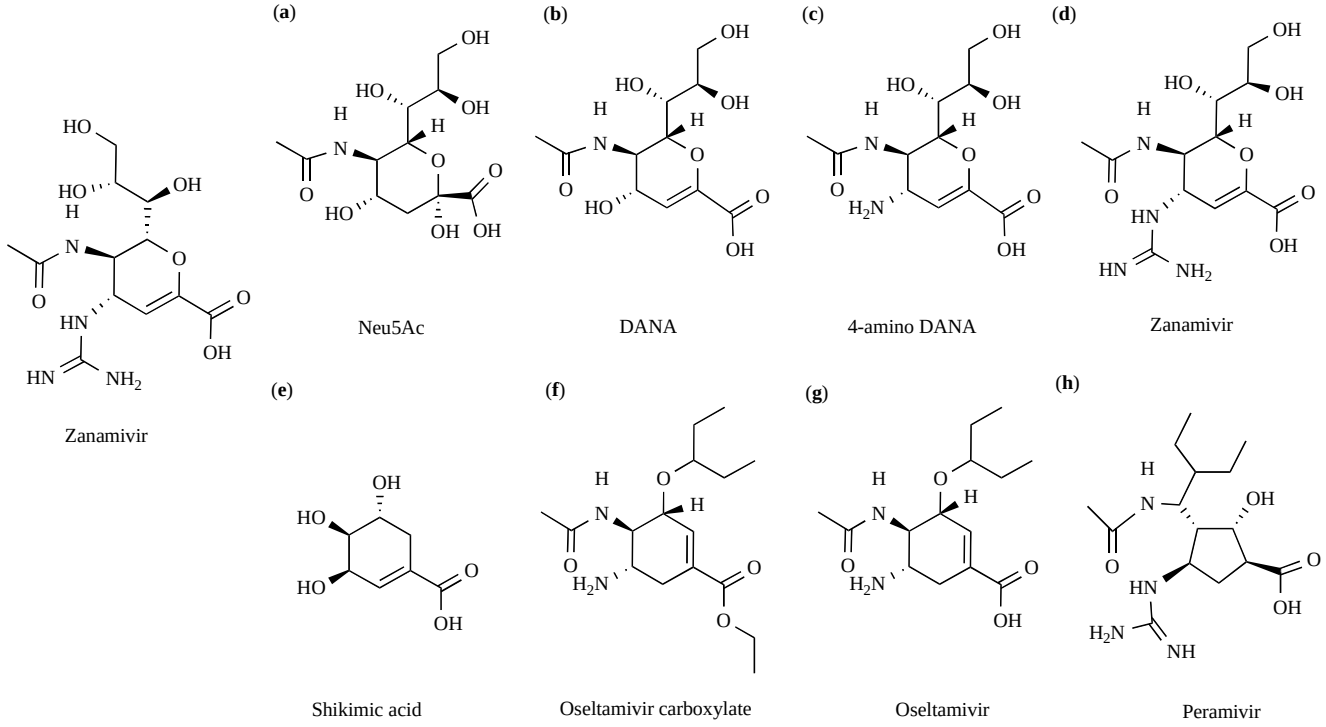


Fig. (5). Neuraminidase inhibitors; (**a**) Neu5Ac, (**b**) DANA, (**c**) 4-amino DANA, (**d**) Zanamivir, (**e**) Shikimic acid, (**f**) Oseltamivir carboxylate, (**g**) Oseltamivir, (**h**) Peramivir.

Based on computational analysis of the binding fusion of DANA in the catalytic site of NA, there is a negatively charged amino acid residue that is aligned with the C4 hydroxyl group of DANA [46]. Replacing this hydroxyl group with a positively charged amino group, the 4-amino DANA showed a 100-fold better inhibitory effect compared to DANA [47]. This is the result of a salt bridge formation with a conserved Glu119 in the active site [46].

Oseltamivir is a pro-drug ethyl ester and is converted to the active carboxylic acid form (oseltamivir carboxylate; Fig. **5f**) by human carboxylesterase (HCE) 1 in the liver [48]. It is the most commonly used antiviral drug and is prepared from shikimic acid (Fig. **5e**). Neu5Ac and its derivatives such as DANA and zanamivir have a glycerol group and C8 and C9 hydroxyl groups that form a bidentate interaction with Glu276 in the active site of NA [See the review in Ref 43]. In contrast, oseltamivir has pentane 3-oyl group at the same position instead of a glycerol group. Within the catalytic site of NA, the pentane 3-oyl group induces a conformational change in Glu276 to make a stable charge-charge interaction to Arg224. This induced fit is essential to achieve the potent antiviral activity. In this reorientation, His274, a neighboring basic amino acid helps the conformational change of Glu276. Therefore, mutation of His274 to Tyr274 results in oseltamivir-resistance. Other amino acid changes in the NA catalytic site have also been reported for avian influenza virus (H5N1) [49], seasonal influenza A viruses such as H1N1 and H3N2, and for influenza B virus [see the review in Ref. 50]. In mouse studies, oseltamivir has normally been administered orally as a broad-spectrum neuraminidase inhibitor of influenza A viruses.

In one study using an H5N1 mouse model, oseltamivir administered at 1 and 10 mg/kg/d prevented mortality in infected mice [51]. It was also found that therapy could be delayed for up to 36 h post-virus exposure and still be efficacious. However, it was observed that various influenza A H5N1 virus strains differed in their sensitivity to oseltamivir. For example, the A/Vietnam/1203/2004 strain was more resistant to oseltamivir than was the original highly pathogenic A/Hong Kong/156/97 virus [52]; treatment twice a day for 8 days was required to achieve efficacy against the Vietnam strain compared to 5 days of treatment for the A/Hong Kong/156/97 virus. Oseltamivir can also inhibit H3N2, H1N1 seasonal viruses [53], and ceratain pandemic H1N1 viruses [54] when orally administered at doses of 1-10 mg/kg/d.

With increased oseltamivir resistance being detected in many currently circulating strains of seasonal and pandemic H1N1 viruses, a number of combination studies have been attempted to determine if combination therapy (oseltamivir plus another drug) would be useful in treating currently circulating viruses [19, 21-23, 33, 34, 45, 51, 55, 56]. Synergy was detected using suboptimal doses of ribavirin and oseltamivir against influenza A/Vietnam/1203/2004 (twice daily ribavirin at 37.5 mg/kg/day and oseltamivir at 1 mg/kg/day) and A/Turkey/15/06 (twice daily ribavirin at 37.5 mg/kg/day and oseltamivir at 10 mg/kg/day) [33]. Synergy was also shown for combinations of oseltamivir and peramivir when treating mice infected with influenza A/NWS/33 (H1N1) virus; the combination (twice daily oral oseltamivir (0.4mg/kg/day) plus twice daily intramuscular peramivir (0.1 and 0.2mg/kg/day)) performed better than suboptimal doses of each compound alone [56]. In addition, the combination of twice daily amantadine (30 mg/kg/day) and oseltamivir (10 mg/kg/day) protected 90% of H5N1 infected mice versus 10-11% survival for mice receiving single treatments [21]. The combination of twice daily administered oseltamivir (0.2 mg/kg/day) and rimantadine (7.5 mg/kg/d) was resulted in 87% survival of mice infected with influenza A/Aichi/2/68 (H3N2) virus [55]. Each treatment alone was largely ineffective in preventing mortality. Treatment of an H3N2 infection (A/Victoria/3/75) with a combination of oseltamivir plus favipiravir administered twice daily at 25 mg/kg/day for each drug increased survival to 90%; treatment with each drug alone at 25 mg/kg/d did not prevent mortality [45]. Although data on the effects of combining suboptimal doses of zanamivir plus oseltamivir to enhance survival of mice infected with lethal dose of influenza virus have not been published, a human case study suggests that this combination (oral oseltamivir and inhaled zanamivir) was not be effective in treating critically ill patients infected with pandemic influenza A (H1N1) virus [57]. In another

study, in treating adults with seasonal influenza A, mainly due to H3N2 virus, the combination of oseltamivir and zanamivir was less effective than oseltamivir monotherapy, and was not significantly more effective than treating patients with zanamivir alone [58]. Thus, it would appear that this combination is not warranted for treating most human influenza A infections.

Zanamivir is the first approved neuraminidase inhibitor and has a guanidinyl group instead of a hydroxyl group at the C4 position of DANA. Zanamivir showed higher antiviral activity compared to 4-amino DANA, because the guanidinyl group preferentially binds to the carboxylate of Glu227 and Glu119 of NA [59]. Although numerous examples of oseltamivir-resistant influenza viruses have been reported, to date there have been few examples of zanamivir-resistant H5N1 virus, pandemic H1N1 influenza virus, or H3N2 seasonal influenza virus isolated from patients [60]. Thus, zanamivir remains of interest due the fact that some of the more common mutations involving neuraminidase resistance such as the H274Y and N294S multidrug resistance phenotypes, do not confer drug resistance to zanamivir [61]. One of the more interesting *in vivo* studies has involved the use of zanamivir for treating an oseltamivir-resistant influenza A/Hanoi/30408/2005 H5N1 virus infection of ferrets [49]. Treatment with zanamivir resulted in decreased nasal virus titers, but not treatment with oseltamivir. Often patients with severe ARDS are subjected to prone positioning ventilation as part of the treatment regimen, which decreases absorption of drugs that require enteral administration [62]. Thus, it has been postulated that intravenous zanamivir might be an appropriate alternative drug for such patients [62]. Using a hollow fiber cell culture system as a model for intravenous treatment of pandemic influenza H1N1 infections in humans, zanamivir, at a dose of 600 mg given twice daily, inhibited the replication of oseltamivir-resistant influenza viruses throughout the course of the experiment [63]. These data suggest that there might not be cross-resistance for oseltamivir and zanamivir although they have the same mechanism of virus inhibition.

In a study to ascertain the benefit of combining FDA-approved anti-inflammatory agents with zanamivir, BALB/c mice were infected with influenza A/Vietnam/1194/04 and treated individually or in combination with zanamivir, celecoxib, gemfibrozil, and mesalazine 48 h after virus exposure [64]. There were marginally significant improvements in survival rate ($P = 0.02$), survival time ($P < 0.02$), and a decrease in inflammatory markers ($P < 0.01$) in the group treated with the triple combination of zanamivir, celecoxib, and mesalazine when compared with zanamivir treatment alone. However, the addition of the immunemodulators to zanamivir treatment did not improve the reduction of virus loads compared with zanamivir treatment alone. The authors suggested that the immunomodulators might have improved the clinical outcome in mice by their synergistic effects in reducing cytokine dysfunction and preventing apoptosis. Finally, as mentioned above, in a controlled human clinical trial, the combination of zanamivir with oseltamivir does not seem to be warranted since the combination was less effective than oseltamivir and zanamivir monotherapies [58].

Peramivir has a cyclopentane template, while other neuraminidase inhibitors, such as oseltamivir and oseltamivir carboxylate have cyclohexene templates. Peramivir retains activity against various zanamivir and oseltamivir-resistant A and B viruses [65]. The dissociation half-life of peramivir from N9 enzyme was more than 1 day, while that of oseltamivir and zanamivir was 1.25 h [65]. In one of the initial studies using peramivir to treat H5N1 viruses, Govorkova *et al.* (2001) were able to protect mice from a lethal infection with A/Hong Kong/156/97 (H5N1) virus using peramivir administered p.o. at doses of 0.1 and 10 mg/kg/d [66]. Peramivir significantly protected animals against mortality, ameliorated weight loss due to virus infection, and reduced virus lung titers. No brain virus was detected. The compound could be administered as late as 60 h post-virus exposure and still promote survival of mice. Peramivir was also significantly inhibitory to an infection in mice induced by influenza A/NWS/33 (H1N1) virus when given by oral gavage at 5 mg/kg twice daily for 5 days up to 60 h after virus exposure [67]. In the same study, peramivir (12.5 mg/kg given p.o. twice daily for 5 days beginning 4 h pre-virus exposure) was also found to significantly protect mice from challenge with lethal doses (LD70-LD100) of influenza A/Shangdong/09/93 (H3N2) virus. Mortality induced by influenza A/Bayern/57/93 (H1N1) and B/Lee/40 viruses in mice was also significantly inhibited when infected mice were given peramivir at 100, 10, and 1 mg/kg per day using the treatment regimen described above [67]. Bantia *et al.*. (2006) have also reported efficacy against a seasonal influenza A H1N1 virus. A single i.m. administration protected mice up to 48 h post-virus exposure [65]. The efficacy of i.m. administered peramivir, was recently shown in a study of a pandemic influenza A/California/04/2009 H1N1 virus in mice [54]. A single i.m. injection (30 mg/kg) of peramivir given 1 h prior to virus exposure, significantly reduced weight loss (p <0.001) and mortality (100% survival) compared to mice in the vehicle group. Peramivir administered 24, 48, or 72 h after infection as a single i.m. injection at 50mg/kg, also significantly protected infected mice against death and weight loss; survival was 100, 40, and 50%, respectively. Thus, peramivir has been shown to be a broad-spectrum inhibitor of influenza A and B viruses in mouse models.

As has previously been mentioned, synergistic effects in a mouse model have been seen for combinations of oseltamivir and peramivir when treating mice infected with various influenza A viruses [56]. In addition, one study evaluated the efficacy of various dosing combinations of ribavirin and peramivir to treat an infection in mice caused by influenza A/NWS/33 (H1N1) virus [35]. Mice were treated with ribavirin at 20 and 6.25 mg/kg/day combined with peramivir at 1, 0.32, or 0.1 mg/kg/day, or used alone twice daily for 5 days starting 4 h before exposure to virus. Most drug combinations significantly increased survival compared to the survival rate for the placebo group. The combination of the two inhibitors produced additive to synergistic interactions in these mouse experiments with no enhancement of toxicity in the host. Although peramivir and rimantadine combination therapy has been tested *in vitro* and found to be synergistic [68], combination studies in mice or ferrets have been few. In a combination study using i.m.-administered peramivir and orally administered rimantadine, mice were infected with a sublethal dose (<40% mortality) of influenza A/Victoria/3/75 (H3N2) model, and compounds were administered for 5 days beginning 1 h before viral inoculation [69]. The peramivir and rimantadine doses ranged from 0.3-3 mg/kg/d and 5-30 mg/kg/d, respectively. Efficacy was determined by amelioration of weight loss. The combination of 1 mg/kg/d peramivir with 5 and 10 mg/kg/d rimantadine significantly ameliorated weight loss (p <0.05 vs. vehicle and individual agent); weight loss was limited to only 1.69 and 0.69 g, respectively, compared to the over 5 g weight loss detected for the control mice. The combinations of 1 mg/kg/d peramivir with 5, 10 and 30 mg/kg/d rimantadine and 3 mg/kg/d peramivir with 5, 10 and 30 mg/kg/d rimantadine were considered synergistic for amelioration of weight loss.

Thus, limited studies have been reported on the efficacy of combination therapy with peramivir and other influenza inhibitors to lessen the chance of drug-resistant viruses arising in the population when peramivir becomes approved for clinical use. Therefore, it is of concern that peramivir-resistance has been confirmed in mutant viruses showing the H274Y and N294S phenotypes, which are also associated with oseltamivir resistance [61]. In addition, the newly discovered I222V mutation seemed to potentiate the resistance to oseltamivir/peramivir in the H274Y mutant virus [61]. Ominously, the former mutation seemed to also compensate for reduced viral fitness of single mutation virus, suggesting the possibility of the emergence of multiple drug resistant replication competent viruses that could easily disseminate into a susceptible population.

CONCLUSIONS

Anti-influenza A virus agents target the viral proteins that are required for adsorption, membrane fusion, un-coating, replication, amplification, and budding during the viral life cycle. As viruses obtained drug-resistance mutations, chemists continue to design drug candidates based on structural analysis and synthesize novel type of antiviral agents. In addition, virologists have proven the effectiveness of combinations of antiviral agents *in vitro* and *in vivo* and have proposed therapeutic courses for clinical settings. Future collaborations between these experts will provide the necessary solutions in the fight against the next pandemic.

CONFLICT OF INTEREST

None.

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